

World Clinical *Stem Cells*

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Core progresses in stem cells in 2014

It took 138 years from the first mentioning of the term "Stammzelle" ("stem cell") describing the "ancestor unicellular organism from which all multicellular organisms evolved" by German biologist Ernst Haeckel in 1868 and his notion to also name the fertilized egg "(embryonic) stem cell" in 1877, to Japanese scientists Shinya Yamanaka and Kazutoshi Takahashi to convert mature adult cells by means of reprogramming technology to embryonic-like "induced pluripotent stem cells" in 2006. In these 138 years, many other seminal discoveries on stem cells have been made resulting in our current understanding that these cells come in all shapes, such as toti-, pluri- or multipotent, embryonic, adult, or "reprogrammed". Importantly, the more we discover the biology of stem cells, the more we also appreciate their remarkable plasticity and their potential for therapy development, or as diagnostic and prognostic biomarkers. In fact, there is great hope in stem cells as a source for the treatment, or the discovery of treatments, for a variety of diseases, and in some cases this has already become reality. Nevertheless, the stem cell field at large is still in its infancy and there is much more to learn before many of the proposed implications will enter routine clinical application.

The current issue of *World Clinical Stem Cells* e-books (ISBN 978-0-9861420-2-4), provides a comprehensive summary of the latest developments in translating stem cells to clinical application. In seven sections (advances in adult stem cells; advances in embryonic stem cells; advances in fetal stem cells; advances in hematopoietic stem cells; advances in neoplastic stem cells; advances in neural stem cells; advances in pluripotent stem), the reader will find a diverse collection of articles that cover the entire spectrum of stem cells in various organs and their (therapeutic) implications in disease.

Adult stem cells, and in particular mesenchymal stem cells (MSC), have been of interest because of their relative easy accessibility, maintenance, and their broad range of applicability. Two contributions by Michalis Mastroi, Huey Lin, and Techung Lee and Shihori Tanabe review in depth their broad biological function and therapeutic potential, and a novel mechanism in MSC functions through purinergic receptors is discussed by

Sonia Scarfi. In addition, differences in MSC populations are discussed by Tokiko Nagamura-Inoue and Haiping He, and Mustapha Zeddou, Biserka Relic and Michel G Malaise who compare umbilical cord-derived MSC with MSC derived from other tissue. A more detailed discussion on the role of MSC in individual tissue types and organs with respect to disease is provided in a series of articles listed below.

Several contributions address MSC in context of bone and cartilage tissue. While the use of MSC for osteoarthritis is discussed by Kristin Uth and Dietmar Trifonov, their osteogenic and therapeutic potential for periosteum and cartilage repair or regeneration is reviewed by Concetta Ferretti and Monica Mattioli-Belmonte, Mohamadreza Baghaban Eslaminejad, and Elham Malakooty Poor, and Dean T Yamaguchi. In contrast, MSC can also be part of disease processes, and a role of MSC in the pathophysiology of myeloma bone disease is summarized by Antonio Garcia-Gomez, Fermin Sanchez-Guijo, M Consuelo del Cañizo, Jesus F San Miguel, and Mercedes Garayoa. Another set of articles deals with adipose MSC. While Eun-Hee Kim and Chan Yeong Heo, Wakako Tsuji, J Peter Rubin and Kacey G Marra, and Patrick C Baer cover their general biology and broad therapeutic potential, Anna Park, Won Kon Kim, and Kwang-Hee Bae highlight in depth their role in adipose tissue development, Han-Tsung Liao and Chien-Tzung Chen compare their osteogenic potential with those of bone marrow-derived MSC, and Cecilia Romagnoli and Maria Luisa Brandi discuss their use for bone repair in combination with scaffolds.

MSC have also implication in several other biological systems. These include their potential role as immune-modulatory cells reviewed by Justin D Glenn and Katharine A Whartenby, as well as Dobroslav Kyurkchiev, Ivan Bochev, Ekaterina Ivanova-Todorova, Milena Mourdjeva, Tsvetelina Oreshkova, Kalina Belezova, and Stanimir Kyurkchiev. A more in depth discussion in context of immune-modulation in corneal wound repair can be found in the article by Fei Li and Shao-Zhen Zhao, and a related article by Jinny J Yoon, Salim Ismail, and Trevor Sherwin reviews the presence of limbal stem cells as a secondary stem cell reservoir

on the corneal surface. The cardio-vascular system is also a target of MSC. As being anti-inflammatory, immunosuppressive, and actively involved in tissue repair, Aika Yamawaki-Ogata, Ryotaro Hashizume, Xian-Ming Fu, Akihiko Usui, and Yuji Narita discuss a role of MSC for treatment of aortic aneurysms, and Poay Sian Sabrina Lee and Kian Keong Poh review the function of endothelial progenitor cells in vasculogenesis and as therapeutics in cardiovascular diseases. A more general discussion on the classification of osteoprogenitors in context of arterial calcification is provided by Francesco Vasuri, Silvia Fittipaldi, and Gianandrea Pasquinelli. MSC also play a role in acute kidney injury management, which is discussed by Francesca Bianchi, Elisa Sala, Chiara Donadei, Irene Capelli, and Gaetano La Manna, and Elvin E Morales and Rebecca A Wingert highlight their potential role in kidney repair by providing a review on the use of reprogramming differentiated renal cells for tissue regeneration. Additional articles cover intestinal stem cells in the pathogenesis of celiac disease by Anna Chiara Piscaglia, MSC therapy for skin flaps necrosis by Fu-Gui Zhang and Xiu-Fa Tang, the use of MSC in pancreatic island transplantation for treatment of type 1 diabetes by Marina Figliuzzi, Barbara Bonandrini, Sara Silvani, and Andrea Remuzzi, and MSC differentiation into gonad and adrenal steroidogenic cells by Takashi Yazawa, Yoshitaka Imamichi, Kaoru Miyamoto, Akihiro Umezawa, and Takanobu Taniguchi.

Finally, MSC have also implications in neurological disorders of both the central and the peripheral nerve system, and five contributions address their potential from different angles. A general overview of MSC in neurological and retinal diseases is provided by Tsz Kin Ng, Veronica R Fortino, Daniel Pelaez, and Herman S Cheung, while more in depth discussion on MSC in the treatment of autism and spinal cord injury can be found by contributions from Dario Siniscalco, James Jeffrey Bradstreet, Nataliia Sych, and Nicola Antonucci, and Venkata Ramesh Dasari, Krishna Kumar Veeravalli, and Dzung H Dinh, respectively. One of the most advances in translating the MSC paradigm to clinical application has been their use in traumatic brain injury and an update on clinical trials is provided by Ana Maria Blanco Martinez, Camila de Oliveira Goulart, Bruna dos Santos Ramalho, Júlia Teixeira Oliveira, and Fernanda Martins Almeida. Last but not least, Florence Appaix, Marie-France Nissou, Boudewijn van der Sanden, Matthieu Dreyfus, François Berger, Jean-Paul Issartel, and Didier Wion discuss the plasticity of brain perivascular MSC in cancer formation and tumor progression.

Hematopoietic stem cells, which are also classified as adult stem cells, have long been used to understand and treat diseases of the blood. Four articles discuss their disease as well as therapeutic properties. While Jianbiao Zhou and Wee-Joo Chng review the pathogenic biology of leukemia stem cells, their identification and separation, and strategies for their eradication, Michael J

Ricci, Jeffrey A Medin, and Ronan S Foley, and Abraham S Kanate, Marcelo C Pasquini, Parameswaran N Hari, and Mehdi Hamadani summarize the current state and advances in hematopoietic cell transplantation for acute myeloid leukemia and other high-risk hematological malignancies. In addition, Melhem Solh discusses the advantages and disadvantages of umbilical cord blood as an alternative source for hematopoietic cell transplantation, and on a more fundamental note, a contribution by Bruno C Huber, Ulrich Grabmaier, and Stefan Brunner reviews the therapeutic use of parathyroid hormone on mobilizing bone marrow-derived stem cells.

Two sections address advances in embryonic and pluripotent stem cells, which in recent years have rapidly evolved as one of the most studied stem cell sources - last but not least as a consequence of the introduction and commercialization of reprogramming technologies that have tremendously facilitated their production. A fundamental issue in translating the pluripotent stem cell paradigm to future clinical application is the understanding of stem cell biology and their differentiation in concert with understanding human disease pathogenesis. In this process, animal models and in particular mouse models, are vitally important. As for disease modeling, two contributions by Kiyomasa Nishii, Yosaburo Shibata, and Yasushi Kobayashi, and Michiko Ichii, Kenji Oritani, and Yuzuru Kanakura discuss how knowledge from connexin mutations or B lymphocyte development in mouse models can be translated to studying human disease and B lymphopoiesis in embryonic stem cells, respectively. An additional article provided by Lisa Mohamet, Natalie J Miazga and Christopher M Ward addresses how pluripotent stem cell technologies can be used to model Alzheimer's disease. As for understanding pluripotent stem cell biology and differentiation, Kate Hawkins, Shona Joy, and Tristan McKay give an update on the cell signaling pathways that are involved in stem cell reprogramming, while Mykyta V Sokolov and Ronald D Neumann discuss the effects of genotoxic stress on altering their gene expression profiles. Yan Li, Meimei Liu, and Shang-Tian Yang review procedures on how to produce dendritic cells from pluripotent stem cells in a large scale, and Anne-Laure Hafne and Christian Dani on how to generate brown and white adipocytes. Pluripotent stem cell-derived neurogenesis is discussed by Masahiro Otsu, Takashi Nakayama, and Nobuo Inoue who review the generation and clinical applications of differentiated neural stem cells, as well as Yan Li, Meimei Liu, Yuanwei Yan, and Shang-Tian Yang, who focus on the role of natural and synthetic extracellular matrixes in neural differentiation protocols. In the section Advances in Neural Stem Cells additional contributions are from Shuchen Zhang and Wei Cui and Koji Shimozaki, who discuss Sox2 transcription networks in the regulation of pluripotency and neural differentiation, and in context of disease, a role of brain cell stem cells in glioma forma-

tion is reviewed by Aram S Modrek, N Sumru Bayin, and Dimitris G Placantonakis, while Julie Chao, Grant Bledsoe, and Lee Chao summarize the use of the kallikrein-kinin system in stem cell therapy, including brain diseases.

There are two major characteristics of stem cells whose understanding is crucial for therapy development, their regenerative/therapeutic and their neoplastic capacities. Fetal stem cell transplantation has a long history to treat various human conditions and an overview of its past, present, and future is provided by Tetsuya Ishii and Koji Eto. In contrast, stem cells of all sources can be the source of malignancies, and an update on their role in cancer of different tissues can be found in the section Advances in Neoplastic Stem Cell. Three articles cover the nerve system with contributions by Nermin Sumru Bayin, Aram Sandaldjian Modrek, and Dimitris George Placantonakis who discuss the molecular characteristics and therapeutic implications in glioblastoma stem cells, Shengwen Calvin Li, Mustafa H Kabeer, Long T Vu, Vic Keschrumrus, Hong Zhen Yin, Brent A Dethlefs, Jiang F Zhong, John H Weiss, and William G Loudon who evaluate the distribution properties of stem cells to target malignant brain tumors, and Mojdeh Safari and Alireza Khoshnevisan who give an overview of the role of cancer stem cells in tumors of the osseous spine. Six more articles address a role of neoplastic stem cells in various other tissues, including ovarian cancer by Christen L Walters Haygood, Rebecca C Arend, J Michael Straughn and Donald J Buchsbaum, head and neck squamous carcinoma by

John M Le, Cristiane H Squarize, and Rogerio M Castilho, hepatocarcinoma by Lei-Bo Xu and Chao Liu, gastrointestinal cancer by Sameh Mikhail and Amer Zeidan, thyroid cancer by Parisha Bhatia, Koji Tsumagari, Zakaria Y Abd Elmageed, Paul Friedlander, Joseph F Buell, and Emad Kandil, and a role of microRNA-140 in context of cancer stem cells in breast cancer by Benjamin Wolfson, Gabriel Eades, and Qun Zhou.

In summary, this issue provides a comprehensive overview of our current knowledge about the various sources of stem cells and their contribution to the understanding and treatment of human disease. Although we are still at the very beginning of exploring the full potential of stem cells at large, continuous progress in the field fuels hope that using these remarkable cells can in future be one of the most prominent therapeutic option for many disease entities.



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Limbal stem cells: Central concepts of corneal epithelial homeostasis

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Abstract

A strong cohort of evidence exists that supports the localisation of corneal stem cells at the limbus. The distinguishing characteristics of limbal cells as stem cells include slow cycling properties, high proliferative potential when required, clonogenicity, absence of differentiation marker expression coupled with positive expression of progenitor markers, multipotency, centripetal migration, requirement for a distinct niche environment and the ability of transplanted limbal cells to regenerate the entire corneal epithelium. The existence of limbal stem cells supports the prevailing theory of corneal homeostasis, known as the XYZ hypothesis where X represents proliferation and stratification of limbal basal cells, Y centripetal migration of basal cells and Z desquamation of superficial cells. To maintain the mass of cornea, the sum of X and Y must equal Z and very elegant cell tracking experiments provide strong evidence in support of this theory. However, several recent stud-

ies have suggested the existence of oligopotent stem cells capable of corneal maintenance outside of the limbus. This review presents a summary of data which led to the current concepts of corneal epithelial homeostasis and discusses areas of controversy surrounding the existence of a secondary stem cell reservoir on the corneal surface

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Key words: Limbal stem cell; Corneal epithelium; XYZ hypothesis; Corneal homeostasis; Corneal wound repair

Core tip: It is a long held belief that stem cells reside only at the limbus. However, there are recent reports that present evidence of corneal repair and maintenance independent of limbal involvement. These findings call to light the possibility of previously undiscovered reservoirs of corneal stem/progenitor cells located at the central and peripheral cornea. A new secondary reservoir of stem cells has a significant clinical implication as new therapeutics for corneal degenerative disorders. This review outlines the historic evidence for limbal stem cells and discusses the role of these putative central and peripheral corneal stems cells in corneal homeostasis.

Original sources: Yoon JJ, Ismail S, Sherwin T. Limbal stem cells: Central concepts of corneal epithelial homeostasis. *World J Stem Cells* 2014; 6(4): 391-403 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i4/391.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i4.391>

INTRODUCTION

The transparent front surface of the eye, the cornea (Figure 1A) overlies the iris, pupil and anterior chamber. The structures that compose the anterior chamber are

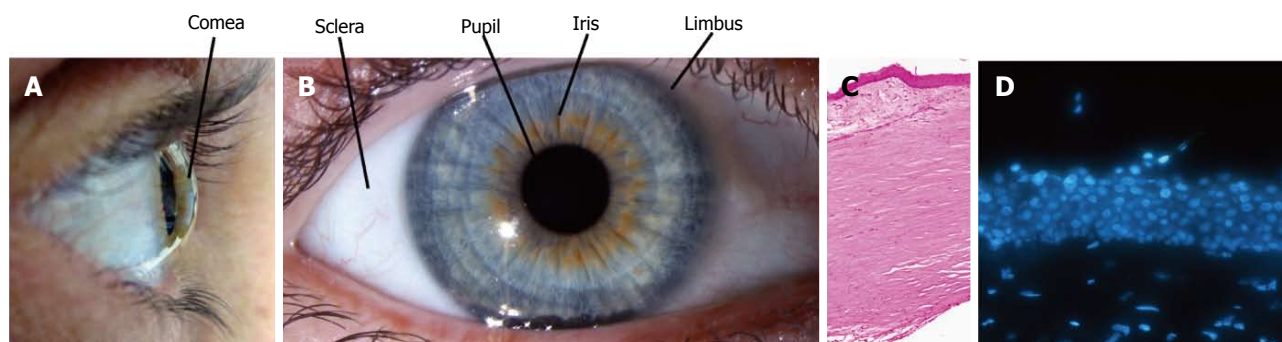


Figure 1 Anatomy of the eye. The cornea (A) comprises the colourless front portion of the eye immediately anterior to the iris and pupil (B). The limbus, located at the corneoscleral junction (B) is the transitional zone where the corneal and conjunctival epithelia merge, is shown in section using Haematoxylin and Eosin stain (C) and is considered a reservoir of stem cells which migrate centripetally to form the 5-7 cell layer corneal epithelium (DAPI fluorescence to highlight cell nuclei in corneal section, D).

surrounded by the white opaque sclera with the tissues meeting at the limbus. Maintenance of corneal integrity is imperative to light entry and refraction onto the correct position on the retina.

The anterior-most ocular surface is composed of corneal and conjunctival epithelia with the limbus at the transition zone between the two (Figure 1B and C). The corneal epithelium undergoes continuous renewal throughout life (Figure 1D). The central dogma of corneal homeostasis states that the mass of the epithelium remains constant so that the rate of cellular addition must equal that of cellular loss^[1]. The predominant theory for corneal homeostasis is the XYZ hypothesis proposed by Thoft *et al.*^[2] in 1983. This theory proposes that the limbus serves as a reservoir of ocular stem cells. Asymmetric division of these stem cells produces a stem-like daughter cell which remains within the limbus and a transient-amplifying cell (TAC) (Figure 2A) which migrates centripetally and anteriorly (Figure 2B). TACs undergo multiple rounds of replication and progressively lose “stemness” (Figure 2C) as they migrate anteriorly and progress to post-mitotic suprabasal wing cells, and then terminally differentiated superficial squamous cells (Figure 2D). The superficial cells are lost from the surface by normal exfoliation (squamation) or traumatic injury (Figure 1E). Therefore anterior migration from cells of the basal epithelium “X” and centripetal migration from the limbus “Y” equals desquamation from the surface “Z”. The entire human corneal epithelium is renewed in 9 to 12 mo^[3].

Whilst the research underpinning the limbus as the main reservoir for corneal epithelial stem cells has been consolidated with sophisticated cell tracking assays, an additional emerging view of the existence of stem cells outside of the limbus is supported by findings from several independent groups. This review analyses the data in support of limbal stem cells (LSCs) and looks at the possibility of a secondary reservoir of stem cells for the corneal epithelium.

LIMBAL EPITHELIAL STEM CELLS: HISTORICAL REVIEW

Studies reporting differences between central corneal and

limbal cells were published as early as the 1940s. These early studies showed increased frequency of mitoses in the basal layer of peripheral cornea using mitotic figure counts and radiated thymidine^[4,5]. Centripetal migration of cells expressing melanin pigment was observed in rabbit as well as human corneas, suggesting the limbus as a source of new cells^[6,7]. Since then, various studies have established the limbus as the location of corneal epithelial stem cells based on a set of unique properties observed within this cell population:

Slow cell turnover rate

DNA label-retention studies have shown the limbus contains cells in a growth-arrested or slow cycling state. Retention of radiated thymidine or 5-bromo-2'-deoxyuridine (BrdU) has been reported in limbal cells of mice cornea *in situ*^[8-10], human limbal explant cultures^[11] and whole cornea organ cultures^[12]. The retention of DNA label was observed for up to nine weeks in these studies. The labelling index, or the percentage of BrdU-retaining cells, was 1%-4% in mice corneas^[9,10,13], and approximately 4% in human limbal explant cultures^[11]. The nuclear label was lost progressively as the labelled cells moved towards the central cornea, indicating increased cell division during centripetal migration^[8].

Slow turnover rate in the limbus has also been demonstrated by resistance to 5-fluorouracil (an anti-metabolite which specifically targets proliferative cells)^[14], cytoplasmic staining for cyclins D, E and A (indicator of a growth-arrested state)^[15] and susceptibility to malignant transformation^[16-18]. The susceptibility to tumour formation is thought to be a property of stem cells as oncogenic mutations are more likely to accumulate in cells with long life span^[19].

Clonogenicity and proliferative potential

Life-long maintenance of any stratified epithelium necessitates a self-renewing pool of stem cells, asymmetric division of precursor cells and a rapid proliferative response upon injury^[20]. Studies have suggested that these attributes are unique to the limbal cell population.

Self-renewal capacity or clonogenicity of limbal cell populations has been shown by their ability to form

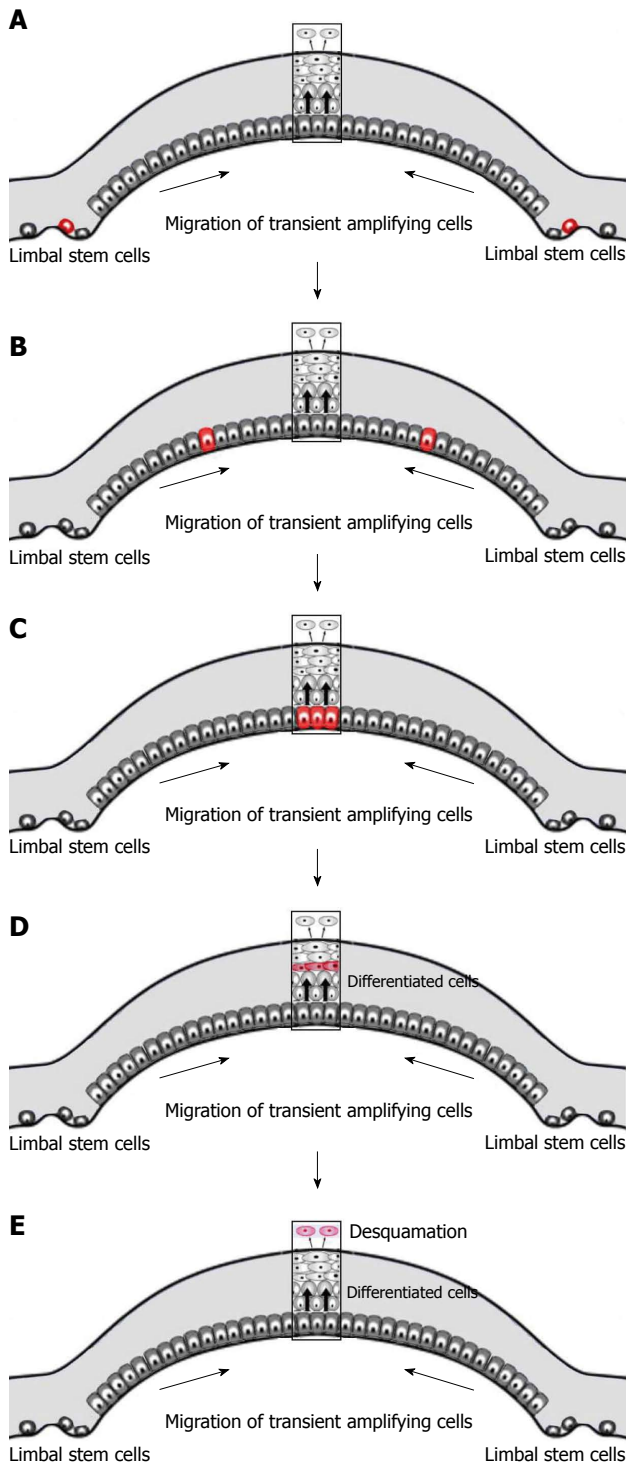


Figure 2 The X, Y, Z hypothesis of corneal maintenance. Limbal stem cells at the peripheral cornea divide and give rise to transient amplifying cells (TACs) (A). These TACs migrate centripetally through the basal epithelium (B) and undergo a limited number of divisions on the central cornea (C). The differentiated daughter cells move anteriorly to replenish the upper layers of the cornea (D) where they are eventually shed from the corneal surface (E). Hence the sum of X (proliferation and anterior migration) and Y (centripetal migration) must equal Z (desquamation of superficial cells) for corneal maintenance. Red cells: Continuum of transient amplifying migrating and/or differentiated cells.

sphere colonies on a 3T3 fibroblast feeder layer^[21]. These authors showed that the holoclone, meroclone and paraclone colony formation system previously identified in

human skin could be translated into spheres derived from human corneal biopsies. The single cell-derived sphere colonies from the limbus (equivalent to holoclones) were capable of undergoing 80 to 100 cell division cycles and could be propagated up to 14 passages before senescence. Single cell isolates from central cornea only formed paraclones (mostly consisting of terminally differentiated cells and capable of 15 cell divisions at maximum) and meroclones (intermediate form between holoclones and paraclones).

Asymmetric cell division has been suggested by uneven distribution of cell fate determinants across the corneal epithelium. Molecules implicated in asymmetric cell division and early cell fate decision, such as Musashi-1^[22], Notch-1^[23], p75^[24], C/EBP δ ^[25] and Δ Np63 α ^[26] have been almost exclusively localised in the mouse and human limbus.

Proliferative potential of limbal cells has been demonstrated by both *in vitro* and *in vivo* studies. Primary human limbal epithelial cell cultures showed high proliferative potential with a mean of 23 population doublings *in vitro*, while central corneal cells could not be propagated^[27]. Explant cultures of human limbal epithelium showed larger outgrowth and higher mitotic rate compared to explants from central epithelia^[28,29]. When transplanted into the flanks of athymic mice, single cell suspensions from limbal cell culture produced cysts which had more organised structure and longer life span than those derived from central corneal cell suspensions^[30]. Furthermore, *in vivo* animal studies have shown that the slow cycling limbal basal cells can rapidly divert to proliferative status upon damage to cornea^[8,13].

Cellular morphology

Morphological differences between limbal and corneal cells have been highlighted using a variety of imaging technologies including synchrotron infrared microspectroscopy^[31], morphometric analysis of DAPI-stained nuclei^[9], transmission electron microscopy^[32,33], *in vivo* confocal microscopy and flow cytometry^[34]. These studies commonly identified cuboidal cells 10 μ m in diameter with a high nucleus-to-cytoplasm ratio in the limbal basal layer. The sparse cytoplasm in these cells appears smooth due to the paucity of organelles and intracellular junctions, another indicator of low metabolic activity and protein turnover. In contrast, basal cells of the central epithelium are more columnar and have a lower nucleus-to-cytoplasm ratio^[31].

Biochemical characteristics

The identification of exclusive biochemical markers of corneal stem cells has been for many years a highly desirable endeavour. A number of putative stem cell markers have been suggested based on the biochemical transition that takes place in the basal cell layer of the corneo-limbal junction^[35-37]. Limbal basal cell layers preferentially express certain structural proteins (vimentin, cytokeratin 14, 15 and 19), cell adhesion molecules (integrin α 6, β 1,

$\beta 4$, P-cadherin and N-cadherin), enzymes (α -enolase, aldehyde dehydrogenase, cytochrome oxidase, Na^+/K^+ -ATPase and carbonic anhydrase), metallothionein, growth factor receptors (KGF-R and NGF-R), cell fate/cycle regulators (notch-1, Musashi-1, $\Delta\text{Np}63\alpha$, p75, Bmi-1 and C/EBP δ) and ABCG2, an ATP-binding cassette transporter protein. ABCG2 has been shown to be responsible for the efflux of the nuclear dye Hoechst 33342, enabling isolation of ABCG2-positive cells using flow cytometry^[38]. This dye efflux property is an established marker of a stem cell in many cell lineages including haematopoietic^[39], neuronal^[40], muscle^[41], and epithelium^[42]. The ABCG2 proteins are thought to protect LSCs from oxidative stress by transporting small regulatory molecules required for their proliferation, differentiation and apoptosis^[43]. ABCG2-positive cells are termed side population (SP) cells, and only a small proportion of limbal basal cells are SP cells. The SP cells have been shown to possess a number of stem cell properties including up-regulation in response to central corneal wounding^[44], small cells with high nucleus-to-cytoplasm ratio, slow cycling, expression of $\Delta\text{Np}63\alpha$ and ABCG2, absence of cytokeratin 3, 12 and involucrin, and increased colony-forming efficiency and growth capacity^[45,46].

As limbal basal cells migrate out of the limbus, their protein expression profile gradually changes. Central corneal epithelium is characterised by the loss of α -enolase and melanin pigmentation and the expression of cytokeratin 3 and 12, connexin 43 and 50, involucrin and *CLED*, a Ca^{2+} -linked protein associated with early epithelial differentiation. The expression of a large amount of metabolic enzymes and proteins in the central corneal cells is thought to contribute to the increase in cell size^[47]. Furthermore, increase in cell size has been correlated with loss of colony-forming efficiency^[48].

Centripetal migration

Centripetal migration of corneal epithelial cells is a well-documented phenomenon^[49,50]. Imaging studies have directly visualised centripetal migration of limbal cells towards the centre of the cornea. One of earliest studies used India ink to mark limbal cells which then migrated centripetally over the wounds of the mice cornea^[51,52]. Centripetal migration was observed in rabbit lamellar keratoplasty model where the host corneal epithelial cells invaded the grafted donor tissue^[53]. Similar results were obtained in the explants of human donor corneal buttons, where all donor corneal epithelial cells were replaced by recipient cells as early as three months post-penetrating keratoplasties^[54]. Both Collinson *et al.*^[55], and Nagasaki *et al.*^[56] used transgenic mice with reporter genes to visualise centripetal migration in normal mice cornea. Interestingly, Matsuda *et al.*^[57] and Srinivasan *et al.*^[58] found that wounds close to the limbus or repeated insult to the central epithelium accelerated the healing rate, the latter implying that rapidly dividing TACs of the periphery have moved to more central areas after the first trauma and respond more quickly to the second.

The chemotactic signal for centripetal migration may be provided in the form of cytokines and/or the difference between the composition of extracellular matrix between the limbus and the cornea^[59]. KGF, a paracrine hormone secreted by stromal cells, has been shown to enhance outgrowth in rabbit limbal explant culture on human amniotic membrane^[60]. While the inflammatory cytokine interleukin-6^[61], fibronectin^[62], and hyaluronan^[63], all of which are highly up-regulated upon injury, have been shown to play a role in drawing rabbit limbal cells towards the wound.

Recently, a very elegant study by Di Girolamo *et al.*^[64] has shown the centripetal movement of cells generated in the limbus using inducible multicolour tagging technology *in vivo*. Furthermore, this study linked the inducible multicolour tagging system with K14, one of the cytokeratin molecules that has been shown to mark an association with limbal stem cells. This study clearly showed that coloured K14 positive cells originated from the basal limbal epithelium and formed narrow corridors of epithelial cells that radiated centripetally onto the corneal surface. These authors do acknowledge that K14 is not an absolute limbal stem cell marker and that they could not exclude the existence of stem cells outside the limbal niche as K14 was targeted because of its limbal location.

Multipotency

Limbal basal cells characteristically lack differentiation markers indicating they are in an undifferentiated state. Several studies however, have implied a high multipotent differentiation potential when appropriate combinations of cellular signalling molecules are encountered: Rabbit limbal epithelial cell sheets transformed into fibroblasts when transplanted onto limbal stroma^[65]; during the culture of human limbal explants, the limbal epithelial cells which invaded into the stroma underwent epithelial-mesenchymal transition^[66]; mouse limbal epithelial cells expressed opsin when transplanted onto mice retina, indicating their potential to differentiate into rod photoreceptors^[67]; and the potential to transdifferentiate to neuronal cells was demonstrated by Zhao *et al.*^[68]. In their study, rat limbal cell isolates maintained in growth factor-driven culture system expressed neuronal progenitors, β -tubulin, nestin and neurofilament. When subject to serum-containing differentiation medium, the limbal cell isolates expressed glial markers such as GFAP and O4. The limbus-derived neuron-like cells not only expressed neuronal markers and neurotransmitter receptors, but also exhibited electrical responses to GABA and kainic acid^[69].

Stem cell niche

A stem cell niche is an anatomically defined area that is thought to provide a variety of intrinsic and extrinsic factors such as the physical protection, survival factors and cytokines and deemed essential to the maintenance of a stem cell population while preventing entry into differentiation^[70,71]. Over the past decade, much progress

has been made in characterising the putative niche in the limbus. The limbal areas are rich in melanin pigments, highly innervated, well-vascularised and have a different array of extracellular matrix components than the central epithelium. Melanocytes or melanin granules within the cytoplasm of progenitor cells are thought to play a role in protection against ultraviolet radiation^[8,72]. Blood-derived growth factors and nutrients provide for the active cell division^[8,73].

The epithelial-stromal interface in the limbus differs from that in the central cornea. Bowman's layer, a densely interwoven collagen sheet lying between the basement membrane of the central corneal epithelium and the stroma, is absent in the limbus^[74]. In the limbus, stroma directly underlies the epithelial basement membrane. The limbal epithelial basement membrane also differs from that of central cornea in its composition^[75-80]. The limbal basement membrane labelled positive for type IV collagen $\alpha 1$ chain, laminin $\alpha 2$, $\beta 1$, $\beta 2$, $\gamma 1$, $\gamma 3$ chains, nidogen, agrin, BM40/SPARC, tenascin-C and thrombospondin-4, whereas central cornea showed positive immunoreactivity to type IV collagen $\alpha 3$ chain, type V collagen, thrombospondin-1 and endostatin. Limbal-specific basement membrane components were co-localised with putative stem cell markers such as ABCG2, p63 and cytokeratin 19, but not with differentiation markers including cytokeratin 3, connexin 43, desmoglein and integrin $\alpha 2$. In addition, the cornea-limbal transitional zone showed strong immunostaining to type XVI collagen, fibrillin-2, tenascin-C/R, vitronectin, bamacan, chondroitin sulfate and versican, and were co-localised with vimentin-positive cell clusters.

To date, four anatomic structures have been proposed as the corneal stem cell niche; Palisades of Vogt, limbal epithelial crypts, limbal crypts and focal stromal projections. The Palisades of Vogt are ridges of epithelium in the limbus that extend centripetally from the bulbar conjunctiva, and are easily visible by slit lamp microscopy, especially in young donors or those with dark skin^[7,81,82]. More recently, Shanmuganathan *et al.*^[83] and Dua *et al.*^[84] identified limbal epithelial crypts located at the interpalisade epithelial rete ridges of the Palisades of Vogt. The limbal epithelial crypts radiate either peripherally into conjunctival stroma or circumferentially into limbal stroma. Shortt *et al.*^[85] proposed two additional niches using *in vivo* confocal microscopy; limbal crypts which are projections of limbal epithelium from the peripheral cornea into the limbal stroma, and focal stromal projections which are finger-like projections of limbal stroma with central blood vessels extending upward into the epithelium. These papillary structures offer physical protection for the deeply seated cells from injuries and shearing forces, and a large surface area that can accommodate increased cell numbers, blood vessels, and other supportive cells such as melanocytes, macrophages and stromal cells. Limbal crypts and focal stromal projections predominantly occur within regions of the cornea normally covered by the eyelids, which is a potential protec-

tive mechanism of these proposed niches^[85]. Some of the putative stem cell features such as expression of ABCG2, p63 and p75, and high nucleus-to-cytoplasm ratio have been identified in the limbal basal cells lining these papillary structures^[24,77]. In patients with limbal stem cell deficiency (LSCD), these four proposed niche structures are absent^[84,85].

Recent studies have identified stromal stem cells which are directly subjacent to limbal basal cells^[86,87]. An arising view of the limbal niche environment is that the limbal basal cells, stromal stem cells and the extracellular matrix molecules function as one unit to maintain the reservoir of ocular stem cells^[88-90]. Human limbal epithelial cells co-cultured with stromal stem cells produced colonies with average diameter five times as large as those obtained with murine 3T3 feeder layer, indicating enhanced proliferation of limbal cells in the presence of stromal stem cells^[91]. Recently, it was shown that limbal epithelial cells actively merge with stromal cells *via* chemokine receptor-mediated signalling in sphere-forming conditions, and this interaction seemed crucial for the maintenance of stem cell phenotype^[92].

Limbal stem cell deficiency

The ability of limbal cells to regenerate corneal epithelium is robust evidence for the existence of stem cells in the limbus. Limbal stem cell deficiency (LSCD) is a complex corneal disorder resulting from functional and/or anatomical loss of limbus due to chemical or thermal burn, radiation, genetic/autoimmune disorders, multiple surgeries, contact lens use, infection or drug use^[93,94]. Signs and symptoms of LSCD include conjunctivalisation, corneal vascularisation, pain, tear, redness, oedema, poor vision and blindness, which are thought to be associated with failure of epithelial regeneration^[95,96]. Similar symptoms and a delayed wound healing response could be reproduced in rabbits by surgically removing the limbus^[95,97]. The degree of loss of limbal tissue has been shown to correlate with the severity of pathology^[98]. Clinical studies have shown that LSCD can be successfully treated with application of limbal cells^[99-102]. Currently the sources of limbal cells are limbal autograft for unilateral LSCD, allogenic limbal graft from living related or cadaveric donors and *ex vivo* expanded limbal cells on transplantable substrate^[93]. The overall success rate of limbal cell transplant is estimated at 76%, ranging from 50% to 100%^[103]. The success rate varies between studies because outcome parameters, *ex vivo* expansion protocol, length of follow-up and aetiology of LSCD are different in each study^[103]. Standard corneal transplants do not appear to provide a cure for patients with LSCD^[104].

LIMBAL STEM CELL CONUNDRUMS

The body of evidence for the presence of stem cells at the limbus is impressive and convincing if largely circumstantial. The final piece of the jigsaw that remains to be revealed is the identification of an absolute stem cell

marker that is definitive of stem cell functionality. Likewise the body of evidence of the origin of epithelial cells at the limbus and their contribution to corneal epithelial homeostasis through the centripetal movement over the corneal surface has been elegantly shown by several research groups in several mammalian systems both *in vitro* and *in vivo*. However, despite this body of evidence, the proof that stem cells of the corneal epithelium reside only at the limbus and nowhere else is lacking and several pieces of knowledge remain unexplained by our current understanding of corneal maintenance by limbal stem cells:

Specificity of putative LSC indicators, criteria and markers

The traditional defining features of stem cells of the corneal epithelium include slow turnover rate, clonogenicity, proliferative potential, characteristic morphology, expression of certain proteins, centripetal migration *in vivo*, multipotency, specialised niche structures and ability to regenerate corneal epithelium. Despite the obvious biochemical changes at the cornea-limbal junction, selection of a consensus LSC marker has not been straightforward because each of these candidate markers has limitations resulting in inevitable ambiguities in separating stem cells from early progenitors^[33,105]. In fact, there is mounting evidence showing that some of the putative markers of LSCs are not unique to the limbal basal cells.

Slow turnover rate has been demonstrated by label retaining studies in animal models. However, there are several pitfalls related to the use of label retention as a marker of stem cells^[106]. The duration of the DNA labelling period was typically less than one week in most label retaining studies^[8-11]. Cells quiescent during the labelling period will not take up DNA label and never be identified by this method. On the other hand, cells that have undergone a few rounds of cell division may still show DNA label albeit at a weaker level. Furthermore, label retention is not an essential property of stem cells as stem cells such as those underlying mammalian intestinal mucosa have short cycle time^[107]. Not all label retaining cells are stem cells and vice versa.

The slow cycling property of the limbal cells has also been inferred from their resistance to 5-fluorouracil and predisposition to cancer. However, cells resistant to 5-fluorouracil are also found in the central epithelium although smaller in number than in the limbus^[14]. Predisposition to cancer is also common in cells at the transitional zone where two types of epithelia unite in non-ocular tissue systems. The endo-ectocervical and oesophagus-stomach junctions are such examples.

Clonogenicity and asymmetric division are not unique properties of the limbal cells. Central corneal cells isolated from various mammalian species including humans have been shown to form clonogenic spheres *in vitro* although the number of spheres formed was smaller than when limbal cells isolates were used^[108,109].

Asymmetric division as a means of self-renewal of

stem cells is a widely accepted concept, but is difficult to show in experimental settings, and therefore it is as yet largely hypothetical due to a lack of compelling evidence. Recent evidence suggests mitotic spindle orientation and direction of asymmetric division are under the influence of specific environmental cues from the limbus rather than intrinsic polarity^[110,111]. Possible environmental cues include growth factors, adhesion molecules and components of basement membrane that are specifically found in the limbus^[112].

In terms of morphological criteria for LSCs, different groups have reported contradictory results. The amount of melanin granules^[8,32,33], prominence of nucleoli and basal membrane invaginations^[9,32,33,73] appear to vary from study to study. The reason for this contradiction is unknown but the lack of clear morphological distinction between stem cells and TACs could be responsible. As yet, TACs cannot be distinguished from true stem cells based on cellular morphology alone.

The expression of the protein markers of the LSCs either occurs in other cell types of the ocular surface, or is subject to change depending on environmental input. Cytokeratin 19, a well-established marker of limbal basal cells is also expressed in conjunctival epithelial cells^[113]. Δ Np63 α was identified in the corneal panni excised from patients with LSCD using western blot^[114]. The free-floating spheres generated from human central corneal cells expressed Δ Np63 α and ABCG2^[109]. ABCG2 was found to be weakly expressed in the central cornea with what appeared to be an increasing gradient of expression towards peripheral cornea and finally the limbus^[109,115].

Furthermore, the link between limbal location and stem cell indicators is further compounded as several studies have indicated that the components of the niche influence the expression of LSC markers. Espana *et al*^[116] transplanted rabbit central corneal or limbal epithelial sheets onto either limbal or corneal stroma, and investigated the expression profile of two differentiation markers, cytokeratin 3 and connexin 43. Regardless of the type of epithelium transplanted, corneal stroma promoted expression of cytokeratin 3 while limbal stroma suppressed it. Expression of connexin 43 and apoptosis only occurred when corneal epithelium was cultured on corneal stroma. Li *et al*^[87] showed that when human limbal epithelial cells were co-cultured with stromal stem cells, p63 α was up-regulated and cytokeratin 12 down-regulated. The opposite expression pattern was observed when corneal fibroblasts were used instead of stromal stem cells. Kurpakus *et al*^[117] showed that bovine conjunctival cells on corneal substrate expressed the differentiation marker cytokeratin 12 only when the basement membrane was left attached to the substrate, suggesting corneal basement membrane may encourage differentiation.

Since there is not one consensus marker for LSCs, a combination of functional, morphological and immunohistochemical markers is perhaps the most useful identifier for LSCs at present. To date, the “SP” property is the only marker that has been aligned with functionality.

ABCG2-positive cells in the limbus exhibited proliferative capacity, label retention and clonogenicity. However, heterogeneity exists even within the limbal SP cells as suggested by the lack of intracellular complexities in 60% to 80% of limbal SP cells^[47].

At the time of writing this article, a newly published study in *Nature* has defined a new gene, ABCB5, as a novel limbal stem cell marker^[118]. The authors have shown ABCB5 positive cells were predominantly BrdU label retaining cells from the limbus and co-localised with Δ Np63 α in both mice and humans. Furthermore, the authors showed that ABCB5 positive cell numbers were reduced in LSC deficient patients and that ABCB5 positive cells isolated from mouse and human corneas had the ability to rescue the cornea in LSC deficient mice in both syngeneic and xenogeneic transplant models. Finally, the paper demonstrated that ABCB5 knockout mice showed disorganised corneal epithelial organisation and reduced wound healing capabilities, although bizarrely the knockout mouse was indistinguishable from wild type littermates by physical examination and contained all anterior and posterior segment components.

This appears to be the first description of a molecular limbal marker with stem cell functionality, and may be the missing jigsaw piece required to define limbal stem cells beyond doubt.

Limbus-independent corneal maintenance

A number of independent studies have challenged the long held belief that the limbus is the sole repository of stem cells in the corneal epithelium. These studies show that wound healing and normal corneal homeostasis can take place in the absence of limbus.

In 1994, Sandvig *et al.*^[119] showed that small lesions made in the rat central corneas did not evoke proliferative responses in the limbus, while medium-sized and large lesions did. This suggests wound healing of small lesions does not require limbal input. Our laboratory developed a “donut” excimer laser ablation model to demonstrate that human corneal epithelial regrowth occurs bi-directionally from both central and peripheral cornea^[115]. In our model, the cell proliferation and migration response to wounding appeared to be as rapid from the central cornea as from the limbus, with central corneal epithelial cells fully capable of corneal epithelial regeneration. When the limbus was also ablated to remove any LSCs, re-growth occurred from the remaining central corneal epithelium and extended right out to the limbus.

Corneal maintenance without limbal input has also been observed by several other researchers. Huang *et al.*^[97] created a rabbit LSCD model by performing 360° cornea-limbal peritomy. After six months, two thirds of the corneas were completely normal while one third showed mild vascularisation. Kawakita *et al.*^[120] blocked communication and migration between the limbus and the cornea by transplanting a stainless steel ring on rabbit peripheral corneas. In their study, the isolated central corneas remained free of epithelial defects for at least

six months. In a mouse LSCD model where the limbus was cauterised, the corneas remained transparent for four months^[108]. In this study, portions of athymic mice limbus were excised and replaced with limbal grafts from β -gal-ROSA26 mice whose cells were β -galactosidase labelled. After four months they observed that β -galactosidase-labelled limbal cells never migrated out of the grafts and hence made no contribution to corneal homeostasis. However, when the eyes with limbal transplants were chemically or physically wounded, the labelled cells rapidly migrated out of the graft, along with unlabelled recipient limbal cells, to create a mosaic in the resulting healed corneal epithelium.

One criticism that these studies commonly face is that their observations may be due to the result of a TAC response as the periods of observation were rather short. If stem cells do exist in the central cornea, one would expect to see long-term corneal maintenance in the animal LSCD models.

Indeed, long-term corneal maintenance in the absence of limbal input has been described in a few case reports. Some patients who had 360° LSCD were found to have normal corneas for up to 12 years^[121]. Also in LSCD patients who received *ex vivo* expanded limbal cell transplants, donor limbal cells that only lasted for 28 wk^[122] or 9 mo^[123] still resulted in the long-term restoration of the central corneal epithelium. What is maintaining the central cornea in these cases? Assuming desquamation of superficial cell layer occurs constantly, there are a few possible scenarios; (1) the amount of limbal stem cells remaining is undetectable but just enough to maintain homeostasis; (2) TACs in the basal cell layer of the central epithelium have an unexpected life span and a greater than previously thought proliferative potential; or (3) a self-renewing pool of precursor cells exist in the central cornea. Two independent groups have proposed the existence of a conceptual type of cell in the central corneal epithelium which is a TAC with more stem cell-like characteristics^[121,124]. Further research efforts are required to explore and clarify these possibilities although a TAC cell with more stem cell-like characteristics sounds uncommonly similar to a stem cell. Thus the question arises - is there a different type of stem cell that exists on the corneal surface that may be activated by different mechanisms, may serve different purposes and may be defined by different markers than the limbal stem cells?

Ex vivo expansion of LSCs on amniotic membrane

A further strong argument against the existence of stem cells in the central cornea is the absence of anatomic niche structure in the central cornea to maintain stemness. However, there is evidence for survival and self-maintenance of LSCs outside of the described limbal niches.

The most frequently used substrate for limbal stem cell expansion is human amniotic membrane, the innermost wall of the placenta consisting of an epithelial monolayer, basement membrane and avascular stroma^[125].

Isolated limbal cells, when cultivated on amniotic membrane, formed stratified epithelium much resembling cornea *in situ* and exhibited limbal stem cell phenotype such as increased expression of Δ Np63, p75, p63, ABCG2, integrin β 1, Pax6, cytokeratin 3 and 19, decreased expression of connexin 43, increased resistance to phorbol ester-induced differentiation^[126], label retention and clonogenicity^[127]. Paulkin *et al.*^[128] analysed corneal buttons from LSCD patients who had previously received limbal cell transplants on amniotic membrane. The regenerated epithelial specimens had normal stratified structures and expressed central corneal markers cytokeratin 3 and 12 but not 19. These techniques provide evidence that limbal stem cells can survive, proliferate and expand outside of their niche which has been previously thought to be necessary for LSC maintenance.

It is not fully understood how an avascular structure like amniotic membrane can maintain the phenotype and metabolic needs of the LSCs^[36,129]. The amniotic basement membrane is thought to promote adhesion, migration and differentiation of limbal epithelial cells, while amniotic stroma provide growth factors and anti-angiogenic and anti-inflammatory cytokines such as KGF, HGF, NGF, TGF- β and bFGF that prevent apoptosis and help maintain the stem cell phenotype.

Cytokine signalling is becoming increasingly recognised as a key component of a niche, regulating stem cell morphology and behaviour^[130]. The Wnt/ β -catenin signalling system has been shown to be responsible for preventing apoptosis of limbal cells *in vitro*^[131]. The authors suggested that as long as survival factors are present, limbal stem cells are likely to survive outside their niche. Indeed, in a mouse model, LSCD was successfully treated with human limbal fibroblast-conditioned culture medium but not with skin fibroblast-conditioned medium, again emphasising the importance of chemical signals produced in the limbus^[132].

There are studies which question the longevity of *ex vivo* expanded limbal epithelial cells. Li *et al.*^[66] showed progressive loss of clonogenicity and proliferative potential of limbal explant cultures on intact amniotic membrane in subsequent passages. The reason for this contradictory result is unknown but slight differences in expansion protocol and donor tissue variability might be responsible.

Furthermore, one study has proposed the existence of compound niches of cells that exist in the limbus of the mouse in unwounded corneas^[133]. However, after wounding these compound niches were able to migrate onto the surface of the cornea and express corneal epithelial cytokeratins while also retaining both features of the compound niche and features of goblet cells. This study serves to illustrate that a niche may not be an immovable structure to which cells attach but may be inherent to the cellular components and therefore able to migrate with those components.

Developmental origin of limbus

Epithelia of skin, gut wall and cornea are outer most coverings of our body and share the same developmental

origin. In all types of epithelia, with the exception of cornea, desquamated cells are replaced with newly generated cells from stem cells located in the basal layer^[8]. Only corneal epithelium is thought to be renewed from a distant repository of stem cells. This is somewhat peculiar in evolutionary sense especially when the directly adjacent conjunctiva is maintained in the same way as any other epithelia^[134].

In fetal eyes, adult LSC markers are found in the basal layer across the cornea^[135,136] and it is unknown how the markers become segregated in the limbus during development. Investigation of limbal organogenesis has raised a possibility that the limbal papillary structures are mere developmental remnants. The limbus does not develop until eyelids open and the ocular surface is exposed to amniotic fluid^[135,136]. The papillary structures of the limbus do not form until post-natal life^[137]. The question remains as to why a microenvironment essential for the support of stem cell maintenance only appears after birth and why stem cells can be maintained on the central cornea prior to birth.

CONCLUSION

A strong body of evidence has accumulated over the past few decades, showing that markers of stemness are exclusively localised at the limbus. Furthermore the centripetal migration of corneal epithelial cells after generation at the limbus has been definitively shown. Therefore, the limbus has been designated as the single repository of stem cells of the corneal epithelium. However, there is mounting evidence showing that the expression of the stem cell markers are largely determined by extrinsic signals provided by the regional microenvironment^[130,138], and the markers themselves do not indicate intrinsic stemness. As shown by the clinical success of LSC transplant on amniotic membrane in LSCD, a niche structure is not an absolute requirement for the survival of ocular stem cells, as long as the right survival signals are provided. The existence of the limbus as the sole repository of corneal epithelial stem cells also does not explain a number of clinical observations which have demonstrated corneal wound healing without limbal input and also does not explain the developmental origin of the limbus.

A vast majority of studies consider central cornea as a lineage-committed, post-mitotic tissue, but some groups have independently suggested a possibility that stem cells exist outside the limbus. Until more definitive data becomes available, the possibility of the existence of progenitor cells outside the limbus should not be excluded as central cornea may provide a new source of stem cells that can serve as a sustainable repository of high quality, evaluated, optimised tissue for the treatment of corneal degenerative disorders.

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Renal stem cell reprogramming: Prospects in regenerative medicine

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Abstract

Stem cell therapy is a promising future enterprise for renal replacement in patients with acute and chronic kidney disease, conditions which affect millions worldwide and currently require patients to undergo lifelong medical treatments through dialysis and/or organ transplant. Reprogramming differentiated renal cells harvested from the patient back into a pluripotent state would decrease the risk of tissue rejection and provide a virtually unlimited supply of cells for regenerative medicine treatments, making it an exciting area of current research in nephrology. Among the major hurdles that need to be overcome before stem cell therapy for the kidney can be applied in a clinical setting are ensuring the fidelity and relative safety of the reprogrammed cells, as well as achieving feasible efficiency in the reprogramming processes that are utilized. Further, improved knowledge about the genetic control of renal lineage development is vital to identifying predictable and efficient reprogramming approaches, such as the expression of key modulators or the regulation of gene

activity through small molecule mimetics. Here, we discuss several recent advances in induced pluripotent stem cell technologies. We also explore strategies that have been successful in renal progenitor generation, and explore what these methods might mean for the development of cell-based regenerative therapies for kidney disease.

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Key words: Kidney; Regeneration; Induced pluripotent stem cell; Reprogramming; Differentiation; Stem cell; Renal progenitor

Core tip: The identification of regenerative therapies to treat kidney disease is an exciting but challenging area of ongoing scientific investigation. Cellular reprogramming may provide a tractable means to replace damaged renal tissue, and current researchers have pursued a number of innovative ways to produce renal cell types. Here we explore the issues confronting several reprogramming technologies, recent advances in reprogramming renal cells, and discuss areas of future scrutiny that are needed to help develop cell-based therapies for various kidney disease conditions.

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INTRODUCTION: KIDNEY DISEASES AND THE NOTION OF THERAPEUTIC USES OF INDUCED PLURIPOTENT CELLS FOR RENAL REPLACEMENT THERAPY

Kidney organs perform essential physiological roles in

excretion and homeostasis^[1]. Kidney diseases can arise during development, juvenile, or adult life. Types of renal disease include acute kidney injury (AKI), which is the abrupt loss of renal function that can often become permanent, and chronic kidney disease (CKD), the progressive loss of renal function that culminates in organ failure known as end stage renal disease (ESRD)^[1,2]. The need for new treatments for kidney disease, the 8th leading cause of death in the United States^[3], is a growing concern in the medical field. For example, there are approximately 31 million people in the United States diagnosed with CKD^[4]. Unfortunately, kidney diseases are a global health problem as well, and have continued to increase in incidence in correlation with the rise in aged populations and escalation in conditions like diabetes that often negatively impact renal health^[5-8]. At present, kidney disease treatments deal with symptom management through the renal replacement therapies of dialysis or organ transplant. However, formulating therapies that repair kidney structure and restore compromised functionality is of the utmost importance considering the limited numbers of viable kidneys available for transplant, as well as the complications that can arise in organ recipients^[9-13]. To identify innovative ways to combat kidney diseases, numerous research groups have focused their energies on the identification of adult renal stem cells^[14-17]. However, this has remained a controversial topic despite the multitude of studies performed to date^[14-17]. In addition to the search for endogenous renal stem cells, the study of renal lineage specification during kidney organogenesis has been pursued—knowledge which can be applied toward the development of cell-based therapies for the purpose of kidney regeneration that would not necessitate the employment of adult stem cells.

One such cell-based alternative is the use of induced pluripotent stem cells (iPS) derived from the patient's own tissue. iPS cells can be used to study development and cell differentiation without the need for embryonic stem (ES) cell lines, whose cell source carries with it a surplus of ethical concerns, and can provide a resource to help researchers with disease modeling and drug development^[9]. Using iPS cells from the patient's renal tissue can serve to circumvent the need for a kidney transplant and avoid the use of lifelong immunosuppressant drug treatments. Thus, the notion of iPS-based regenerative medicine has many appealing benefits if the paramount challenges associated with realization of such cell-based therapies can be overcome. Utilizing integrating viral vectors containing the “Yamanaka factors” to reprogram cells has shown substantial success in generating iPS cells (approximately 0.1%), but the fact that these viral vectors integrate into the genome (sometimes in large copy number) has been a serious cause for debate as to their toxicity and their relative capability to be used in a clinical setting. Researchers have also investigated other avenues such as the use of non-integrating vectors so as to make the iPS cells safer to use in cell therapies, but with limited success, as evidenced by the very low induction rates and relative efficiency of the reprogramming method

(approximately 0.001%). Making safer and more controllable iPS cells is an integral part of developing cell-based therapies for the treatment of diseases and injuries. For example, Abad *et al.*^[18] shows evidence of how uncontrolled reprogramming can affect the body in the form of teratomas developing in multiple organs of transgenic mice transiently expressing the four “Yamanaka factors”. Other alternatives to the use of reprogramming factors are also being investigated, such as the use of microRNAs (miRNAs) to generate iPS cells. This method shows much promise, even though the cells' behavior *in vivo* still has to be controlled (approximately 10% efficiency reported in previous studies)^[19].

For the purposes of treating kidney disease, researchers have been assessing different ways of obtaining renal progenitor cells, and one such way involves partial reprogramming of differentiated renal cells into a renal progenitor state. Experimental evidence has supported the notion that the more closely related the start and end cells types are, the more efficient the reprogramming process will be. Although the method proved to be better than most at producing reprogrammed cells (approximately 0.875%)^[20], the overall amount of progenitors produced is still not cost-effective enough to be of applicable merit for therapeutic purposes. Another drawback to this partial reprogramming method is the thorough screening process that has to be applied in order to find the adequate combination of genes that will successfully reprogram the kidney cells into a progenitor-like state, which would be both time-consuming and costly. A method of obtaining renal progenitors that has received significant attention is the directed differentiation of iPS cells. Typically done with growth factors (which are rather expensive), exciting recent reports have now suggested that certain low-cost chemical compounds can be used to achieve the same goal of directing iPS cells towards a specific renal cell lineage with an approximate 90% conversion rate in one week. Although still dependent on the production of iPS cells, directed differentiation into renal progenitors is still a promising method that can be applied in tandem with a more optimized, efficient, and safer reprogramming protocols. In the following sections we further discuss these and other recent advances, as well as their general impact in the medical field.

REPROGRAMMING METHODS: REVERSE ENGINEERING TO OBTAIN STEM AND OTHER PROGENITOR CELLS FROM DIFFERENTIATED CELLS

Current therapies directed towards the treatment of kidney disease focus on symptom management instead of treating and hopefully curing the overall condition, and because of this researchers are working on alternatives that may now aid in the restoration of normal kidney function. As aforementioned, one alternative to current methods is the use of reprogrammed cell-based therapies

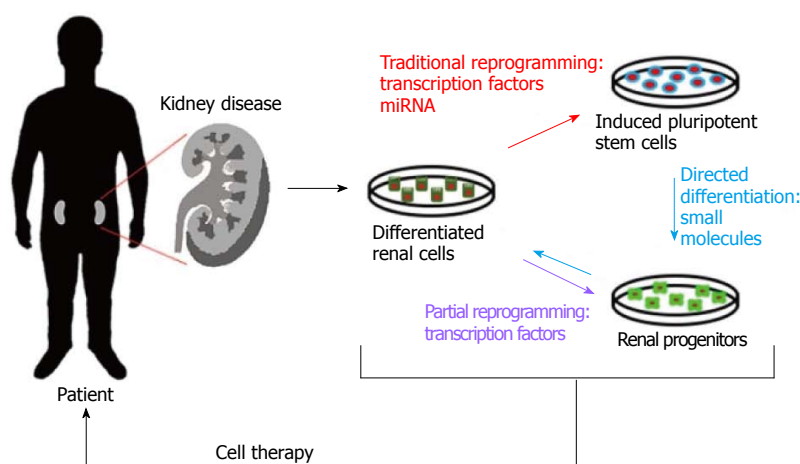


Figure 1 Renal cell reprogramming methods. (Red) Traditional reprogramming involving the use of transcription factors or miRNAs to generate pluripotent stem cells; (Purple) partial reprogramming with transcription factors to obtain multipotent progenitors; (Blue) directed differentiation into cells with a specific phenotype by treating induced pluripotent stem cells with small molecules. These newly reprogrammed/differentiated cells can then be used therapeutically to replace lost cell types within the injured kidney.

in order to restore damaged or diseased kidneys. Two of the most prominent reprogramming strategies currently being used involve either the conversion of different sources of stem cells into renal progenitors, or the reprogramming of differentiated renal cell populations into a more pluripotent state (Figure 1).

Traditional cell reprogramming involves the overexpression of developmental genes in differentiated adult cells in order to induce an earlier developmental and pluripotent phenotype. The typical factors that are overexpressed for cell reprogramming, discovered by Takahashi *et al.*^[22] and Yamanaka *et al.*^[22] back in 2006, are OCT4, SOX2, c-MYC, and KLF4 (now deemed “Yamanaka factors”), these factors are typically transfected into cells through the use of lentiviral vectors, which insert these exogenous genes into the host genome. At first, a cocktail of four viral vectors, each one containing one of the previously mentioned “Yamanaka factors” was introduced into the cell in order to promote a change in cell phenotype. However, these techniques lacked efficiency due to many non-specific genomic integrations, as well as the heterogeneous population that resulted from the process (some cells were only partially reprogrammed because not all of the vectors integrated)^[21,22]. In terms of kidney disease, producing iPS cells from cells of renal origin would contribute greatly to the development of cell therapies and treatments as they would be predicted to integrate more readily into the diseased kidney due to their conserved epigenetic memory^[23].

Interestingly, Zhou *et al.*^[24,25] were able to generate iPS cells from human exfoliated renal epithelial cells found in urine, something that can be collected without the need for surgical intervention, which would help in the development of therapies for kidney disease due to their epigenetic memory of renal origin. Using a cocktail of four different retroviruses containing the four “Yamanaka factors” they were able to create iPS cells (in about 16-25 d after transduction) from the previously mentioned cell source (cultured for about a week from 13 different test subjects, which means that the complete protocol would last about a month to produce iPS cells) with varying degrees of reprogramming efficiency (0.01%-4.0%) and

ability to differentiate, something that is to be expected when you use multiple integration vectors, mainly because the researcher cannot assess if the cells have incorporated all of the four reprogramming factors, or if the integrated vector copy number is low enough for the reprogrammed cells to be viable for therapeutic purposes (something that was not investigated in this study). Although a reprogramming efficiency of 4.0% is relatively high compared to other studies, the fact that there is great variation between the iPS cells produced (evidenced by the varying degrees of reprogramming efficiencies between the different donors, and that not a lot of the iPS cells produced could differentiate into other cells types) greatly diminishes their clinical applications, and provides further evidence that utilizing multiple integration vectors to reprogram cells is not an effective method for producing iPS cells for therapeutic purposes.

Researchers have tried to address this issue by trying create a better method of reprogramming that could decrease the number of genomic integrations, and assure that all of the factors necessary for reprogramming are being expressed within the cell. Sommer *et al.*^[26] managed to do just this by creating a single lentiviral vector containing all of the “Yamanaka factors” which is able to convert mouse postnatal fibroblasts into iPS cells. Not only were they able to ensure that all four transcription factors were integrated into the cell’s genome, but they were able to reduce the amount of genomic integrations to a mean of about 1.5-2.8 proviral copies^[26]. Compared to the multiple vector approach, this single vector method has an increased efficiency of 0.5%, which is about 50 times more efficient (relative efficiency of the multiple vector method is about 0.01%-0.05%), but this number might vary between cell types due to the many unique properties found in the various tissues throughout the body. Fortunately iPS cells have already been created from cell sources from cells of distinct embryonic origins (endoderm, mesoderm, ectoderm). This will benefit the development of regeneration cell therapies as iPS cells derived from the affected or injured organ will work more effectively in cell therapies that intend to regenerate the particular organ of interest, because of the genome-

wide epigenetic memory of the differentiated adult cell that is to be reprogrammed^[27].

Working under the previously stated premise (specifically in the case of renal disease), Wang *et al.*^[23] were able to generate iPS cells from mouse renal tubular epithelial cells (RTECs) using a single lentiviral vector containing the previously mentioned “Yamanaka factors” in about 21 d. The cells produced in this study were relatively indistinguishable from mouse ES cells, as confirmed by morphological, immunocytochemical, genetic expression, and karyotype analysis^[23]. Not only did these cells adopt an ES-like morphology and were able to express undifferentiated ES cell markers such as fibroblast growth factor 4 (FGF-4) and NANOG, but they were also able to differentiate into cell types of all three germ layers, as evidenced by the presence of AFP, desmin, and nestin (endoderm, mesoderm, and ectoderm markers respectively) in embryoid bodies formed from said cells^[23]. The cells also exhibited a normal 40XY karyotype and once reprogrammed, the viral transgenes were largely silenced which is necessary if there is any chance of applying this method in clinical applications, mainly to avoid problems during differentiation that might result in tumor development; the relative efficiency of this method, however, leaves something to be desired (0.1%)^[23].

Another reprogramming strategy that researchers have pursued is partial reprogramming of cells into a more multipotent phenotype that can produce cell lineages of a specific organ structure, which in the case of Hendry *et al.*^[20] would be embryonic nephron progenitors (NPs). The efficiency of the reprogramming process is correlated to the lineage relationship between the start and end cell types, in other words, the more closely related the start and end cells types are, the more efficient the reprogramming process^[20]. Hendry and colleagues investigated this premise by trying to generate NPs from HK2 cells line (human kidney cell line; adult proximal tubule cells)^[20]. Through combinatorial screening of 15 different transcription factors associated with the specification of the nephron progenitor phenotype they were able to identify 6 (*SIX1*, *SIX2*, *OSR1*, *EYA1*, *HOXA11*, *SNAIL2*) genes that would recapitulate the network of genes associated with the cap mesenchyme (CM)^[20]. Each factor was packed into individual lentiviral constructs accompanied by green fluorescent protein (GFP) to identify successfully infected cells, and successful reprogramming events were defined by significant morphological changes as well as robust expression of *SIX2* and Cbp/P300-interacting transactivator 1 (CITED1) protein, CM-specific markers^[20]. Reprogrammed cells showed upregulation of *Matrix metalloproteinase 9* and *2* (*MMP9* and *MMP2*), epithelial-to-mesenchymal transition (EMT) markers, as well as repressed expression of epithelial cadherin (*E-CADHERIN*), which suggests the occurrence of an EMT event within these cells^[20].

Further evidence of the cells’ conversion into nephron progenitors can be seen in a recombination assay that was developed to test the induced NPs’ potential in *ex*

vivo organoid cultures^[28,29]. They found that the induced progenitors were able to integrate with the endogenous NP field, and failed to integrate into the uretic bud compartments (a cellular population that the CM does not make). The overall efficiency of this partial reprogramming process is about 0.875%, which is substantially better than many of the techniques discussed so far (most likely due to the close relation between adult proximal tubule cells and NPs), however, the use of multiple lentiviral constructs makes the use of these cells quite toxic; therefore integrating all of these factors into a single construct might increase the efficiency of the reprogramming quite drastically, as well as their potential for use in therapies.

TANGENTIAL AND NON-INTEGRATION METHODS OF REPROGRAMMING

The use of integrating viral vectors has become quite widespread in the field of cell reprogramming, but because of various concerns that have arisen during their use (interruption of the cell’s genome and/or the risk spontaneous reactivation of the viral genome that might lead to tumor formation) researchers are actively looking for different alternatives so as to decrease the risk of using reprogrammed cells in the treatments of diseases such as end-stage renal disease (ESRD). Nightingale *et al.*^[30] (2006) were able to produce a non-integrating lentiviral vector that was able to transiently express GFP in about 90% of cultured human T lymphoid cells for approximately 20 d, which speaks to the potential of non-integrating vectors^[30]. In 2008, Stadtfeld *et al.*^[31] were able to generate mouse iPS cells from fibroblasts and liver cells by using non-integrating adenoviruses that transiently expressed the four “Yamanaka factors”. The cells were showed distinct characteristics of pluripotency such as the expression of endogenous pluripotency genes, demethylation of *Oct4* and *Nanog* promoters, and the ability to produce teratomas *in vivo* and contribute to all three germ layers^[31]. Even though the infection efficiency of the adenoviral vectors was relatively high (50%-60% for quadruple infected cells), the overall reprogramming efficiency this non-integrating method was between 0.0001% to 0.001% (significantly lower than integrating viral methods; 0.1% on average)^[31], something that is probably due to the rapid dilution of the adenoviruses during cell division which results in the cells not being exposed to the reprogramming factors for an adequate amount of time so as to induce a successful change in phenotype.

Another example of iPS cells created by non-integrating vectors can be seen in Guarino *et al.*^[32]. Yu and colleagues were able to create human iPS cells by utilizing three modified episomal vectors containing different combinations of six reprogramming factors (*OCT4*, *SOX2*, *NANOG*, *LIN28*, *c-MYC*, and *KLF4*) and the SV40 large T gene (*SV40LT*) to counteract the toxic effects of *c-MYC* expression, a cis-acting oriP element and an Epstein-Barr nuclear antigen 1 (EBNA1) gene^[32]. The

latter of these elements provided the vector with the ability to be transfected without the need for viral packaging and to be stably replicated outside of the chromosome^[32]. The factors packaged inside the vectors were linked by the internal ribosome entry site 2 (IRES2), and this was done in order to increase reprogramming efficiency by coexpressing them^[32]. Utilizing these vectors researchers were able to make iPS cells that exhibited typical ES cell colony morphology and gene expression profile, and they were able to produce teratomas *in vivo* that contained differentiated derivatives of all three germ layers^[32]. Subclones of the reprogrammed cells showed no signs of the vector or transgene sequences other than the change in phenotype, which is an incredible accomplishment, the reprogramming efficiency of the method however, is rather low (about three to six colonies per 10⁶ input cells)^[32].

Although non-integrating vectors are a good alternative in order to produce safer iPS cells for use in treatments, they are not very cost-efficient considering that these methods and vectors produce very low amounts of reprogrammed cells. Another alternate method that has seen a lot of attention in recent years is the use of miRNAs instead of exogenous transcription factors as a means of reprogramming^[19]. Wang *et al*^[19] used a lentiviral vector containing *miR302/367*, a unique cluster of miRNAs that is highly expressed in EM cells, in order to produce iPS cells from human embryonic kidney (HEK) 293T cells and found that these reprogrammed cells generated ES-like colonies, showed increased expression of ES cell markers (SOX2, KLF4, c-MYC, OCT4, LIN28 and NANOG), could form embryoid bodies, and could differentiate into germ-like cells *in vitro* and *in vivo*. So as to improve the differentiation potential of the miRNA-induced iPS cells researchers cultured the HEK293T cells in serum-free media, as well as in the presence of two small molecules: vitamin C and fibroblast growth factor (bFGF) so as to better shape the morphology of the reprogrammed cells^[19]. Although the overall efficiency of the reprogramming method described is yet to be determined, previous reprogramming studies with miRNAs have demonstrated this type of approach to be more efficient than the standard reprogramming factor methods (10% *vs* 0.1%, respectively)^[33], making this type of method a promising candidate for further studies.

FORWARD THINKING: OBTAINING RENAL PROGENITORS USING LOW-COST AND EFFICACIOUS SMALL MOLECULES

In the endeavor to create renal progenitors, controlled differentiation of iPS cells has become a good alternative to partial reprogramming of differentiated cells. One particular technique that stands out is the use of small molecules in order to induce a more renal-specific pluripotent state. Lam *et al*^[34] created an intermediate mesoderm (IM)-specific differentiation platform around

the small molecule CHIR99021, a glycogen synthase kinase-3 β inhibitor (CHIR). This inhibitor manages to recapitulate mesendoderm formation during development in human pluripotent stem cells (hPSCs), as evidenced by the compounds ability to produce cell lineages that transiently expressed various primitive streak genes such as *BRACHY*, *MIXL1*, *FOXA2*, *EOMES*, and *GSC*^[34]. The transient expression pattern of these genes in the CHIR-treated cells during a 72 h period is also consistent with that found in cells during the course of gastrulation, which means that CHIR99021 imitates normal developmental mimetics^[34].

Utilizing this compound researchers were able to screen various exogenous factors in order to determine the minimum requirements needed promote differentiation of these CHIR-induced mesendoderm-like cells toward IM^[34]. They reported that fibroblast growth factor-2 (FGF2) in combination with retinoic acid (RA) was able to induce IM differentiation in the mesendoderm-like cells. This conclusion was drawn from the fact that the treated cells were both *PAX2* and *LHX1* positive, two markers for which coexpression in the same domain has only been described in the developing kidney and dorsal spinal cord. Further evidence that these *PAX2*⁺*LHX1*⁺ cells were directed to an IM state and that they could produce IM-derived cell populations and tissues came in the form of tubule structures (with primary cilia) expressing proximal tubular markers once the exogenous FGF2 and RA were removed from the culture media. One of the many differentiated kidney markers whose expression was evaluated in the *PAX2*⁺*LHX1*⁺ cells was *SIX2*, a multipotent nephron progenitor cell marker. This nephron progenitor population composes what is known as the CM and these give rise to nearly all epithelial cell types in the nephron tubule, with the exception of those from the collecting duct. Lam *et al*^[34] were able to use the double positive IM-like cells in order to screen different growth factors so as to identify the conditions that promote and sustain a *SIX2*⁺ cell population, and they were able to determine that the addition of FGF9 and Activin A could do just this, as well as induce the expression of other CM markers such as *SALL1* and *WT1*. Although researchers were able to effectively produce IM-like cells that are able to differentiate into subsequent renal cell populations, the need for exogenous growth factors is still an issue due to that fact that these very same growth factors are incredibly expensive, and therefore not very cost-effective to use in clinical applications.

Araoka *et al*^[35] on the other hand, utilized a combination consisting of only small molecules, as opposed to small molecules and growth factors, in order reach the same goal. In this particular strategy the mesendoderm stage is skipped altogether and the hPSCs are differentiated directly into an IM state. Using high throughput chemical screening they were able to identify two compounds that increased induction of *Odd-skipped related 1* (*Osr1*), a transcriptional regulator that is expressed in the embryonic day 7.5 IM until kidney organogenesis and

therefore a good marker to utilize in order to identify IM cells. The two compounds identified were AM580 and TTNPB, RA receptor antagonists (RAR) that induce differentiation of hPSCs into OSR1⁺ IM cells with relatively high efficiency (> 60% and > 50% respectively) when compared to positive controls. To further optimize OSR1 induction researchers combined each RAR with CHIR, which resulted in an increased induction rate of around 80% in only 5 d utilizing only two chemicals in a serum – free environment.

As mentioned before, one of the main differences between the methods used Araoka *et al.*^[35] and Lam *et al.*^[34] is that the former can skip the mesendoderm stage altogether. This was demonstrated when researchers analyzed mesendoderm markers (*BRACHYURY*, *GOOSECOID*, and *MIXL1*) in the small molecule-treated hPSCs, and found that the induction rate for *BRACHYURY*⁺ cells was around 6%, and that expression levels for said markers were very low in cells produced from the small molecule method when compared to cells produced with CHIR and growth factor activin A. The ability of these IM-like cells to produce the various IM-derivative cell types was also evaluated, and after additional days of differentiation researchers found that these induced IM cells did in fact produce cells expressing marker genes for various IM-derivative cell types such as *FOXD1*, *SALL4*, *GATA4*, among others. These cells also had the ability to give rise to the derivative cell types *in vivo*, as well as renal tubule-like structures positive for renal tubule markers such as *Lotus tetragonolobus* lectin (LTL), E-CADHERIN, and laminin *in vitro*.

Both of these studies^[34,35] provide evidence that utilizing small molecules in order to produce renal progenitors for cell therapies is a viable option in the field of regenerative medicine, and the various benefits that this type of method provides makes it a good alternative to explore. Utilizing these chemical compounds is not only less costly, but more efficient in terms of number of cells converted to the desired phenotype (even though the reprogramming efficiencies for the template iPS cells were not stated in either one of the studies). Unfortunately there is still some variability between studies that needs to be addressed before any progress can be made on any viable therapeutic solution.

Both of the methods described above are highly efficient for IM differentiation of hPSCs in terms of the time the procedure takes, the markers analyzed, and the compounds used^[34,35]. Lam *et al.*^[34] utilizes a method that has both chemical compounds and growth factors, but only takes 3 d to produce IM cells. Araoka *et al.*^[35] on the other hand only use chemical compounds, but take about two more days in order to reach the same goal. In terms of markers utilized the former uses a combination of LHX1 and PAX2 (a pair of markers that, as stated previously, are only found in the developing kidney and dorsal spinal cord), while the latter uses an engineered *OSR1-GFP* human iPS cell line to verify if the cells have reached an IM state, a gene that is also expressed in the

lateral plate mesoderm and can therefore provide some heterogeneity to the sample that might alter the results of future studies.

CONCLUSION

Recent progress in knowledge about cellular reprogramming has rapidly advanced prospects for the development of regenerative therapies for the medical treatment of many conditions, among them being kidney diseases, making this a very exciting time in the field of nephrology. Here, we discussed a number of research studies in the field of stem cell reprogramming. We explored how such methods have been utilized to reprogram renal lineages, and thus might be used to develop therapies to treat kidney disease. Additionally, iPS cells can be used for disease modeling to identify targeted therapeutics for heritable conditions^[36]. Moving forward, there are a number of complex issues to further resolve about the therapeutic application of iPS cells for disease treatment, and most assuredly other issues yet to be identified, which apply both to the kidney and other organs within the body (Figure 2).

Issues involved in the therapeutic application of reprogrammed cells include the number and type of cells needed, along with the identification of an appropriate delivery system for the condition to be treated. Currently, there are various ongoing clinical trials in the United States that are using stem cells to treat a wide range of conditions such as age-related macular degeneration to polycystic kidney disease^[37]. The amount of cells utilized by these studies can fluctuate between the stem cells type and the way they are used (50000-200000 human embryonic stem cells in retinal cell transplants and 2×10^6 mesenchymal stem cells (MSCs)/kg of patient's weight in kidney disease treatments^[37,38]), but even so the amount of pluripotent cells produced by the methods mentioned in this review are still relatively low when compared to the amount used in the before-mentioned trial therapies.

Other issues that still need to be addressed are cell quality (can you isolate healthy renal cells to reprogram as opposed to the diseased ones?) and downstream processing, a problem because, due to ethical reasons, many of the pluripotency tests that are usually performed on reprogrammed cells can't be done with human iPS cells, which might create some heterogeneity within the human iPS cell lines. Also, we have barely scratched the surface of how epigenetic memory affects iPS cell differentiation patterns. All of these concerns still need to be investigated before adequate therapies can be developed (Figure 2).

Although there remains a sizable amount of work to be done in order to optimize the efficiency of these methods, they still represent a promising alternative to current therapies, mainly because they have the potential to provide the affected patient with the means to regain kidney function without the need for a kidney transplant or dialysis. It would be interesting to see how these

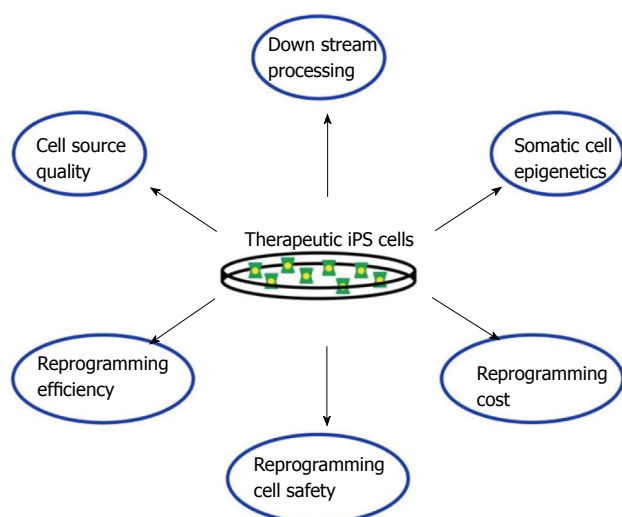


Figure 2 Challenges faced when developing induced pluripotent stem cell-based therapies. Many issues still have to be overcome before any effective cell therapies can be used.

methods would be affected if they were done with other animal models, such as in the zebrafish, an organism that has the capacity to regenerate renal tissue^[39-41], and what type of information can be learned from animal models about how reprogramming methods can be optimized or the nature of renal progenitors^[42]. As more insights continue to be gathered about the genetic mechanisms of renal lineage development and regeneration in various vertebrate models, as represented for example by recent reports in the zebrafish^[43-45], frog^[46], and mouse^[47], crucial information may be elucidated about potent methods to regulate renal reprogramming or even to promote pathways of endogenous cell regeneration in the damaged kidney.

Moving forward, there may be significant challenges for cell-based therapies posed by the microenvironment in the damaged kidney—termed by some as the “seed and soil” dilemma. Namely, the importance of an appropriate microenvironment, or niche, the so-called “soil”, is essential for the prosperity and normal growth of the stem cells, or “seeds”, to be administered in a putative treatment^[48]. The complexity of renal anatomy and composition alone may pose significant hurdles to cell-based therapies, and can be further complicated if the environment due to the disease state is refractory to the success of the regenerative therapy. In sum, altering the microenvironment to facilitate success of the cellular therapy is likely to be vital.

One promising avenue is the utilization of other stem cells, *e.g.*, MSCs, which have been shown in a number of contexts to stimulate a local, if not organismal, humoral environment that facilitates regeneration^[37,38]. The kidney is in fact among such organs whose status can be improved by MSCs in some disease settings^[49]. In animal models of AKI, administration of MSCs has provided renoprotective effects^[50-53]. Notably, a limitation that has been recognized is the inability of MSCs to mediate im-

provements in chronic renal disease states^[54]. These observations indicate that much remains to be learned about how to facilitate cell-based therapies with approaches that address the complex variables associated with any given disease state. Thus, it is imperative that future research is performed to better understand the relationships and physiological impacts of disease states within organisms. Nevertheless, the progress in stem cell biology to date continues to fuel enthusiasm that methods like reprogramming can be harnessed to improve quality of life and relieve suffering in the decades to come.

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Arterial calcification: Finger-pointing at resident and circulating stem cells

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Abstract

The term "Stammzelle" (stem cells) originally appeared in 1868 in the works of Ernst Haeckel who used it to describe the ancestor unicellular organism from which he presumed all multicellular organisms evolved. Since then stem cells have been studied in a wide spectrum of normal and pathological conditions; it is remarkable to note that ectopic arterial calcification was considered a passive deposit of calcium since its original discovering in 1877; in the last decades, resident and circulating stem cells were imaged to drive arterial calcification through chondro-osteogenic differentiation thus opening the idea that an active mechanism could be at the basis of the process that clinically shows a Janus effect: calcifications either lead to the stabilization or rupture of the atherosclerotic plaques. A review of the literature underlines that 130 years after stem cell discovery, antigenic markers of stem cells are still debated and the identification of the osteoprogenitor phenotype is even more elusive due to tissue degradation occurring at processing and manipulation. It is necessary to find a consensus to perform comparable studies that implies phenotypic recognition of stem cells antigens. A hypoth-

esis is based on the singular morphology and amitotic mechanism of division of osteoclasts: it constitutes the opening to a new approach on osteoprogenitors markers and recognition. Our aim was to highlight all the present evidences of the active calcification process, summarize the different cellular types involved, and discuss a novel approach to discover osteoprogenitor phenotypes in arterial wall.

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Key words: Osteocalcin; Osteoprogenitor; Stem cells markers; Arterial calcification; Resident and circulating

Core tip: We review state of art on active arterial calcification, introduce new insight in arterial osteoprogenitors (OPs) phenotypes and the concept of amitosis. Analysis of literature of all markers used to define mesenchymal stem cells and OPs revealed the evident incongruity between the actual studies: each research has its own panel of antigen markers. Still, osteocalcin resulted the most promising marker of resident and circulating OPs. A new technique allows maintaining DNA/RNA integrity in highly calcified or ectopic bone formation: new studies should consider this technique and the particular division of OPs to identify them.

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INTRODUCTION

Physiological and pathological mechanisms of vascular calcification

Previously considered passive and degenerative, vascular

calcification is now recognized as a pathobiological process sharing many features with embryonic bone formation^[1]. Vascular cell differentiation responds to microenvironmental and mechanical cues, since substrates of great stiffness, such as fibronectin, promote osteochondrogenic differentiation, whereas distensible substrates, such as laminin, promote smooth muscle or adipogenic differentiation^[2]. The biomineralization process begins from the so-called crystallization nucleators, which trigger the formation of a primary crystal nucleus, together with the removal of the mineralization inhibitors [ankylosis protein, nucleotide pyrophosphatase, matrix glutamyl protein (MGP)]. The extracellular matrix vesicles contain deposits of calcium and alkaline phosphatase (ALP), pyrophosphatase, *etc.*, which increase the inorganic phosphates in the vesicles^[3]. They also stimulate the production of osteopontin, another nucleation inhibitor^[4]. During the vessel calcification there are active processes similar to those in the bone biomineralization. In depositions in both tunica interna and media of the vessel wall, matrix vesicles have been identified^[5]. Post-mortem studies have shown that vessel wall may contain a typical bone, cartilage or adipose tissue, with bone as the predominating type of metaplasia (10%-15% of samples), appearing in various morphological forms, from amorphous calcium deposits to mature bone tissue^[6].

The increasing interest in vascular calcifications derives from the fact that in the atheromatous disease they were considered a form of plaque regression, while more recently the extent of calcification was associated with a worse prognosis, albeit the real impact of calcification within a specific lesion is unclear^[7]. Moreover, vascular calcification is commonly seen during other systemic disease, such as diabetes, end-stage renal disease and calciphylaxis, and it is generally considered as a bad outcome predictor^[8]. In the coronary arteries the extent and dimensions of the calcification seem to play a key role, since small depositions increase the probability of atherosclerotic plaque rupture, especially on their edges, while with individual, large calcification foci such risk is even likely to decrease^[8,9]. In a study on 10 stabiles and 10 ruptured coronary artery *post-mortem* specimens, calcifications did not significantly affect the stability of the atheroma, in contrast with the significant reduction in stability associated with the lipid content. Removing the calcification led to a statistically insignificant change in stress^[7]. Anyway, vascular calcification is considered a worsening factor, probably due to the association with the general risk factors: a study by Iribarren *et al.*^[10] found that aortic arch calcification was associated with coronary heart disease risk both in men and women. Thus aortic arch calcification may reflect the general burden of disease or be a marker of a more aggressive disease.

Histological patterns of vascular calcification

Histologically, arterial calcifications can be classified in calcifications of the tunica intima, principally related to atherosclerosis and, calcifications of the tunica media,

unrelated to atherosclerosis (Monckeberg's type)^[6,11].

The intimal atherosclerotic calcifications are the most common form of arterial calcification. Calcium accumulation is initiated by an increase in the plaque of modified lipids, pro-inflammatory cytokines, phosphate and lipoprotein complexes, as well as foci of necrosis^[1,6]. *In vitro* studies have shown that pro-inflammatory cytokines, oxidized low-density lipoprotein (LDL) or other macrophage release products promote the osteogenesis and the calcium accumulation^[12-14], while some studies correlated the vascular calcification with the duration of the hypercholesterolemia^[15] and with inflammation *in vivo*^[16]. The so-called punctate deposits start in the deeper intimal regions, adjacent to the media, but very large deposits, involving the whole intima, can be seen^[11]. In this tissue, hematopoietic, osteoblast-like and osteoclast-like cells were described^[17,18]. A finest and more diffuse pattern of calcification, involving the whole intima was recently described due to processing techniques that do not require decalcification^[19].

The medial calcification was firstly described by Monckeberg more than a century ago^[20]. Since then, the "rail-road track" medial calcification was observed in patients with diabetes and chronic renal disease^[21,22], as well as in young patients without substitute patent with evident patent metabolic disorders^[23,24]. In aging, medial calcification may develop by unknown etiology, or result from associated conditions such as chronic renal failure, diabetes, neuropathies and denervations^[11,25]. In any case, these calcifications are likely to occur in not-atherosclerotic arterial segments^[26].

Premises for a stem cell origin of vascular calcification

Classically, the heterotopic calcifications that can be found in the atheromatous plaques, in not-atheromatous arteries, as well as in many tissue, have been subdivided in active and passive^[11]. The active calcifications follow different (and still unclear) mechanisms that can lead to a true ossification of the vessel wall^[27,28].

While very rare in veins^[29], ectopic calcification in arteries has been noted for many decades. In 1877, Howse found bone-formation in the wall of a ruptured axillary artery^[30]. Until recently, however, this phenomenon was simply viewed as a passive consequence of aging^[31]. However, as already observed in the 1900s, this condition was reported in the aorta of a girl eight years of age, aorta of adults between the ages of sixteen and twenty-four years, in an infant of fifteen months old and in an ossified aorta in a child of three years^[30].

Regardless to the arterial layer, calcifications are found in different vessels as coronaries, distal arteries and aorta. As stated above, clinical outcome depends mainly on the degree and the location of calcification, additionally to the underlying disorder^[32]. Several models postulating mechanisms for the formation and inhibition of calcification have now been proposed^[33]. These are the active model; the passive physicochemical model; and the arterial osteoclast-like cells model. One model doesn't exclude

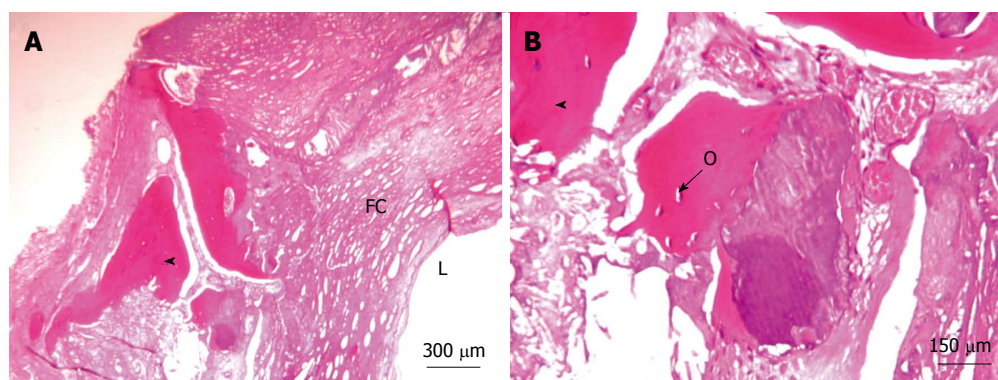


Figure 1 Carotid artery calcifications, hematoxylin and eosin staining. A: Sheet-like calcifications; B: Osteocytes are visible within the bone lacunae-like mature structure in development with lamellar bone. L: Lumen; FC: Fibrous cap; Arrowhead: Ossification; O: Osteocytes.

the other.

In some cases arteries can evolve into mature bone tissue histomorphologically indistinguishable from skeletal bone^[11]. In our practice, this evolution occurs in at least 5% of diseased arteries. In Figure 1, a Haematoxylin-Eosin staining of a section of carotid atherosclerotic plaques revealed the presence of osteocyte cells within bone lacunae-like mature structure in development; lamellar bone is also visible.

One of the most recent mechanism proposed in order to shine a light on active vascular calcification is the possible role of stem/progenitor cells, either resident in the vessel wall or circulating cells deriving from the bone marrow. In addition, chondrocyte-like cells, typically not expressed in normal arteries, osteoblast-like cells and multinucleated osteoclast-like cells (OLCs) are found in calcified arteries^[17,33]. These cells are recognizable thanks to their peculiar morphology and positivity to specific histological markers; osteoprotegerin, osteopontin (OPN), osteocalcin (OCN), MGP and bone matrix protein (BMP)^[34].

The present review focuses on the current and most recent knowledge on the mechanisms of active vascular calcification ascribable to resident and circulating cells that acquire the plasticity of the stem/progenitor cells and that trigger or participate to the vascular calcification processes.

CIRCULATING STEM CELLS

The passive model of vascular calcification has been progressively abandoned, since evidence of a genetic and active process has been observed.

Bone marrow (BM)-derived mesenchymal stem cells (MSC) have the ability to differentiate into many stromal cell types, as myocytes, fibroblasts, astrocytes, adipocytes, chondrocytes and osteocytes; the last two are referred as osteo-progenitors^[34].

Progenitors are proliferative cells with a limited capacity for self-renewal and are often unipotent. Accumulating evidence indicates that the mobilization and recruitment of circulating or tissue-resident progenitor cells that give rise to endothelial cells (ECs) and smooth muscle

cells (SMCs) can participate in atherosclerosis, neointima hyperplasia after arterial injury, and transplant arteriosclerosis^[35]. Specifically progenitor cells can contribute to calcification: BM contains both osteoblast and osteoclast precursors termed as osteoprogenitors (OPs) associated with bone remodeling^[36]. This novel mechanism was named “circulating cell theory”: the bone marrow derived cell population may seed the arteries and contribute to disease or repair^[37]. The mobilization is the process under the regulation of cytokines in which immature cells from the BM are recruited to the blood^[38].

Another common mechanism that can explain the recruitment of circulating OPs in arteries is homing^[39]; in response to stress signal, injury, inflammation, repair or abnormal cytokine signalling, circulating cells cross the endothelium and invade the target tissue^[40]. The endothelial phenotype selectively modulates bone marrow-derived stem cells homing: indeed different endothelial phenotypes hold functional differences. As an example, coronary artery endothelium enables the fastest bone marrow stromal cells integration. Transmigration requires the interaction of vascular cell adhesion molecule-1, very late antigen-4, $\beta 1$ integrins, metalloproteinases (MMP) secretion and cytokines^[40].

Recently, a primitive CD14-positive cell population was defined and named monocyte-derived multipotent cells (MOMCs). These cells show a fibroblast-like morphology and the expression of several stem cell markers such as CD14, CD45, CD105, CD34 and type I collagen, but lack expression of CD117 (c-kit) or CD133. These characteristics are quite peculiar^[41]. Due to this hybrid phenotype, a subpopulation of these cells is likely to overlap the endothelial progenitor cells (EPC) originally described by Asahara *et al.*^[42], characterized by the co-expression of CD14 and CD34. Conversely, the so-called monocyte-derived endothelial progenitor cells are described as a MOMC subpopulation positive for CD14 but with low expression for CD34. These cells have the ability to differentiate also into osteoblasts, adipocytes, or neuronal cells^[43].

Another subset of these cells showed bone resorption capacity on dentine slices and expression of genes for ca-

thepsin K and calcitonin receptor, characteristic of functional osteoclasts^[41]. MOMCs express receptor activator of nuclear factor- κ B ligand (RANKL), which is required for osteoclast formation from mononuclear precursors. These results indicate that human MOMCs can express RANKL and differentiate into functional osteoclasts without RANKL-expressing accessory cells.

Under specific stimulations (PDGF: Platelet-Derived Growth Factor, interleukin IL-4, IL-13) CD14⁺ monocyte precursors can also differentiate into fibrocytes^[44]. Discovered in 1994, fibrocytes are bone marrow-derived mesenchymal progenitors that co-express hematopoietic stem cell genes, markers of the monocyte lineage, and fibroblast products. Fibrocytes constitute another source of circulating cells able to differentiate in fibroblasts, myofibroblasts and adipocytes^[45].

In valve and arteries, myofibroblasts contribute to cardiovascular ossification; Vattikuti observed that adventitial activated myofibroblasts cells are diverted to the osteoblasts lineage: the hypothesis is that myofibroblasts, responding to vascular smooth muscle cell osteopontin production contributes to calcification in diabetes. Moreover pericytic myofibroblasts expressed BMP-2, a powerful bone morphogen^[46].

RESIDENT STEM CELLS

Mesenchymal stem cells

Bone marrow-derived MSC which reside in the vessel wall can differentiate in several cell types, including osteoblasts, chondrocytes and endothelial cells^[47-51].

Previous results from our group showed that it is possible to isolate and culture spindle-shaped resident cells with the characteristics of MSC directly from the vessel wall of thoracic aortas harvested from multiorgan and tissue donors. These vessel-wall MSC (vw-MSC) are CD45⁺ and show low expression for CD34, but most co-express CD44, CD90 and CD105, like the bone marrow-derived MSC^[52]. Moreover, at reverse transcription polymerase chain reaction these cells express transcripts of embryonic stem cell (OCT4, IL6 and BCRP-1) and hematopoietic stem cell (c-Kit, BMI-1)^[52]. Years after we confirmed that vw-MSC expressed the stemness markers Stro-1, Notch-1 and OCT4, and that they were able to differentiate into adipogenic, chondrogenic and leiomyogenic lineages, when cultured in induction media^[53]. Recently, Klein *et al.*^[54] described a CD44⁺ population of “vascular wall-resident multipotent stem cells”, expressing also CD90 and CD73, and negative for CD34 and CD45. Moreover, vw-MSC were also isolated and cultured from arterial specimens frozen up to 5 years, and showed positivity for HLA-G, Stro-1, Oct-4 and Notch-1, in addition to the above mentioned^[55].

Recently it was hypothesized that MSC might play a role in the pathogenesis of atherosclerosis, and it was demonstrated that, under particular conditions, MSC in culture acquire an osteoblastic phenotype *via* the activation of the Wnt pathway^[56]. In hyperlipidemic rats treated

with angioplasty to have a vascular damage, MSC started the vessel wall remodelling and triggered calcification, mediated by paracrine BMP-2^[57], which is considered one of the main mediators in the differentiation of MSC (and others) along the osteoblastic lineage^[58,59]. Interestingly, MSC cultured in a uremic serum for one month (mimicking partly the renal failure stimuli) hyperexpressed alkaline phosphatase, osteopontin, Runx2 and showed an up-regulation of BMP-2^[60].

SMC

SMC of the human arterial wall show a great phenotypic plasticity, since it was demonstrated that in culture they can differentiate in almost all mesenchymal lineages (except adipocytic), and in particular conditions they can calcify^[61,62]. These cells were originally described as calcifying vascular cells (CVC), *i.e.*, SMC that under cAMP stimulus undergo osteoblast differentiation (with expression of alkaline phosphatase, type I collagen and matrix glutamyl protein), aggregate and form mineralized nodules^[12]. The matrix carboxyglutamic acid protein (MGP)-deficient mice are a well-known animal model characterized by a progressive calcification of not-atherosclerotic arteries: in these mice vascular SMC were replaced by mineralizing chondrocyte-like cells^[63]. The possibility of a phenotypic transition by the cells of the arterial wall opened new possibilities in the theories of the active calcification model.

Steitz *et al.*^[64] demonstrated the phenotypic transition of cultured bovine aortic smooth muscle cells into mineralizing cells: after 10 d from the administration of β -glycerophosphate, the smooth muscle cells lost their contractile properties (and the smooth muscle α -actin expression) and acquired an osteocalcin- and osteopontin-positive phenotype. Years later, researchers from the same group demonstrated that vascular SMC from MGP-knock-out mice expressed *Runx2/Cbfa1* and gave rise to osteogenic precursors^[65]. In SMC from human arteries, an increased expression of osteo- and chondrogenic transcription factors (Cbfa1, Msx2, Sox9) was observed concomitantly with a decreased expression of muscle markers^[66]. SMC cultured in 2D scaffolds and treated 2 wk with lyso-phosphatidylcholine (LPC) underwent trans-differentiation to CVCs by up-regulation of the *Runx-2* gene^[67], while more recently the same authors demonstrated that using 3D cultures (a more reliable model of *in vivo* conditions) the growth and mineralization of cultured SMC is even more efficient, and adjustable by external factors such as LPC (enhancer) and Schnurri-3 (inhibitor)^[68].

Neoangiogenesis and endothelial cells

According to several observations, neoangiogenesis and vascular calcification are closely correlated: first of all, neovessels can simply be considered as means of transportation for progenitor cells in the tissue, but endothelial cells are able to produce cytokines that can stimulate osteoprogenitor cells, *in vitro* and *in vivo*. Moreover, many

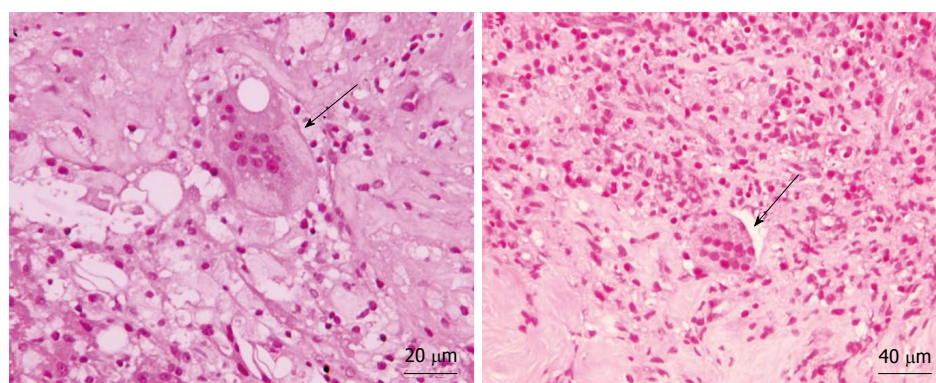


Figure 2 Osteoclasts-like giant cells admixed with inflammatory infiltrate. Arrows point osteoblast cells.

growth factor (such as FGF-2 and VEGF) can stimulate both neoangiogenesis and the activation of osteoblasts and osteoclasts^[8]. Endothelial cells cultured under pro-atherogenic stimuli produce pro-osteogenic factor, such as BMP-2^[69]. This is particularly interesting, considering that most of plaque neoangiogenesis derive from adventitial *vasa vasorum*, and can drive many progenitor cells, pericytes, and inflammatory stimuli, including cytokines, in the media and intima layers^[70,71].

The potentiality of endothelial cells to become directly a source of stem cells was demonstrated in a diabetic mouse model by Yao *et al.*^[72], who found that the stimulation with BMP-4 induced endothelial-to-mesenchymal transition and expression of osteogenic markers in aortic endothelial cells. In cultured human aortic endothelial cells, high glucose concentrations cause the acquisition of a “chondrocyte-like” phenotype, with the expression of *STRO-1*, *CD44* and *SOX9*^[73]. Previous *in vivo* data from our group have demonstrated that quiescent *vasa vasorum* in normal arteries from healthy subjects express markers of progenitor cells, namely Nestin and WT1, thus showing proliferative potential^[74]. The same phenotype is expressed by intraplaque neoangiogenesis, and particularly Nestin is correlated with complicated plaques^[75].

Osteoclast-like giant cells

Like in the normal bone tissue, the calcification of the vessel wall and/or atheromatous plaque is likely to depend on a balance between pro-osteogenic and anti-osteogenic stimuli. In this setting the so-called osteoclast-like giant cells (Figure 2) play a role in calcium reabsorption, as it was demonstrated decades ago by the findings that apoE-knockout mice lacking also the gene for macrophage colony stimulating (*M-CSF*, a cytokine involved in osteoclast survival) developed massive arterial calcifications^[76]. The origin of the OLC in the vessel wall are not clear yet, and whether they derive from resident stem cells, from circulating hematopoietic precursors, from a differentiation of mononuclear cells or from other cells not yet established is still to be clarified. The mononuclear cells commonly found in atheromatous plaques share many phenotypic and genetic features with osteoclasts and they have a hematopoietic origin, while many circulating precursor cells express receptors for RANK

and M-CSF, both essential for the osteoclast activity^[11].

Pericytes and macrophage progenitor cells

Pericytes share several phenotypic markers with CVCs, including α -actin, β -actin, and the 3G5 epitope of monoclonal antibody-defined ganglioside antigen^[8]. The putative role of pericytes as a “reservoir” of progenitor cells, and their potential to differentiate into several cell types, including osteoblasts, is well known^[66,77,78]. In the last three decades, using different models, a lot of evidence have been adduced that pericytes can undergo chondro- and osteogenic differentiation^[50,79,80]. After 8 wk of culture, pericytes have been shown to proliferate and form multicellular clumps with a mineralized matrix containing type I collagen, Gla protein, osteocalcin and osteopontin^[81,82]. Furthermore, culturing these cells in a chondrogenic media (TGF- β 3: Transforming growth factor β 3) pericytes undergo chondrogenic differentiation^[50]. Other authors hypothesize that adventitial pericytes (expressing activating *Msx2* and other osteoblastic transcription factors) might also be able to stimulate the production of alkaline phosphate, the Wnt pathway activation and the β -catenin nuclear activation in medial cells (SMC)^[83]. This represents an interesting example of indirect stimulus towards calcification mediated by the synergic cross-talk between different cells of the vessel wall. Indeed, arterial adventitia contain different progenitor cells, as it was demonstrated in murine aorta, where a population of Sca-1+/CD45+ macrophage progenitor cells has been recently described, which represents a *reservoir* of non-circulating precursors cells^[84].

The role of the adventitial cells in the regulation of the functions of the vessel wall, both physiologically and in pathological conditions including calcifications, surely deserves future in-depth analyses.

DEFINITIONAL CRITERIA OF OSTEOGENIC LINEAGES

Osteoblastic “profile” and mechanisms

As shown above, several *in vitro* and animal models have demonstrated that a main mechanism of vascular calcification is represented by BMP-2 and 4. BMP-2 upregu-

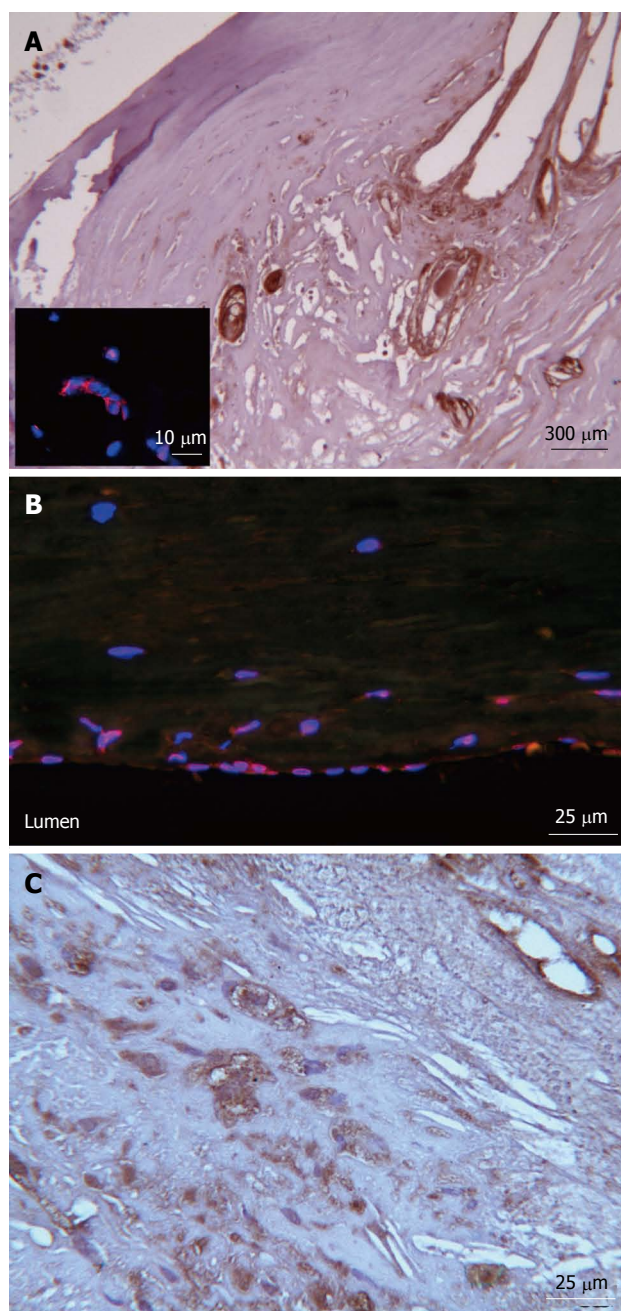


Figure 3 Osterix and osteocalcin expression in carotid plaques. A: Osterix immunohistochemistry (IHC) positivity in vessels single-label immunofluorescence micrographs representing Osterix (red) detectable in the nucleus of endothelial cells of a single vessel; B: Osterix (red) is also seen in the carotid endothelium layer, nuclei (blue) were counterstained with DAPI (4',6-diamidino-2-phenylindole); C: Cytoplasmic and extracellular matrix osteocalcin IHC pattern.

lates *Runx2*, which induces the production of type I collagen and alkaline phosphatase^[85,86]. As demonstrated in murine models, MGP is the principal inhibitor of BMP-2, and a loss of MGP leads to tissue calcification^[63]. One of the master genes essential for driving differentiation of mesenchymal cells into terminally differentiated osteoblasts is Osterix^[11], that can be also found expressed in endothelial cells of the diseased arterial wall (Figure 3).

Recently, the receptor activator of NF- κ B ligand (RANKL) was identified as another key molecule in the

differentiation of osteoblasts and osteoclasts: in apoE^{-/-} mice, the immunostaining for RANKL was diffusely positive in activated chondrocytes involved in the vascular ossification process^[87], and its serum level seems to increase with ageing proportionally to the risk of cardiovascular events^[88].

Osteopontin is a normal component of the bone and plays a role in the regulation of the mineralization. In calcified human plaques, OPN is expressed in SMC, endothelial cells and macrophages^[89,90].

Osteocalcin is one of the most studied markers of osteoblast lineage. OCN is synthesized by osteoblasts and is the major component of the bone matrix (1%-2%). OCN is capable of binding hydroxyapatite (HA) thanks to his glutamyl (GLA) residues. Five Ca²⁺ ions are bound by 3 GLA residues carboxylated by vitamin K1^[91], thus the OCN can dock on the HA and add calcium and growth crystal leading to the grow of bone. Transcription of OCN is regulated by Vitamin D3. In addition to binding to hydroxyapatite, OCN functions in cell signaling and the recruitment of osteoclasts and osteoblasts^[92].

In patients with peripheral artery disease, the percentage of circulating bone marrow-derived OPs, positive to OCN, increased with the severity of aortic calcification^[93]. Wang *et al*^[94] demonstrated that in injured arteries the release of TGF β mobilize MSC from the blood stream to the neointima. In a mouse model LDLR^{-/-}, Nestin+/Sca+ cells were all recruited in the calcified arteries were OCN+ osteoblastic cells were seen: they observed that MSC generated OCN+ osteoblastic cells in the calcified lesions and that the migration of MSC to the lesions depends on TGF β production from the lesions. Finally, when TGF β receptor 1 was inhibited in mice there was a decrease of the number of MSC in the blood concomitant to their recruitment to the arterial lesions at the calcified lesions.

Different studies correlate the amount of circulating OCN-positive cells to the presence of coronary disease. Flammer *et al*^[95] counted with flow cytometry the blood circulating population of cells positive to both immature EPC markers CD133+, CD34-, KDR+ and OCN. They observed that this fraction of cells, OCN+ EPC, increased in patients with cardiovascular risk factors compared to patients with a stable coronary artery disease history. Of note that the blood circulating cells expressing OCN have been shown to be able to calcify *in vitro* and *in vivo*^[96]. In a similar study, Gössl *et al*^[97] compared the fraction of EPC circulating cells CD34+/KDR+/OCN+ in 3 groups; the control group (normal coronary arteries/no endothelial dysfunction) versus two groups with coronary atherosclerosis: early coronary atherosclerosis (ECA: Normal coronary arteries but with endothelial dysfunction) and late coronary atherosclerosis (LCA: Severe, multi-vessel coronary artery disease). The number of CD34+/KDR+/OCN+ cells were increased by -2-fold in the ECA patients, with smaller increases in the LCA patients.

The prevalence and extent of calcification seems to

Table 1 Phenotypic markers for circulating and resident progenitors cells

Name of the progenitors	Antigens	Notes (origin)	Ref.
MSC, EPC and CEC in human			
MSC	CD105+, CD73+, CD90+, CD14-, CD34-, CD45-, CD79-, and CD19-	Resident from various tissues	Dominici <i>et al</i> ^[103]
vw-MPSC	CD44+, CD90+, CD73+, CD34-, CD45-	Resident from arterial adventitia	Klein <i>et al</i> ^[54]
vw-MSC	CD44+, CD90+, CD73+, CD105+, CD29+, CD166+, Stro-1, Notch-1 and Oct-4	Resident from aortic arches, thoracic and femoral arteries	Pasquinelli <i>et al</i> ^[52,53]
Circulating MSC	CD105+, CD166+, CD54+, CD55+, CD13+, CD44+; CD34-, CD45-, CD14-, CD31-, CD133-	Circulating and resident from bone marrow; cartilage; synovial membrane; peripheral blood; umbilical cord blood; teeth	Qian <i>et al</i> ^[104]
Circulating EPC	CD34+, CD133+, VEGFR2+ (KDR)	Circulating and resident from bone marrow; peripheral blood; umbilical cord blood; hematopoietic stem cells; hemangioblast; fat tissues	
Circulating total PC	CD133+; CD34+; CD133+/CD34+	Circulating	Baker <i>et al</i> ^[105]
Circulating EC	CD146+/CD31+	Circulating	
Circulating EPC	CD34+ VEGFR+ CD133+	Circulating	
Circulating EPC	(CD34+/CD133-/KDR+/CD45-) expressing VDR+ or OCN+, and VDR+ and OCN+	Circulating (in chronic kidney disease)	Cianciolo <i>et al</i> ^[106]
Circulating EPC	CD133+, CD34+, KDR+	Circulating (in rheumatoid arthritis with coronary Calcification)	Yiu <i>et al</i> ^[107]
OPs in human			
OP	CD44+, CD63+, CD146+, Stro-1+	Resident bone marrow	Gronthos <i>et al</i> ^[108]
CVC	3G5+	Resident from arteries (Pericytes markers)	Bostrom <i>et al</i> ^[109]
Circulating osteocalcin-positive mononuclear cells	OCN+	Circulating bone marrow derived	Pal <i>et al</i> ^[93]
Circulating progenitor cells (Pro-calcific differentiation)	CD34+/OCN+; CD34+/BAP+; CD34+/OCN+/BAP+; OCN+/KDR+ ratio; BAP+/KDR+ ratio; OCN+/BAP+/KDR+ ratio	Circulating (in diabete mellitus)	Fadini <i>et al</i> ^[110]
Circulating EPC expressing osteocalcin	OCN+/CD133+/CD34-/KDR+	Circulating (in cardiovascular disease)	Flammer <i>et al</i> ^[95]
Circulating EPC expressing osteocalcin	OCN+/CD133+/CD34-/KDR+	Circulating (in coronary atherosclerosis, plaques instability)	Gössl <i>et al</i> ^[97]
Circulating T cells	CD28- CD8 +T cells	Circulating (in calcific aortic stenosis)	Winchester <i>et al</i> ^[111]
OPs in mouse models			
Mesenchymal OPs	CD45-/TER119-/ Sca-1+/PDGFRa+	Resident in the mouse bone marrow	Morikawa <i>et al</i> ^[112]
Bone marrow-derived calcifying cells	Sca-1+/PDGFRa- and Sca-1+/PDGFRa+	Resident from mice aorta	Cho <i>et al</i> ^[113]
Circulating osteogenic cells	Sca-1+, PDGFRa+, CD45-, CD44+, CXCR4+	Circulating in ectopic bone formation in a mouse model	Otsuru <i>et al</i> ^[114]

MSC: Mesenchymal stromal cells; EPC: Endothelial progenitors cells; CEC: Circulating endothelial cells; vw-MPSC: Vascular wall-resident multipotent stem cells; vw-MSC: Vascular wall-resident multipotent mesenchymal stem cells; PC: Progenitors cells; CVC: Calcifying vascular cells; OPs: Osteoprogenitors; CD: Cluster of differentiation; Oct-4: Octamer-binding transcription factor 4; VEGFR: Vascular endothelial growth factor receptor; OCN: Osteocalcin; VEGFR2 (KDR): Kinase insert domain receptor; VDR: Vitamin D receptor; BAP: Bone alkaline phosphatase; Sca-1: Stem cells antigen-1; PDGFR: Platelet-derived growth factor receptor; CXCR-4: C-X-C chemokine receptor type 4.

have a genetic component that appears to be partially independent of those involved in atherogenesis. Specific genes that have been linked to arterial calcification in humans are also involved in atherosclerosis and include angiotensin I-converting enzyme, apo E, E-selectin, MMP-3, MGP, CC chemokine receptor 2, and estrogen receptor α ^[11].

New processing techniques of calcified tissue

Due to the tissue composition, morphological analysis of calcified or bone-like tissue is often incomplete: the decalcification procedure degrades the 3D structure of cells and hydrolyses the nucleic acid molecule^[98]. Decalcification procedure with ethylenediaminetetraacetic acid or chloride acid put significant limitations to the study of

ectopic tissue calcification. Based on this consideration, we recently decided to apply a new technique to preserve nuclear morphology and nucleic acid content, whilst preserving the 3D cellular structure. This protocol was patented at the Massachusetts Institute of Technology of Cambridge (Patent number WO2006009860 A3)^[99,100]. Thanks to this method, a new set of cells missed for almost 100 years^[101] were discovered: the shape of the nucleus was difficult to spot because of the standard 2.5 μ m cut. Metakaryotic cells, also called bell-shaped cells, were identified first in developing fetus, then in adult cancerous tissue and finally in vascular tissue and represent the first possible evidence of stem cells lineages^[53,100,102].

Briefly, the spreading protocol^[99,100] is based on the digestion of Carnoy fixed tissue with of a collagenase

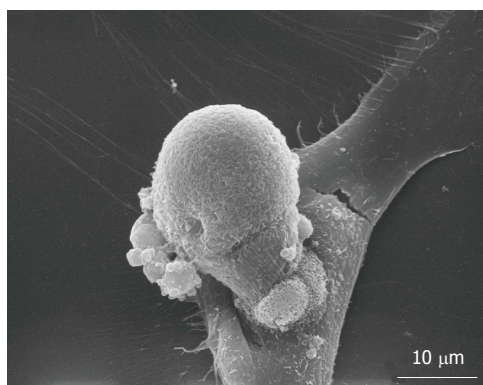


Figure 4 Scanning electron microscopy. Budding process in vascular wall-resident multipotent stem cells *in vitro*.

type II enzyme that slowly disaggregate calcified tissue, after maceration in acetic acid, tissue are spread on a slide in a single monolayer of cells. At this point, standard immunohistochemical and molecular analyses could be performed: the result is that morphology of single cells from mineralized tissues is visible (Fittipaldi *et al* unpublished data).

CONCLUSION

As it is evident from Table 1, the literature of the last 20 years concerning stem cells is characterized by a general incongruity about which marker panel defines the progenitor cells and the different progenitor lineages. This is reflected by the Babel-like terminology used to define the progenitor cells by different groups. This issue is even foggier when it comes to the OPs identification, which became crucial in the last years, with the acceptance of the active model of vascular calcification^[11].

It becomes necessary to uniform the phenotypic definition of osteoprogenitor cells. Paradoxically, the typical morphology of resident osteoblasts and osteoclasts could be of help, since these cells are easily recognizable at optical microscopy. Therefore, the morphology could constitute the basis of the future identification of those resident cells which deserve more attention for the identification of their phenotype. These observations will push towards the study of alternative methods of morphological analysis, including the spreading analysis on calcified tissue, which opens the novel possibility to have more information on DNA and proteins composing bone-like tissue. Twenty years ago, in a study on ectopic bone formation, Solari *et al*^[115] found that osteoclasts undergo amitotic division, and that a budding process is responsible of their division (Figure 4). Recently, these results were fuelled by the finding that some cells with the characteristics of stem cells divide by an amitotic mechanism, using a RNA-DNA intermediate^[116]. We recently found cells with the same characteristics in adult pathological arteries (Fittipaldi *et al* unpublished data).

Most of the definitions (and incongruity) of the stem cells derive from osteo-chondrogenic differentiation

studies on cultured cells. In these *in vitro* models, cells are induced to differentiate by definite exogenous stimuli, which do not correspond to the vessel wall microenvironment during the *in vivo* calcification process. In our opinion, another way to overcome these incongruities in the future, apart from morphology, is the molecular approach, *i.e.*, the identification of one or more markers to locate *in situ* the progenitor cells and the osteogenic precursors in the vessel wall, as well as the definition of the resident amitotic cells. A promising approach to definitely decipher all the markers characterizing the osteoprogenitor cells could be a combined mRNA profiling and gene set analysis, as already performed on the early and late EPC^[117], in order to be able to apply more doable techniques such as immunohistochemistry, immunofluorescence or *in situ* hybridization.

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Stem cell application for osteoarthritis in the knee joint: A minireview

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Abstract

Knee osteoarthritis is a chronic, indolent disease that will affect an ever increasing number of patients, especially the elderly and the obese. It is characterized by degeneration of the cartilage substance inside the knee which leads to pain, stiffness and tenderness. By some estimations in 2030, only in the United States, this medical condition will burden 67 million people. While conventional treatments like physiotherapy or drugs offer temporary relief of clinical symptoms, restoration of normal cartilage function has been difficult to achieve. Moreover, in severe cases of knee osteoarthritis total knee replacement may be required. Total knee replacements come together with high effort and costs and are not always successful. The aim of this review is to outline the latest advances in stem cell therapy for knee osteoarthritis as well as highlight some of the advantages of stem cell therapy over traditional approaches aimed at restoration of cartilage function in the knee. In addition to the latest advances in the field, challenges associated with stem cell therapy regarding knee cartilage regeneration and chondrogenesis *in vitro* and *in vivo* are also outlined and analyzed. Further-

more, based on their critical assessment of the present academic literature the authors of this review share their vision about the future of stem cell applications in the treatment of knee osteoarthritis.

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Key words: Multipotent adult mesenchymal stem cells; Osteoarthritis; Knee joint; Clinical trial

Core tip: Knee osteoarthritis is a common medical condition in the elderly and the obese. Despite the variety of available conventional treatments for this disease, in recent years stem cell therapy has been applied in an ever increasing number of clinical cases. Therefore the aim of this review is to outline the latest advances in stem cell therapy as a non-pharmacologic treatment for knee osteoarthritis. It also emphasizes on some of the challenges associated with stem cell therapy regarding knee cartilage regeneration and chondrogenesis *in vitro* and *in vivo*.

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INTRODUCTION

Osteoarthritis (OA) of the knee is a chronic, indolent disease that affects all genders, ages and races but is known to be most common in the elderly and in obese people. A degenerative disease of the connective tissue, it mainly affects the articular cartilage (Figure 1)^[1]. The definition of knee OA varies in reported studies and includes self-reported knee OA (obtained from a questionnaire), radio-

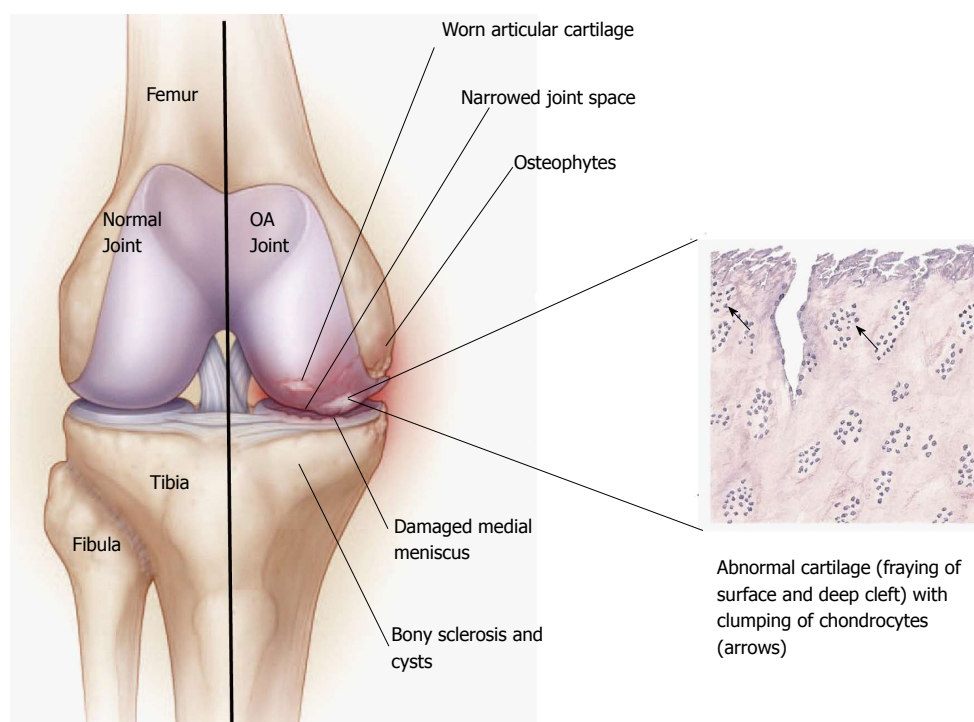


Figure 1 Pathophysiology of knee osteoarthritis. Comparison between a normal and diseased joint (Illustration created after Felson^[3] and Buja *et al*^[4])

graphic definitions of knee osteoarthritis, and symptomatic knee OA (self-reported joint pain and radiographic evidence of OA)^[2]. Symptoms may include joint pain, stiffness and tenderness. Furthermore, as the cartilage substance decreases, the bone surface may also become affected. This results in development of osteophytes (bone spurs) and direct bone-bone contact. In addition to the stiffness of the joint, the patient tries to avoid pain by minimizing joint movement, which leads to muscle atrophy and laxity of the ligaments^[1-4].

The pathogenesis of knee OA have been linked to biomechanical and biochemical changes in the cartilage of the knee joint (*e.g.*, inability to withstand normal mechanical stresses, limited supply of nutrients and oxygen, inadequate synthesis of extracellular matrix components, increased synthesis of tissue-destructive proteinases (matrix metalloproteinases and aggrecanases) and overall apoptosis of chondrocytes)^[4-7]. Recently, synovial inflammation has also been accredited as a factor limiting knee cartilage repair. Moreover, it correlates to clinical signs of knee OA such as swelling of the knee and inflammatory pain^[7,8]. It is believed that synovial inflammation is a response of synovial macrophages to cartilage debris and catabolic mediators entering the synovial cavity^[8,9].

In regards to the epidemiology of knee OA (Table 1), studies indicate that knee osteoarthritis in men aged 60 to 64 is usually found in the right knee (23%) than in the left knee (16.3%), while distribution seems to be more evenly balanced in women of the same age (right knee, 24.2%; left knee, 24.7%)^[6,10]. A variety of endogenous (*e.g.*, age, sex) and exogenous (obesity, patient's lifestyle) risk factors for OA have also been outlined^[2,6,11-14]. Recently, a number

of genome wide association studies (GWAS) (*e.g.*, Rotterdam GWAS^[15], Tokyo GWAS^[15], Chingford Study^[16]) have highlighted the significance of gene mutations (*e.g.*, in *GDF5*) for the development of knee OA^[15-21]. Additionally, cross-sectional studies indicate that the risk of knee OA is 1.9 to 13.0 times higher among underground coal miners when compared to a control population; presumably, due to frequent work in the kneeling or squatting position^[6]. Construction workers, especially floorers, also have a significantly elevated prevalence of knee OA^[6].

As of clinical diagnosis of knee OA, it is complex as during the physical examination of the patient it is needed to confirm and characterise joint involvement, as well as to exclude pain and functional syndromes linked to other causes (*e.g.*, inflammatory arthritis or damaged meniscus)^[3,11,22]. In addition to non-surgical treatments for this condition such as physiotherapy, diet rich in vitamin D and supportive sport (*e.g.*, swimming)^[10,23,24], there are several medicinal and homeopathic products on the market, which promise pain relief and a decrease in symptoms. However, researchers are keen to investigate new treatments to combat OA of the knee.

STEM CELL TREATMENT

Self-regeneration of the cartilage, which includes chondrocytes, ground substance (cartilage matrix) and elastin fibers, is a slow process which results in new cartilage substance that is not stable for intensive burdens. The fluid inside the joint contains mesenchymal stem cells (MSCs) which can differentiate into chondrocytes, but new deposited cartilage is very fragile and can be de-

Table 1 Worldwide prevalence (2005) of knee osteoarthritis

	Knee OA prevalence
Europe	
Western	0.1689
Central	0.1889
Eastern	0.1914
Asia	
Middle East	0.1764
South	0.1563
East	0.1683
Southeast	0.1704
Central	0.1854
Pacific	0.1704
Africa	
North	0.1764
West Sub-Saharan	0.1574
East Sub-Saharan	0.1544
Central Sub-Saharan	0.1528
South	0.1822
Australia and Oceania	
Australia	0.1736
Oceania	0.1813
North America	
United States and Canada	0.1792
Central America	0.1777
Caribbean	0.1756
South America	
Andean	0.1751
Tropical	0.1691
Southern	0.1693

Combined value for male and female, aged 30-100 (Data adapted from March *et al*^[10]). OA: Osteoarthritis.

stroyed by applying a minimal amount of stress on the joint. Additionally there is only a limited quantity of MSCs in the joint available to differentiate and the process of differentiation is slow^[1,25].

STEM CELL MANAGEMENT

The aim in using stem cells is to support the self-healing process of the knee joint cartilage which results in relief from OA symptoms^[26-32]. This treatment should be used in conjunction with additional treatment in order to improve patients' functional status and quality of life. However, osteoarthritis cannot be cured by any radical treatment at the moment.

The stem cell candidates for use in these therapies are multipotent adult MSCs, because they are available in several tissues, including in the fluid inside the joint, and have the ability to differentiate into cells of the chondrogenic lineage^[33,34]. Pittenger *et al*^[35] have described that MSCs could be cultured without losing their multilineage differentiation potential and it has been shown that MSCs are capable of undergoing chondrogenic differentiation both *in-vitro* and *in-vivo*. MSCs can be harvested from bone marrow, periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle and deciduous teeth^[36]. Regardless of their origin they have the capacity to differentiate into many cell types, including cells of connective tissue lineages, including bone, fat, cartilage and

muscle^[26,37]. MSCs were first identified in the pioneering studies of Friedenstein and Petrakova (1966)^[33] and are of major interest of research in the treatment of arthritis, in particular OA.

Multipotent adult mesenchymal stem cells are extensively investigated - in particular their behaviour in cell culture: how do they stay multipotent after several passages; how is chondrogenesis triggered in MSCs^[32]. There are no definitive markers identified for MSCs yet, but the immunophenotype is positive for the proteins and enzymes STRO-1, CD73, CD146, CD105, CD106, CD166 and negative for CD11b, CD45, CD34, CD31 and CD117. These are the most reliable for characterizing MSCs^[34,36].

There are several other criteria which must be considered when growing MSCs in culture. One of the most crucial criteria is the availability of characterized factors which stimulate the anabolic activity in cartilage including transforming growth factor (TGF)- β , bone morphogenetic protein (BMP), fibroblast growth factors (FGF), insulin growth factor (IGF)-1, hedgehog (hh) and Wntless (Wnt) proteins^[26]. These factors are signalling proteins that belong to the tyrosine kinase family of proteins (transmembrane proteins) that activate several downstream processes leading to cell proliferation, survival, growth and a reduction in apoptotic signalling.

Growth factors like FGF2 or transforming growth factor beta induce a positive differentiation of MSCs^[38]. Moreover, the development of methods was required to develop the cartilage phenotype without hypertrophy, fibrinogenesis or ossification. In addition, a delivery system was devised to target cells in a lesion, but without inhibiting their chondrogenic differentiation or the integrity of repaired tissue^[39].

CLINICAL TRIALS

In recent years several clinical protocols for MSCs have been tested^[26-32,40]. In general, MSC related therapeutic approaches have a significant advantage to traditional surgical approaches such as autologous chondrocyte transplantation: no cartilage biopsy is necessary, thus no external stress and cellular damage are applied at the donor-site articular surface^[31]. Moreover, direct intra-articular injection of MSC is perceived as a technically simple way to treat advanced OA of the knee^[32].

Stem cells from patients

MSCs and platelet-rich plasma are harvested from the patient to be treated thus ensuring that the patient's immune system will not reject the cells^[41]. These cells are already specific for the patient's body but they have to be processed before intra-articular injection in the knee joint. This process includes separation of the MSCs by centrifugation and other purification steps. With the aim in mind of increasing cartilage build-up, chondrogenic activity of the harvested cells has to be evaluated, as well as glycosaminoglycan and type II collagen deposition, before reinjection^[29]. The MSCs are tested *in vitro* for their

ability to undergo chondrogenic differentiation under the previous described conditions. Glycosaminoglycan and type II collagen are components of the matrix of cartilage which induces and supports the differentiation of MSCs into chondrocytes. During this procedure it is important that the joint is stressed as little as possible because the newly differentiated cartilage is highly susceptible to damage.

In regards to recent advancements in the field, Neporent^[42] mentioned several pro and contra factors for stem cell injection in the knee joint. MSCs treatment offers the significant advantage of a quick and relatively uneventful recovery. Furthermore the majority of patients became ambulatory within 24 h. There are no reasonable arguments against treatment with the patient's stem cells, but there are several issues that have to be considered that are likely to make it financially less attractive. Firstly, at approximately \$4000 per knee for stem cell reinjection, which will not be covered by health insurance, this treatment is not for affordable by everyone. Secondly, there are several criteria for eligibility for treatment of osteoarthritis with stem cells preparations. For one thing, the body-mass-index (BMI) should not be more than 35. Obesity, as previously mentioned, is a high risk factor for OA, because of the high stress which results on the knee joint. Stem cell treatment is reasonable, if it can be ensured that there would be no high stress on the joint. Furthermore this treatment is applicable only if the degeneration of the cartilage is not complete. As long as cartilage and joint fluid is available, stem cells can differentiate, because of necessary factors are present in the fluid and matrix but in severe cases, with bone-bone contact, stem cell treatment is unlikely to work. Most important for the patient is to minimize physical activity in the immediate period after the therapy because the stress to the joint reduces the chance of successful recovery. Furthermore it is likely that more than one treatment session would be required, meaning a greater investment of time and money.

In addition to the intra-articular injection of MSCs, Nöth *et al.*^[32] also highlighted the use of MSCs as progenitor cells to engineer cartilage implants that can be used to repair chondral and osteochondral lesions, or as trophic producers of bioactive factors to initiate endogenous regenerative activities in the OA joint.

Stem cells from donors

Another potential source of stem cells, which can be used in therapies, is allogeneic MSCs. They are harvested from donated human umbilical cord tissue (HUCT) after normal, healthy births where the mother has been tested for infectious diseases and has a screened medical history. These harvested MSCs are then screened to International Blood Bank Standards (Stem Cell Institute, 2012).

Umbilical cord tissue provides an abundant supply of mesenchymal stem cells avoiding the requirement to harvest stem cells by invasive procedures such as liposuction or bone marrow aspiration. There is evidence show-

ing that mesenchymal stem cells from umbilical cords are more robust than those from other sources such as fat^[43].

Rush University Medical Center^[44], 2013, described the preparation of MSCs harvested from donated umbilical cord tissue: The cells are mixed with hyaluronan, a natural polymer that plays an important role in wound healing and deposition of cartilage, and are subsequently re-injected into the knee joint. In addition they also described a two-year Phase I/ II a clinical study in which a total of 12 participants aged 18 years and older, with a body mass index of less than 35 were enrolled. Initially, six individuals with lesions sized 2 to 5 cm were recruited into the study and an additional six volunteers with lesions larger than 5 cm were enrolled subsequently. Each participant went through an eligibility screening followed by a 12-mo observation period to determine the safety and efficacy of the therapy with an additional long-term follow-up evaluation at 24 mo.

Basically both treatment protocols, both for the MSCs from the patient and from a donor, were identical. Any differences in the MSCs and in some characteristics of the cells arose due to those from the patient themselves, from fat or bone marrow, being "older" than MSCs from umbilical cord and may therefore lack potential for proliferation and/or differentiation.

CONCLUSION

In recent years the role of stem cells in health and disease is a topic of high interest for biomedical research, especially regenerative medicine^[33,45,46], including non-pharmacologic treatment of knee OA^[25,40,47], and drug discovery^[48-50]. At the moment there is an increase in the number of clinical cases utilizing stem cell therapy for knee OA, however, many clinical protocols are still under development^[26,30,40].

Future perspectives about clinical trials with stem cells from patients

Based on the current status of clinical investigations regarding autologous stem cell therapy for OA of the knee some authors have expressed concerns about the issues of dosing, timing of intervention, type of MSCs, mode and route of delivery of MSCs in clinical studies^[51-56]. Therefore the need for a gold standard for autologous stem cell therapy for knee OA arises, which (hopefully) will be the aim of future clinical trials. Another interesting trend is the increased research interest in scaffold assisted or scaffoldless grafts of MSCs as a method to restore the structural and biomechanical characteristics of the OA affected knee^[57-62]. MSC grafts may even prove to be a viable alternative to total knee replacement in the near future. However, we still have to wait for a 100% effective and also low cost clinical procedure to be developed.

Future perspectives about clinical trials with stem cells from donors

The use of human umbilical cord-derived mesenchymal

stem cells (hUC-MSCs) in clinical trials for treatment of knee OA faces the same challenges as clinical trials with other types of MSC in terms of stem cell handling^[43]. There is also the need for more relevant clinical data, so it would be beneficial to have more clinical trials for knee OA, which utilize hUC-MSCs.

Future perspectives about basic research in knee cartilage regeneration and chondrogenesis *in vitro* and *in vivo*

Nowadays basic research in chondrogenesis *in vitro* and *in vivo* is primarily focus on increasing the efficacy of stem cells in terms of tissue repair^[57-62]. However, the issues of stem cell characterization and tumorigenesis *in vivo* are somewhat overlooked.

Until relatively recently, the genomic profile of the stem cell lines maintained *in vitro* was only assessed in terms of ploidy and karyotype, as it was known that cultured cells may exhibit loss or gain of chromosome fragments or whole chromosomes and/or genomic rearrangements^[63-65]. After the introduction of the concept for individual capacity for DNA repair and for maintenance of genomic integrity in research and diagnostic practice, its applicability as a complex marker for the proliferative potential and/or the differentiation capacity of undifferentiated cells has been extensively discussed^[66-69]. Some authors have advised that the minimal panel for characterisation of *in vitro* maintained pluripotent cell lines ought to include markers for individual capacity for repair of genotoxic damage and maintenance of genomic integrity^[69-71]. Some stem cells types (mesenchymal stem cells, haematopoietic cells from bone marrow and iPSC) have been shown to lose *TP53* gene copies during *in vitro* culturing (detected as loss of heterozygosity for markers at the *TP53* locus)^[72]. Shetzer *et al.*^[72] also reported that the cells with loss of heterozygosity were more often than not identified as the origin of the teratoma-like tumours developing after the cells were transplanted in mice.

All those findings in basic stem cell biology will likely influence the development of more advanced (in terms of cell characterization) stem cell culturing and differentiation protocols and lead to the development of a gold standard in clinical trials with MSCs.

Conclusion

In conclusion, stem cell therapy may not become a standard treatment for knee OA till the end of the decade due to various aspects regarding the clinical safety (*e.g.*, risk of complications after surgery, compatibility of donor stem cells) and the affordability of this treatment for the general public. Moreover, there is still no sufficient amount of clinical data on the effectiveness of stem cell therapy when compared with pharmacological treatments for this particular disease^[47]. There is also the emerging application of nutraceuticals as a possible alternative to drugs for knee osteoarthritis^[73,74]. So here comes the question: what will future clinical trials for knee OA and OA in general evaluate: novel pharmaceuticals, novel nu-

traceuticals, improved stem cell therapies?

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Endothelial progenitor cells in cardiovascular diseases

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Abstract

Endothelial dysfunction has been associated with the development of atherosclerosis and cardiovascular diseases. Adult endothelial progenitor cells (EPCs) are derived from hematopoietic stem cells and are capable of forming new blood vessels through a process of vasculogenesis. There are studies which report correlations between circulating EPCs and cardiovascular risk factors. There are also studies on how pharmacotherapies may influence levels of circulating EPCs. In this review, we discuss the potential role of endothelial progenitor cells as both diagnostic and prognostic biomarkers. In addition, we look at the interaction between cardiovascular pharmacotherapies and endothelial progenitor cells. We also discuss how EPCs can be used directly and indirectly as a therapeutic agent. Finally, we evaluate the challenges facing EPC research and how these may be overcome.

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Key words: Endothelial progenitor cells; Cardiovascular

diseases; Hypertension; Diabetes; Dyslipidemia; Therapy; Stents

Core tip: Our review summarizes the important associations between endothelial progenitor cells, cardiovascular risks, drugs and diseases. Current pharmacotherapies may enhance endothelial progenitor cell numbers and function. These and the evolving endothelial progenitor cell-based therapies may be important in the future treatment of cardiovascular diseases.

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INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of mortality in both developed and developing countries^[1]. Angiogenesis, the formation of new blood vessels, has attracted interest in the field of cardiology^[2]. It was believed that angiogenesis could only occur by the new blood vessels sprouting out of pre-existing vessels. Under physiological conditions, vascular endothelium secretes substances that alter vascular tone and “defend” the vessel wall from inflammatory cell infiltration, thrombus formation and vascular smooth muscle cell proliferation^[3]. Indeed, endothelial damage has been implicated in atherosclerosis, thrombosis and hypertension. The balance between endothelial injury and recovery is important for reducing cardiovascular events^[4]. However, mature endothelial cells possess limited regenerative capacity. There is growing interest in circulating endothelial progenitor cells (EPCs) as they may maintain endothelial integrity, function and postnatal neovascularization^[4].

EPC

Differentiation of mesodermal cells to angioblasts and subsequent endothelial differentiation was thought to

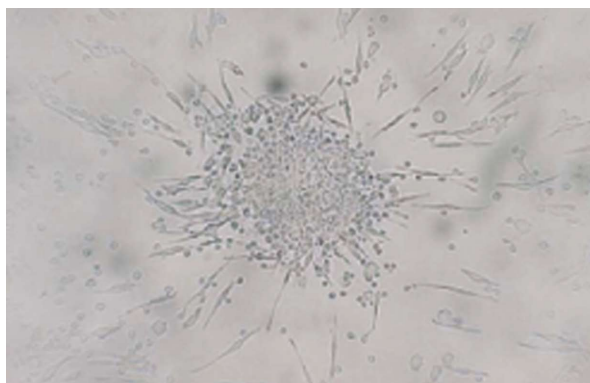


Figure 1 Colony forming unit isolated from human peripheral blood mononuclear cells using commercial colony forming unit-hill assay.

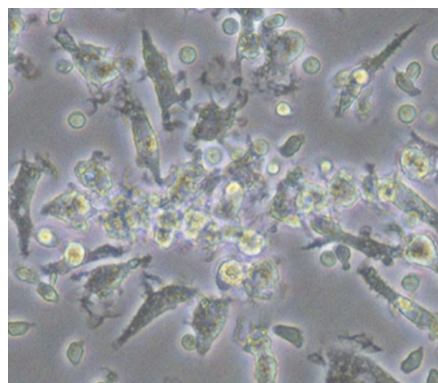


Figure 2 Cobble-shaped outgrowth endothelial progenitor cells from human peripheral blood at day 14.

exclusively happen in embryonic development. This concept was overturned in 1997 when Asahara *et al*^[5] published that purified CD34-positive hematopoietic progenitor cells from adults can differentiate *ex vivo* to an endothelial phenotype. These EPCs showed expression of various endothelial markers and are incorporated into neovessels at sites of ischemia.

EPCs appear to be a heterogeneous group of cells originating from multiple precursors within the bone marrow and present in different stages of endothelial differentiation in peripheral blood. For this reason, the precise characterization of EPCs is difficult because many of the cell surface markers used in phenotyping are shared by hematopoietic stem cells and by adult endothelial cells^[6].

Currently, EPCs are defined as cells positive for both a hematopoietic stem cell marker such as CD34 and an endothelial marker protein such as VEGFR2. CD34 is not exclusively expressed on hematopoietic stem cells but also on mature endothelial cells. Other studies have used the more immature hematopoietic stem cell marker CD133 and demonstrated that purified CD133-positive cells can differentiate to endothelial cells *in vitro*^[7]. CD133, also known as prominin or AC133, is a highly conserved antigen with unknown biological activity which is expressed on hematopoietic stem cells but is absent on mature endothelial cells and monocytic cells^[7]. Thus, CD133⁺VEGFR2⁺ cells more likely reflect immature progenitor cells, whereas CD34⁺VEGFR2⁺ may represent shed cells of the vessel wall^[8]. Controversy remains with respect to the identification and the origin of endothelial progenitor cells which are isolated from peripheral blood mononuclear cells by cultivation in medium favoring endothelial differentiation.

TYPES OF EPC

Although the markers for identification of EPC populations vary between studies, it has been agreed that there are lineage and functional heterogeneities within the EPC population. There are at least two different types of EPCs: the early and late EPCs. Early EPCs are usually

referred to as the angiogenic EPC population obtained from short-term cultures of 4-7 d *in vitro*. These early EPCs form colony forming units (CFU) and possess many endothelial characteristics, such as harboring markers of CD31, TIE2 and VEGFR2^[5]. Hill *et al*^[9] reported a negative correlation between EPCs, measured by CFU and Framingham risk score in 45 men with various cardiovascular risks. They also reported a positive correlation between CFU and brachial flow-mediated dilation, a measure of endothelial function. Late EPCs, often called out-growth EPCs, have different growth patterns and are usually obtained from long term cultures of at least 2-3 wk *in vitro*. Outgrowth EPCs possess additional endothelial characteristics, such as VE-cadherin and von Willebrand factor, in addition to CD31, CD133, CD34 and VEGFR2^[4]. These outgrowth EPCs will further differentiate into mature endothelial cells for angiogenesis and vasculogenesis. These two types of cells have distinct morphology: the early EPCs have a spindle shape (Figure 1) while outgrowth EPCs have a cobblestone-like shape (Figure 2).

Although endothelial dysfunction is associated with the development of atherosclerosis^[10], the utility of EPCs as a prognostic marker has only recently been demonstrated. In a study with 44 patients with coronary artery disease (CAD) and 33 patients with acute coronary syndrome followed up for a median of 10 mo duration, a reduced number of EPCs was associated with a significantly higher incidence of cardiovascular events^[11]. In another larger study with 519 patients with stable CAD, increased levels of endothelial progenitor cells were related to a reduced risk of death from cardiovascular causes, a first major cardiovascular event, revascularization and hospitalization^[12].

However, issues in terms of isolation and identification of EPCs, especially in regards to the characterization or specific cell surface markers of these cells, are still unresolved. In addition, number and/or functionality of EPCs do not adequately describe cardiovascular disease risks. These limitations may be attributable to the inconsistent definitions of EPCs, the number of existing cardiovascular risk factors in different patient populations

Table 1 Effect of peripheral arterial disease on endothelial progenitor cells

Ref.	Subjects	EPCs (number/function)	Findings
Fadini <i>et al</i> ^[14]	55 diabetic without PAD 72 diabetic with PAD	CD34 ⁺ /CD133 ⁺ /KDR ⁺	CD34 ⁺ /CD133 ⁺ /KDR ⁺ is significantly lower in diabetics with PAD compared to diabetics alone
Fadini <i>et al</i> ^[15]	15 healthy controls 30 PAD	CD34 ⁺ /KDR ⁺	CD34 ⁺ /KDR ⁺ is significantly lower in patients with PAD than controls
Delva <i>et al</i> ^[17]	24 healthy controls 45 PAD	CFU CD133 ⁺ , CD34 ⁺ , CD34 ⁺ /KDR ⁺	CFU is significantly increased in patients with PAD compared to controls CD34 ⁺ and CD133 ⁺ are significantly decreased in patients with PAD compared to controls
Morishita <i>et al</i> ^[16]	22 healthy controls 48 PAD	CD34 ⁺ /CD133 ⁺ /KDR ⁺	No difference between groups for CD34 ⁺ /KDR ⁺ CD34 ⁺ /CD133 ⁺ /KDR ⁺ is significantly higher in PAD compared to controls

EPC: Endothelial progenitor cells; PAD: Peripheral arterial disease; CFU: Colony forming units.

and the interaction between EPCs and other hematopoietic progenitor, inflammatory cells or platelets. There is also evidence of varied levels of circulating EPCs that are present in a time dependent manner^[13]. Therefore, depending on when sampling occurs, EPC numbers and functions may be different.

Peripheral arterial disease

Peripheral arterial disease (PAD) is a manifestation of advanced atherosclerosis and affects 20% of the population aged over 65 years. PAD is associated with endothelial dysfunction but there have been limited and inconsistent data available on the number and functional capacity of EPCs in PAD. Fadini *et al*^[14] first demonstrated that the number of EPCs marked by CD34⁺/CD133⁺/KDR⁺ is significantly decreased in diabetic patients with PAD compared to diabetics alone. This finding was further supported by another paper from Fadini where they reported significantly lower CD34⁺/KDR⁺ EPCs in PAD patients compared to healthy controls^[15]. On the other hand, several studies have documented an increased number and functionality of EPCs in PAD patients compared to controls^[15] (Table 1). Both studies reported poor angiogenic response to ischemia and EPC differentiation in PAD patients, together with reduced angiogenesis and low EPC levels^[14,15]. In PAD, EPC mobilization can occur through inflammation and matrix metalloproteinase-mediated mechanisms^[16]. Membrane type 1 matrix metalloproteinase (MT1-MMP) can contribute to vascular remodeling and regulate mobilization of CD34⁺ progenitor cells, while pentraxin-3 is predominantly produced by vascular endothelium and is considered to reflect inflammatory status of endothelium^[16]. There appeared to be an increased number of EPCs and pentraxin-3 and decreased MT1-MMP in PAD patients compared to healthy controls^[16]. Furthermore, cardiovascular events were also significantly correlated with decreased EPC levels and increased oxidative stress. In contrast, the number of EPCs was shown to be significantly higher in severe PAD patients compared to healthy subjects^[17]. These contrasting results may be present due to the different severity of PAD patients recruited in the study and methodological differences

in measuring EPC population which can complicate the interpretation of data. It is also possible that when PAD is only mild, EPC levels correlate to the poor vascular health. However, in severe PAD an elevated number of circulating EPCs may reflect mobilization from the bone marrow to repair endothelial damage. More studies are warranted to investigate these discrepancies in EPC and PAD.

CAD

The presence and extent of endothelial dysfunction predicts the outcome in patients with cardiovascular risk factors and in patients with coronary artery disease. Since endothelial progenitor cells possess the ability to home in on sites of vascular injury, there is emerging interest in the therapeutic use of EPCs related to angiogenesis. In patients with CAD, isolated EPCs had an impaired migratory response and a negative correlation of EPCs with the severity of CAD^[18]. This was likely a result of endothelial dysfunction in patients with CAD^[18], impaired coronary blood flow regulation and the strong association with risk factors for CAD. These risk factors may interfere with signaling pathways regulating EPC mobilization and differentiation, such as those involving granulocyte-stimulating colony stimulating factor (G-CSF) or vascular endothelial growth factor (VEGF). Impaired migratory response affected by downregulation of VEGF may be contributed to by VEGFR2. In addition, several studies documented a decreased number of EPCs in CAD patients^[19-22]. Circulating EPCs are also significantly lower in patients with progression of CAD angiographically^[22]. Exhaustion of endothelial progenitor cells in the bone marrow, reduced nitric oxide bioavailability and long term statin treatment in CAD can also contribute to the reduced number and impairment of EPCs^[21]. However, there are contrasting studies that reported an increased number of EPCs in angiographically significant CAD patients. A significant correlation was observed between the maximum stenosis severity and the number of EPCs from these patients^[23]. Werner *et al*^[24] also observed an inverse association between the level of circulating EPCs and the risk of cardiovascular events among patients with angiographically documented CAD. The

Table 2 Effect of coronary artery disease on endothelial progenitor cells

Ref.	Subjects	EPCs (number/function)	Findings
Vasa <i>et al</i> ^[18]	9 healthy controls 45 CAD	CD34 ⁺ /KDR ⁺ (flow cytometry) Migratory activity	Both CD34 ⁺ /KDR ⁺ and migratory activity were impaired in patients with CAD compared to controls
Eizawa <i>et al</i> ^[19]	36 healthy controls 34 stable CAD	CD34 ⁺ (flow cytometry)	CD34 ⁺ is significantly decreased in patients with stable CAD
Wang <i>et al</i> ^[20]	44 controls 35 mild CAD 25 severe CAD	CD34 ⁺ /KDR ⁺ (flow cytometry) Migratory activity	CD34 ⁺ /KDR ⁺ is the lowest in severe CAD followed by mild CAD Migratory activity is also impaired in CAD patients
Liguori <i>et al</i> ^[21]	15 healthy controls 40 CHD	CFU CD34 ⁺ (flow cytometry) Migratory activity	CFU, CD34 ⁺ and migratory capacity were significantly impaired in patients with CHD CHD is the main predictor which impairs CFU capacity
Briguori <i>et al</i> ^[22]	136 CAD	CFU CD34 ⁺ /KDR ⁺ (flow cytometry)	Low levels of CFU and CD34 ⁺ /KDR ⁺ predict CAD progression
Güven <i>et al</i> ^[23]	24 controls 24 CAD	CD34 ⁺ (flow cytometry)	CD34 ⁺ EPC is significantly elevated in CAD patients compared to controls
Werner <i>et al</i> ^[24]	90 CHD	CFU CD34 ⁺ /KDR ⁺ (flow cytometry)	EPCs is also positively correlated with maximum stenosis CD34 ⁺ /KDR ⁺ and CFU positively correlate with endothelium-dependent vasodilation (acetylcholine infusion)

CAD: Coronary artery disease; CFU: Colony forming unit; CHD: Coronary heart disease; EPC: Endothelial progenitor cells.

HMG-CoA reductase inhibitors (statins)
 Atorvastatin
 Rosuvastatin
 Pravastatin
 Biguanide
 Metformin
 TZDs
 Pioglitazone
 DPP4I
 Sitagliptin
 ARBs
 Losartan
 Candesartan
 Telmisartan
 Valsartan
 ACEI
 Ramipril
 Enalapril
 Zofenopril
 CCBs
 Nifedipine
 Barnidipine

Figure 3 Cardiovascular-related pharmacological therapies which may affect numbers and function of endothelial progenitor cells. TZDs: Thiazolidinedione; DPP4I: Dipeptidyl peptidase 4 inhibitors; ARBs: Angiotensin II receptor blockers; ACEI: Angiotensin converting enzyme inhibitors; CCBs: Calcium channel blockers.

differences in the methodologies are likely to account for the different results. In addition, low frequency of EPCs in circulation and types of EPCs harvested may also contribute to the differences. Moreover, the EPC population may represent a heterogeneous population of endothelial progenitors with differing proliferative capacity. Despite these controversies, the circulating numbers of EPCs appears to predict cardiovascular outcome in patients with CAD^[24] (Table 2).

Congestive heart failure

It has been shown that endothelial dysfunction occurs in patients with congestive heart failure (CHF)^[25-27]. Despite

these observations, limited data are available regarding the pattern of mobilization of EPCs and CD34⁺ cells during HF. In a study of EPC in HF, HF was associated with higher circulating EPC levels compared to healthy controls^[28]. However, the severity of heart failure correlates with circulating EPCs inversely with significantly higher CD34⁺ counts in mild HF compared to severe HF^[29]. CHF may result in hematopoietic progenitor cells migrating to the sites of damage to undergo progenitor cell differentiation. However, a depletion of progenitor cells in the chronic stage of the disease could contribute to the biphasic bone marrow pattern of response to heart failure^[29]. Consistent with numbers, the colony forming unit, one of the functional capacities of EPCs, is an independent predictor for outcomes in CHF and is also negatively correlated with New York Heart Association functional class^[30]. Pertinent studies are summarized in Table 3.

EFFECTS OF CARDIOVASCULAR-RELATED PHARMACOTHERAPIES ON EPC

The presence of conventional cardiovascular risk factors, such as hypertension, dyslipidemia, diabetes and cardiovascular diseases, are associated with endothelial injury and dysfunction. Experimental and clinical studies evaluate endothelial dysfunction as alterations of vasomotor function, such as endothelium-dependent relaxations^[31,32]. Recent research on cell biology has identified circulating EPCs as a useful biomarker of endothelial function and integrity. Cardiovascular pharmacotherapies (Figure 3) have been shown to improve overall numbers and function of EPCs in patients with cardiovascular risks in clinical studies.

Antihypertensive medication

There are many classes of antihypertensives which lower

Table 3 Summary of clinical trials: Effect of heart failure on endothelial progenitor cells

Ref.	Subjects	EPCs (number/function)	Findings
Valgimigli <i>et al</i> ^[28]	45 healthy controls 91 CHF	CD34 ⁺ , CD34 ⁺ /CD133 ⁺ /KDR ⁺ (flow cytometry)	CD34 ⁺ and CD34 ⁺ /CD133 ⁺ /KDR ⁺ are significantly elevated in CHF patients compared to controls EPC number is negatively correlated with NYHA functional class
Nonaka-Sarukawa <i>et al</i> ^[29]	22 healthy controls 16 mild CHF 10 severe CHF	CD34 ⁺ (flow cytometry)	CD34 ⁺ is significantly higher in mild CHF compared to severe CHF
Michowitz <i>et al</i> ^[30]	107 CHF	CFU	CFU is the independent predictor for CHF CFU is also negatively correlated with NYHA functional class

EPC: Endothelial progenitor cells; CHF: Congestive heart failure; CFU: Colony forming unit; NYHA: New York Heart Association.

blood pressure by different mechanisms. Among the most widely used are angiotensin II receptor blockers (ARBs), angiotensin converting enzyme (ACE) inhibitors and calcium channel blockers (CCBs). They all have been shown to modulate EPC number and/or functions.

ARBs: Their main mechanism of action is to act on the renin-angiotensin-aldosterone system for treatment of hypertension. Several studies have explored the effect of ARBs in influencing the number and/or function of EPCs in both experimental and clinical hypertension. Three experimental studies using spontaneous hypertensive rats successfully demonstrated improved EPC numbers and function with ARB treatment^[33-35]. In hypertension, endothelial damage can be caused by reactive oxygen species (ROS) secondary to the increased production of tissue angiotensin II. Since vascular NAD(P)H oxidase is a major source of ROS in the cardiovascular system, ARBs can significantly inhibit major components of NAD(P)H oxidase. Inhibition of oxidative stress in hypertension by ARBs correlated with improvement in EPC numbers and function^[33-35]. These findings were separately validated in the clinical setting where a similar improvement in EPC numbers and function were observed in healthy subjects and those with CAD^[36,37] (Table 4). EPCs cultivated from healthy volunteers treated with telmisartan had a significantly higher number and improved function of EPCs compared to cells not treated with telmisartan^[36]. However, the increase of numbers and function of EPCs was inhibited by specific peroxisome proliferator-activated receptor- γ (PPAR- γ) inhibitor, GW9662. This suggests that telmisartan-induced EPC proliferation is likely *via* the PPAR- γ -dependent pathway. Furthermore, it has also been shown that telmisartan is a ligand of PPAR- γ ^[38]. In a double-blinded study, CAD patients with no history of hypertension receiving 80 mg of telmisartan for 4 wk had a significantly higher absolute number of EPCs compared to the placebo group. This was further supported by improvement in endothelial function in the treatment group^[37]. The improvement on EPCs in these patients with CAD was independent from the antihypertensive action of telmisartan as reduction in blood pressure was not statistically different between the groups. Therefore, ARBs may be able to induce improvement in numbers and function

of EPCs *via* pleiotropic effects. The several mechanisms include inhibition of NAD(P)H oxidases and stimulation through the PPAR- γ pathway.

ACE inhibitors: Similar to ARBs, ACE inhibitors are used to treat hypertension and congestive heart failure through inhibition of angiotensin converting enzyme which is part of the renin-angiotensin-aldosterone system. Generally, there was a positive trend towards improvement in EPC numbers^[39] and function^[39,40] with ACE inhibitors (ACEI) in patients with stable CAD and in hypertensive patients (Table 4). The administration of ramipril increased the number and improved the functional capacity of EPCs in patients with CAD within 1 wk of treatment. The improvement was further enhanced after 4 wk. Bradykinin B2 receptor pathway which activates endothelial nitric oxide synthase (eNOS) and is involved in neovascularization of EPCs may have contributed to the beneficial effects of ramipril. Indeed, nitric oxide levels were increased *via* activation of bradykinin. This effect was independent of any impact on blood pressure^[39]. Further comparison between enalapril and zofenopril demonstrated that EPCs were increased after 1 and 5 years of follow up^[40]. ACE inhibition is reported to stimulate nitric oxide (NO) activity and decreases oxidative stress in human endothelial cells^[41]. Zofenopril increases NO production in endothelium, decreases atherosclerotic development and reduces ROS^[42]. Similar to ARBs, ACE inhibition improves the number and function of EPCs independently of the blood pressure lowering effect and acts *via* the endothelial NO pathway.

CCBs: CCBs decrease blood pressure by inhibiting L-type voltage-gated calcium channels to decrease intracellular calcium. It acts on vascular smooth muscle to induce vasodilation and therefore decreases blood pressure. Preliminary results from two studies reported favorable outcomes on EPC numbers and function with CCBs in patients with essential hypertension^[43,44] (Table 4). Men with stage 1 hypertension who were treated with nifedipine had a significantly improved number and angiogenic-related function of EPCs^[43]. The improvement may have been driven by increased VEGF release from vascular smooth muscle cells by nifedipine. It was also shown that nifedipine-treated EPCs had greater resistance to ROS-

Table 4 Effect of antihypertensive medications on endothelial progenitor cells

Ref.	Subjects	Drugs	Duration	EPCs (number/function)	Findings
Angiotensin II receptor blockers					
Yao <i>et al</i> ^[33]	42 SHR-SP rats	Losartan (10 mg/kg per day) <i>vs</i> Placebo	2 wk	CFU CD34 ⁺ (flow cytometry) Migratory activity	Losartan improved EPC number and function from SHR-SP rats compared to WKY rats
Yu <i>et al</i> ^[34]	18 SHR-SP rats	Candesartan (1 mg/kg per day) <i>vs</i> Tempol, Trichlormethiazide	2 wk	CFU	The highest CFU count was observed in candesartan treatment group
Yoshida <i>et al</i> ^[35]	12 SHR-SP rats	Valsartan (300 mg/L) <i>vs</i> Hydralazine	2 wk	CFU Migratory activity	Treatment with valsartan stimulated increase in CFU and migration activity in SHR-SP rats compared to hydralazine-treated rats
Honda <i>et al</i> ^[36]	15 healthy controls	Telmisartan (1 μmol/L) <i>vs</i> Valsartan	4 d	CFU Proliferation activity	CFU and EPC proliferative activity are significantly increased in cells treated with telmisartan <i>in vitro</i>
Pelliccia <i>et al</i> ^[37]	40 CAD	Telmisartan (80 mg/d) <i>vs</i> Placebo	4 wk	CD34 ⁺ /CD45 ⁺ /KDR ⁺ (flow cytometry)	CD34 ⁺ /CD45 ⁺ /KDR ⁺ is significantly elevated in patients treated with telmisartan
Angiotensin converting enzyme inhibitors					
Min <i>et al</i> ^[39]	20 CAD	Ramipril (5 mg/d) <i>vs</i> Placebo	4 wk	EPC number Migratory activity Proliferation activity Adhesion activity	There was 1.5 fold increase in EPC number after 1 wk of treatment Followed by 2.5 fold increase in EPC count after 4 wk Migration, proliferation and adhesion activities were also significantly improved with ramipril
Cacciatore <i>et al</i> ^[40]	36 HT	Enalapril (20 mg/d) <i>vs</i> Zofenopril (30 mg/d)	1 yr and 5 yr	CFU Migratory activity	Increased CFU count for both treatment groups at 1 yr and 5 yr No difference for migratory activity
Calcium channel blockers					
Sugiura <i>et al</i> ^[43]	37 HT	Nifedipine (20 mg/d) <i>vs</i> Untreated	4 wk	CD34 ⁺ /CD133 ⁺ (flow cytometry) Migratory activity	EPC number and function were significantly improved in the nifedipine group
de Ciuceis <i>et al</i> ^[44]	29 essential HT	Barnidipine (20 mg/d) <i>vs</i> Hydrochlorothiazide (25 mg/d)	3 and 6 mo	EPC number	EPC number was significantly elevated in patients treated with barnidipine compared to hydrochlorothiazide

CAD: Coronary artery disease; CFU: Colony forming unit; EPC: Endothelial progenitor cells; HT: Hypertension; SHR-SP: Spontaneous hypertensive rats-stroke prone; WKY: Wistar-Kyoto.

mediated oxidative stress and apoptosis. In addition, improvement of endothelial function by nifedipine may be partially due to increased proliferation and angiogenic activities of EPCs. Another CCB, barnidipine, also demonstrated a similar beneficial effect on EPCs in patients with essential hypertension^[44]. Thus, CCBs along with ARBs and ACEI may result in better vascular health in CAD patients with and without hypertension.

CHOLESTEROL LOWERING MEDICATION

HMG-CoA reductase inhibitors (statins)

Statins or HMG-CoA reductase inhibitors reduce cholesterol levels through inhibition of HMG-CoA reductase, an important enzyme in the synthesis of cholesterol in the liver. There is evidence to demonstrate that statins play an important role in the primary prevention of CVD^[45]. The data on statins in primary and secondary therapy in CVD is overwhelmingly consistent. Different doses of different statins have been reported to be useful in increasing EPC numbers^[46-51] and function^[46,52] for a treatment period of 3-16 wk. Studies have reported

that statins exert beneficial effects on EPCs by enhancing EPC proliferation and differentiation *via* the Akt pathway. This can result in activation of the eNOS pathway and VEGF-induced endothelial cell migration^[46,50]. However, there was a study which reported a contrasting outcome where 40 mg/d of statin long term resulted in a decrease in EPC numbers and continuous statin therapy is inversely correlated with EPC numbers^[53] (Table 5). It was put forth that EPCs may be unable to adequately respond to a continuous stimulus of a chronic dose of statins. This may result in desensitization. However, function of EPCs measured by CFU was not altered by statin treatment. Although long term statin therapy may result in a reduced EPC count, the beneficial effects of statin therapy in improving EPC and endothelial function is consistently documented.

ANTI-DIABETIC MEDICATION

Thiazolidinedione/metformin

Thiazolidinedione (TZD) and metformin are important oral medications in the management of type 2 diabetes

Table 5 Effect of HMG-CoA reductase inhibitors (statins) on endothelial progenitor cells

Ref.	Subjects	Drugs	Duration	EPCs (number/function)	Findings
Vasa <i>et al</i> ^[46]	15 CAD	Atorvastatin (40 mg/d)	4 wk	CD34 ⁺ /KDR ⁺ (flow cytometry) Migratory activity	CD34 ⁺ /KDR ⁺ was significantly increased after 4 wk of therapy Migration activity was also significantly improved after 4 wk of treatment
Leone <i>et al</i> ^[47]	40 STEMI	Atorvastatin (80 mg/d) <i>vs</i> Atorvastatin (20 mg/d)	16 wk	CD34 ⁺ /KDR ⁺ (flow cytometry)	Patients who took 80 mg of atorvastatin had higher CD34 ⁺ /KDR ⁺ than those who took 20 mg atorvastatin
Spadaccio <i>et al</i> ^[48]	50 CAD	Atorvastatin (20 mg/d) <i>vs</i> Placebo	3 wk	CD34 ⁺ /CD133 ⁺ (flow cytometry)	Atorvastatin has significantly elevated EPC count after 3 wk
Erbs <i>et al</i> ^[49]	42 CHF	Rosuvastatin (40 mg/d) <i>vs</i> Placebo	12 wk	CD34 ⁺ /KDR ⁺ (flow cytometry)	Rosuvastatin significantly increased EPC count compared to placebo
Tousoulis <i>et al</i> ^[50]	60 SHF	Rosuvastatin (10 mg/d) <i>vs</i> Allopurinol (300 mg/d)	4 wk	CD34 ⁺ /KDR ⁺ , CD34 ⁺ /CD133 ⁺ /KDR ⁺ (flow cytometry)	CD34 ⁺ /KDR ⁺ and CD34 ⁺ /CD133 ⁺ /KDR ⁺ are improved with rosuvastatin treatment compared to allopurinol
Huang <i>et al</i> ^[51]	100 healthy controls 100 ICM	Atorvastatin (10 mg/d) <i>vs</i> Atorvastatin (40 mg/d)	1 yr	CD34 ⁺ (flow cytometry)	CD34 ⁺ count was significantly elevated in patients under 40 mg atorvastatin after 1 yr
Paradisi <i>et al</i> ^[52]	20 healthy controls	Pravastatin (40 mg/d) <i>vs</i> Placebo	8 wk	CFU Tubule formation assay	CFU was increased by 31% in pravastatin group compared to placebo No difference was observed for tubule formation assay between groups
Hristov <i>et al</i> ^[53]	209 CAD (without statin, <i>n</i> = 65, statin 10/20 mg/d, <i>n</i> = 101, statin 40 mg/d, <i>n</i> = 43)	Statin (10/20 mg/d) or 40 mg/d <i>vs</i> Untreated	8 wk	CFU CD34 ⁺ /KDR ⁺ (flow cytometry)	40 mg/d of statin treatment has significantly decreased EPC numbers Continuous statin therapy inversely correlated with EPC numbers

CAD: Coronary artery disease; CFU: Colony forming unit; CHF: Congestive heart failure; EPC: Endothelial progenitor cells; ICM: Ischemic cardiomyopathy; SHF: Systolic heart failure; STEMI: ST-elevated myocardial infarction.

mellitus. TZD activates peroxisome proliferator-activated receptors, while metformin is a biguanide which is effective in reducing glucose production in the liver. Many clinical trials have compared the effects of both TZD and metformin on EPC numbers and/or function. Overall, TZD, metformin or a combination of both drugs has been shown to be beneficial in improving EPC numbers and/or function in diabetic patients^[54-58] (Table 6). In addition, pioglitazone was reported to decrease C-reactive protein (CRP) levels. Since an increased EPC number was significantly correlated to lower CRP, pioglitazone may increase EPC numbers by attenuating the detrimental effects of CRP on EPCs^[54,56,57]. Similar to ARBs, pioglitazone, a PPAR- γ agonist, may directly affect EPCs through PPAR- γ receptors^[54].

Dipeptidyl peptidase 4 inhibitors

Dipeptidyl peptidase 4 (DPP4) inhibitors are new oral hypoglycemic agents and so there is limited data on their effects on EPCs. There is one study that reported increased EPC numbers with sitagliptin after 4 wk of treatment compared to metformin^[59] (Table 6). In addition, besides increased EPC levels, plasma stromal-derived factor-1 α (SDF-1 α) levels were also increased in patients who were on sitagliptin treatment for 4 wk^[59]. The positive effect of DPP4 inhibitors on EPCs is likely driven by SDF-1 α , a physiological substrate of DPP4 and a chemokine which can stimulate bone marrow mobilization of EPCs. SDF-1 α is upregulated and, upon binding to its receptor CXCR4, stimulates the bone marrow to release EPCs.

DPP4 inhibition increases circulating SDF-1 α levels.

EPC AS A THERAPEUTIC POTENTIAL CANDIDATE IN CARDIOVASCULAR DISEASES

Endothelial progenitor cell capture stent

The EPC capture stent is a device which uses the ability of bone marrow-derived EPCs to repair damaged arterial segments. The surface of EPC antibody consists of a covalently coupled polysaccharide intermediate coating with anti-human CD34 antibodies and is then attached to a stainless steel stent. Upon stent placement, the anti-human CD34 antibodies will therefore attract circulating EPCs to differentiate into mature endothelial cells to form a functional endothelium layer. This accelerated healing approach aims to decrease the risk of stent thrombosis and restenosis, as well as reduce prolonged dual antiplatelet therapy in these patients. Effectiveness and safety of this EPC capture stent have been tested in patients with de novo CAD^[60-63] and STEMI^[64-67] and generally these stents are feasible and safe, with major adverse cardiac events reported between 4.2% to 16%. Despite this, there are also contradictory findings which suggest that an EPC capture stent is no better than conventional stents in reducing in-stent restenosis^[62,63]. Preliminary results from a new anti-human CD133 coated coronary stent tested on a porcine model have demonstrated no difference in re-endothelialization or neointima

Table 6 Effect of anti-diabetic medications on endothelial progenitor cells

Ref.	Subjects	Drugs	Duration	EPCs (number/function)	Findings
Thiazolidinedione/metformin					
Wang <i>et al</i> ^[54]	36 type 2 diabetes	Metformin + Pioglitazone (30 mg/d) (<i>n</i> = 24) <i>vs</i> Metformin (<i>n</i> = 12)	8 wk	CD34 ⁺ /KDR ⁺ (flow cytometry) Migratory activity	Both EPC number and migration activity improved with combination of metformin and pioglitazone
Werner <i>et al</i> ^[55]	54 CAD	Pioglitazone (45 mg/d) <i>vs</i> Placebo	4 wk	CFU	Improved EPC number and CFU count with pioglitazone treatment
Makino <i>et al</i> ^[56]	34 type 2 diabetes	Pioglitazone (15-30 mg/d)	24 wk	CD34 ⁺ (flow cytometry)	Number of CD34 ⁺ increased steadily at 12 wk and continued to increase after 24 wk of pioglitazone
Esposito <i>et al</i> ^[57]	110 type 2 diabetes	Pioglitazone (15-45 mg/d) (<i>n</i> = 55) <i>vs</i> Metformin (1000-2000 mg/d) (<i>n</i> = 55)	24 wk	CD34 ⁺ /KDR ⁺ (flow cytometry)	Significant improvement in CD34 ⁺ /KDR ⁺ in patients who took pioglitazone compared to metformin
Liao <i>et al</i> ^[58]	51 healthy controls 46 type 2 diabetes	Metformin (1700-2550 mg/d)	16 wk	CD45 ⁺ /CD34 ⁺ /KDR ⁺ (flow cytometry)	EPC number is significantly lower in type 2 diabetic patients and significantly improved after metformin
Dipeptidyl peptidase 4 inhibitors					
Fadini <i>et al</i> ^[59]	32 type 2 diabetes	Sitagliptin (100 mg/d) (<i>n</i> = 16) <i>vs</i> Metformin (<i>n</i> = 16)	4 wk	CD34 ⁺ /KDR ⁺ (flow cytometry)	EPC number in sitagliptin group significantly improved compared to metformin group by 2 fold

CAD: Coronary artery disease; CFU: Colony forming unit; EPC: Endothelial progenitor cells.

formation with the use of CD133-stents. The existing low number of circulating CD133-positive cells may have resulted in the lack of efficacy of these stents^[68].

Endothelial progenitor cell therapy

Since the successful isolation of adult EPCs in 1997, we now know that bone marrow-derived EPCs may be mobilized to stimulate angiogenesis and may attenuate tissue ischemia for CAD and PAD. Initial pre-clinical studies have reported favorable improvement in left ventricular function in a rat model of myocardial infarction after intravenous injection of *ex vivo* expanded human CD34⁺ cells^[69]. Furthermore, another study examined the effect of catheter-based, intramyocardial transplantation in a swine model of myocardial infarction, providing encouraging outcomes in favoring the application of EPCs as a potential therapeutic therapy in clinical trials^[70].

Recently, there have been several studies using intramyocardial transplantation of autologous CD34⁺ cells in patients with cardiovascular diseases to improve cardiovascular outcomes.

In patients with refractory angina despite medical therapy with antianginal medications and undergoing several revascularization options, including coronary artery bypass graft and percutaneous coronary intervention, intramyocardial transplantation of autologous CD34⁺ cells may be a feasible option. A phase I / II clinical trial^[71] of 24 patients followed by phase IIb^[72] of 167 patients reported a significant improvement in angina frequency and exercise tolerance. An ongoing RENEW study, a phase III trial of 444 patients, will adequately examine the effect of intramyocardial transplantation of autologous CD34⁺ cells in patients with refractory angina^[73]. Besides CAD, there was also a pilot study on the effect of autolo-

gous intramuscular injection of CD34⁺ in critical limb ischemia. The study found that CD34⁺ treatment reduced amputation rates^[74].

CONCLUSION

Endothelial dysfunction secondary to various cardiovascular risk factors can lead to the development of atherosclerosis. As mature endothelial cells possess limited regenerative capacity, there is growing interest in circulating EPCs due to their acclaimed role in maintenance of endothelial integrity, function and postnatal neovascularization. There have been increasing numbers of studies investigating the effects of pharmacotherapies which cardiac patients tend to take on EPC numbers and functions. EPC behavior and mechanisms are also elucidated in patients with CVD, including CAD, HF and PAD. Some studies showed conflicting results and this may be due to the varying definition of EPCs using different methods of identification, different timing of blood sampling, different severity of native disease and concomitant medication and comorbidities that may affect EPC numbers and functions. Besides a biological marker, EPCs have also been shown to be a useful prognostic marker in predicting events in patients with CAD. Lastly, there are several promising studies to suggest EPCs as a novel therapy for CVDs^[74]. However, due to the paucity of circulating cells and the effects of disease on cell quality, investigators need to be mindful of its possible limitations. Possible solutions include enhancing these cell numbers by increasing their mobilization or concentrating them before transplantation and improving their function using *ex vivo* augmentation. Several pilot studies on animals have already shown encouraging results. Further translation to

clinical practice is anticipated.

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WCSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Role of mesenchymal stem cells in cell life and their signaling

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Abstract

Mesenchymal stem cells (MSCs) have various roles in the body and cellular environment, and the cellular phenotypes of MSCs changes in different conditions. MSCs support the maintenance of other cells, and the capacity of MSCs to differentiate into several cell types makes the cells unique and full of possibilities. The involvement of MSCs in the epithelial-mesenchymal transition is an important property of these cells. In this review, the role of MSCs in cell life, including their application in therapy, is first described, and the signaling mechanism of MSCs is investigated for a further understanding of these cells.

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Key words: Mesenchymal stem cell; Differentiation; Stem cell; Application; Self-renewal

Core tip: Mesenchymal stem cells (MSCs) are important cells that have a differentiation and self-renewal capacity and an immune-modulation function. MSCs differentiate into osteogenic-, adipogenic-, chondrogenic- and other cells. The application of MSCs in many situations, such as disease treatment, is full of possibilities for future development. The gene and protein expression and cellular phenotypes of MSCs are described.

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INTRODUCTION

Recent advances in stem cell research have brought about the possibility of stem cell therapies^[1], and new approaches using human genetics have been developed to validate therapeutic targets^[2]. Furthermore, some gene variations may be useful to detect drug effectiveness on these cells^[2]. These novel technologies, when combined, demonstrate their possible application for stem-cell therapeutics. For example, human livers can now be generated from induced pluripotent stem cells (iPSCs) by the transplantation of three-dimensional liver buds that are self-organized *in vitro*^[3]. Furthermore, hematopoietic stem cells can be generated from iPSCs by teratoma formation methods involving co-culturing with OP9 stromal cells, which may be useful for the treatment of hematologic and immunologic diseases^[4]. Even diseased cells, such as sarcoma cells, are now being reprogrammed into stem cells with defined factors, such as Oct4, Sox2, c-Myc and Klf4, to change their cellular phenotype to lack tumorigenicity. Furthermore, sarcoma cells can dedifferentiate into mesenchymal stem cells (MSCs) and hematopoietic stem-like cells, and they can differentiate into connective tissue and erythroid cells^[5,6].

The regulation of differentiation in stem cells involves the expression of several genes; for example, myogenic differentiation 1 induction in immature human iPSCs leads to the differentiation of these cells into mature myocytes^[7]. In hepatic-lineage cell dedifferentiation, cell type-specific transcriptional profiles may correlate to the cell-type transition^[8].

Recently, controlling stem-cell fate using chemical

approaches has been implicated and could lead to new therapies for disease through the understanding of stem cells and regenerative biology^[9]. Chemical therapeutics may induce the self-renewal of cells and target an *in vitro* niche that allows the cells to progress towards cellular differentiation, proliferation, reprogramming and homing^[9]. However, optimization of the chemical structures of these therapeutics is an important factor for developing appropriate cell therapies. Several compounds that regulate cell fate have been selected to bind to nuclear receptors or regulate cellular signaling^[9]. The chemical approach to regulating cells and their niches may open a new door for therapeutic strategies in regenerative medicine, such as targeting self-renewal of stem and progenitor cells as well as differentiation and reprogramming^[9].

Reprogramming using small molecule compounds such as Forskolin, VPA, CHIR99021, 616452, Tranylcypromine and 3-deazaneplanocin A instead of gene transduction has been successful^[10]. In Oct4 promoter-driven green fluorescent protein (GFP)-expressing mouse embryonic fibroblasts, these compounds induce GFP-positive clusters expressing Cdh1 (cadherin 1) (E-cadherin)^[10], which is a marker for the mesenchymal-to-epithelial transition, which has recently been revealed to be an important mechanism for the nuclear reprogramming of mouse fibroblasts^[11]. Chemically iPSCs have gene expression profiles that are similar to those of embryonic stem cells (ESCs)^[10]; therefore, monitoring the gene expression profiles of a variety of iPSCs may be useful for checking the quality of the cells in clinical applications. Additionally, some chemicals important for the self-renewal of ESCs have also been found through screening and may provide insights into the mechanism of stem cell maintenance^[12]. For example, a newly identified small molecule maintains the self-renewal ability of mouse ESCs and functions as a dual inhibitor of a protein kinase, ERK1, and a small GTPase-activating protein, Ras GAP^[12]. Surprisingly, human iPSCs have been developed into a three-dimensional miniature brain, referred to as an *in vitro* cerebral organoid, and this method forecasts the future of organ regeneration^[13,14]. Of the variety of stem cells, MSCs have the potential to differentiate into multi-lineage cells and have other properties such as immunoregulatory functions, which will be discussed in the following sections^[15-19].

ORIGIN OF MSCS

MSCs, which are a type of stem cell, have the ability to differentiate into a variety of cell lineages, such as bone, cartilage, tendon, ligament and adipose tissue^[15,16]. MSCs were originally found as nonhematopoietic cells in bone marrow by the observation of Cohnheim nearly 150 years ago, even before being recognized as MSCs^[20,21]. Osteogenesis, one of the main characteristics of MSCs, was also observed in bone marrow transplantation before the discovery of MSCs^[22,23]. MSCs began to be experimentally recognized and were studied using culture dishes in the 1970s^[24], and during that time, fibroblast colonies were found in bone marrow cultures^[24]. The efficacy of

MSCs *in vivo* has been investigated, and MSCs were found to suppress graft *vs* host disease^[19]. Self-renewing MSCs in bone marrow are rare and are candidates for tissue engineering because of their multilineage-differentiation capacity into various cells^[25]. The phenotype of MSCs is usually described as positive for many molecules, such as CD105 and CD73, and negative for other molecules, such as CD34, CD45 and CD14; however, the MSC phenotype is altered during cultivation^[25-27]. MSCs are promising cells for tissue repair and immunomodulation because they have site-directed and systemic delivery functions^[25]. Furthermore, MSCs have now been considered for the potential use in diabetes mellitus treatment, making the clinical application of MSCs more diverse^[28]. The roles of MSCs are not restricted to disease treatment and include wound repair, as observed in their original discovery. The sources of MSCs now include bone marrow, amniotic fluid, placenta, umbilical cord blood, peritoneum and adipose tissue^[29-35].

The criteria for defining MSCs by the International Society for Cellular Therapy position statement are: (1) plastic adherence under standard culture conditions; (2) expression of CD105, CD73 and CD90 and no expression of CD45, CD34, CD14 or CD11b, CD79A or CD19 and HLA-DR; and (3) capacity to differentiate into osteoblasts, chondroblasts and adipocytes *in vitro*, which is termed the trilineage differentiation potential^[36,37]. MSCs have been reported to be transformed into sarcomas, especially in mice, or to promote tumor growth; however, human MSCs are considered relatively safe for clinical applications^[36,38]. Recently, MSCs have been used for various purposes that utilize their multi-potential abilities to treat disease, and a new cellular model using disease-derived MSCs has been developed^[39]. For the application of MSCs in clinical use, the monitoring of MSC features is essential^[40].

MSC APPLICATION

MSCs have been applied for various purposes, such as the clinical replacement of tissues, and as sources of cells in immune-regulation^[41,42]. Recently, MSCs have been applied as vaccines^[43]. To apply MSCs as a novel vaccine platform, MSCs are expanded *ex vivo* and genetically modified^[43]. However, the quality control of MSCs *ex vivo* is important for the safe application of these cells; therefore, a bank of stored MSCs has been established^[44]. Human iPSCs can be used as sources of MSCs^[45]. The induction of human iPSCs using a small-molecule inhibitor of transforming growth factor (TGF)- β into MSCs has been successful^[45]. In this section, applications of MSCs and differentiated-MSCs in therapeutics are described.

Osteogenic differentiation of MSCs

MSCs are recognized as sources of bone-related regenerative medicine because they can undergo osteogenesis. One of the mechanisms of osteoporosis has been suggested to be an inability of MSCs to differentiate into osteoblasts^[46]. Therefore, a precise investigation for revealing the gene expression profile and molecular signaling

of osteogenic differentiation is needed.

Osteogenic differentiation is often induced with dexamethasone, sodium L-ascorbate, and β -glycerol phosphate^[47], but the roles of microRNAs (miRNAs) in the osteogenesis of MSCs have also been investigated^[48]. Gene expression studies of MSCs using genome-wide association analyses revealed that the EphrinA-EphR pathway for femoral neck bone geometry is coordinated with osteogenesis^[49]. Investigation of bovine MSCs has revealed that the osteogenic differentiation of MSCs was highly induced by ascorbic acid and fetal bovine serum^[50], and upon the osteogenic differentiation of MSCs, mechanical stress could induce the capacity of MSCs^[51]. Hypoxia-mediated signaling in osteogenic differentiation has shown to be mediated *via* regulation of RUNX2 by TWIST^[52]. Moreover, epigenetic regulation is involved in MSC differentiation, and transcription regulation by RUNX2 is important for the osteogenic differentiation capacity of MSCs^[47].

Chondrogenic differentiation of MSCs

One application of MSCs is their use in cartilage repair^[53]. MSCs from adipose tissue have been chondrogenic-differentiated in 3D culture with hydrogel^[53]. Chondrogenic differentiation can be induced by insulin, transferrin, sodium selenite, sodium L-ascorbate, dexamethasone and TGF- β 1^[54]. In some cases, the modulation of MSCs with chemically oversulfated polysaccharide of marine origin up-regulates the TGF- β -dependent chondrogenesis of MSCs^[54]. During chondrogenic differentiation, epigenetic changes have been observed using genome-wide analysis^[55], and the expression of several chondrogenic signature genes were found to be up-regulated. For example, it is known that the trimethylation of lysine 4 of histone 3 (H3K4me3) is up-regulated during chondrogenesis^[55].

Adipogenic differentiation of MSCs

The adipogenesis of MSCs is usually induced by 3-isobutyl-1-methylxanthine, dexamethasone, indomethacin and insulin, but a mechanically induced signal transduction using the depolymerizing drug cytochalasin D has been shown in adipogenesis^[56]. Cytoskeletal mechanisms and signaling molecules such as ERK and AKT are involved in this process^[56]. MSCs have been clinically applied in several situations^[41]. Adipogenesis-related factors may be used for the treatment of obesity and other related disorders^[41]. Importantly, upon adipogenesis of MSCs, the adipogenic differentiation capacity was decreased during *in vitro*, long-term culture^[57]. It is also known that miRNA expression changes upon *in vitro* senescence of MSCs, which suggests that differential miRNA expression might be useful for distinguishing between MSC phenotypes^[57].

Trans-differentiation of MSCs

MSCs also trans-differentiate into non-mesoderm lineages, such as Schwann-like cells which play roles for development, myelination and regeneration in the peripheral nervous system^[58-63]. Adipose-derived stem cells can be differentiated into cells with glial phenotype expressing

GFAP^[58]. Another study has also reported that adipose-derived stem cells can be induced into cells showing neural and glial cell phenotype^[59]. MSCs derived from bone marrow and adipose are shown to express mRNAs and proteins of myelin that is formed by Schwann cells^[64]. MSCs are also suggested to differentiate into myoblast^[65]. On the other hand, MSCs are also used for various diseases including kidney injury, diabetes and brain tumors, although it needs to be elucidated whether the anti-disease effect of MSCs arise from trans-differentiation or other paracrine effects of MSCs^[66-70]. The effect of MSCs for treatment of brain tumors is suggested to be caused by paracrine effect of MSCs towards cancer cells^[70].

MSC effects in the immune response

MSCs are known to be involved in the immune response during circumstances such as the allogeneic transplantation of bone marrow, which mainly causes an immunosuppressive effect. A MSC-induced immunosuppressive effect might be caused by the down-regulation of T-cell differentiation into T helper 17 (Th17) cells and of the function of mature Th17 cells^[71]. This inhibition of mature Th17 cells could occur *via* the cell-to-cell contact mechanism of MSCs and may be mediated by the programmed death-1 pathway^[71]. Allogeneic MSCs have also been demonstrated to regulate the function of Th17 cells derived from rheumatoid arthritis patients^[72]. Co-cultures of MSCs with peripheral blood mononuclear cells (PBMCs) cause a decrease in orphan nuclear receptor gamma (ROR- γ), which is involved in Th17 differentiation and is expressed in PBMCs^[72]. MSCs produce TGF- β and interleukin-6 (IL-6) and regulate the differentiation of T cells into regulatory T cells or Th17 cells^[73]. Therefore, the immune response may be regulated by MSCs *via* Th17 signaling.

MSCs have also been applied as novel vaccine platforms^[43]. MSC vaccination strategies include the modified application of MSCs in anti-microbial or cancer immunization^[43]. Genetically modified MSCs may act as antigen presenters or mediators as well as suppliers of immune-related cytokines^[43]. MSCs from the placenta are known to suppress allogeneic umbilical cord blood lymphocyte proliferation^[74], and it has been suggested that placenta-derived MSCs may be applied in allograft transplantations^[74]. The immunomodulatory properties of equine adult-derived MSCs derived from bone marrow, adipose tissue, umbilical cord blood and umbilical cord tissue have been compared, and it was revealed that lymphocyte proliferation is suppressed by MSCs and secretion of prostaglandin E2 and IL-6 is increased upon allogeneic PBMC or phytohemagglutinin stimulation^[75]. MSCs also decrease the production of tumor necrosis factor- α and interferon- γ ^[75]. The immunomodulatory effect of MSCs on B and T cells have also been studied^[76]. One of the main roles of MSCs on B cells is the inhibition of B cell proliferation, but their effects on B cells are still controversial^[76]. The MSC-induced regulation of the proliferative response of lymphocytes has been reported to be independent of the major histocompatibility complex,

although MSCs mainly induce inhibition and sometimes cause enhancement of the mixed-lymphocyte reaction^[77].

MSC GENE EXPRESSION

MSCs have a variety of gene expression profiles during their developmental stages. Gene expression of MSCs differentiating into adipocytes has been investigated and adenomatous polyposis coli down-regulated-1 (*APCDD1*), chitinase 3-like 1 (cartilage glycoprotein-39) (*CHI3L1*), retinoic acid receptor responder (tazarotene induced 1) (*RARRES1*) and sema domain, immunoglobulin domain (*Ig*), short basic domain, secreted, (semaphorin) 3G (*SEMA3G*) have been identified as potential adipogenic-specific genes^[78]. The expression of these genes is regulated in a time-dependent manner during adipogenesis^[78]. During *in vitro* adipogenic and osteogenic differentiation of MSCs, various genes alter their mRNA expression^[79]. Genes related to cell proliferation and cytoskeleton organization are activated during the osteogenic differentiation of MSCs, whereas genes in PPAR signaling are regulated during the adipogenic differentiation of MSCs^[79].

The gene expression signature is used for the prediction of disease progression or cancer phenotype. The gene expression of patients with breast cancer has been analyzed, and the disease outcomes of young patients have been profiled using predictors^[80]. In anticancer drug discovery, gene expression profiling has been performed on colon cancer cell lines^[81]. MSCs with migration capacity have been used in anti-tumor therapy and must be examined carefully for safety and efficacy^[82]. The gene expression of pluripotency-related genes have been examined in MSCs derived from bone marrow, adipocytes, amniotic membrane and epithelial endometrium-derived stem cells and stroma endometrium-derived stem cells, and these studies suggest that pluripotency-related gene expression varies in different tissues^[82]. Xenograft imaging of mice differentiates between the gene expression patterns of human MSCs and human iPSCs, and the tumor sizes of tumor xenografts of iPSCs are larger than those of MSCs, indicating differences in the migration capacity of MSCs and iPSCs^[82]. Comparison of phenotypic markers and the neural differentiation capacity of MSCs and multipotent adult progenitor cells has been analyzed, and MSCs expressing CD44, CD73 and CD105 have a higher differentiation capacity into neuro-ectodermal lineages than multipotent adult progenitor cells^[83].

The miRNA expression in adipose-derived MSCs has been analyzed, and miR-27b has been identified to be involved in the tolerogenic response^[84]; moreover, it was stated that miR-27b is associated with cell differentiation function^[84]. Another study has revealed that miR-574-3p, which is regulated with Sox9, inhibits the chondrogenic differentiation of MSCs^[85]. The expression of miRNAs, such as the miR-30 family, let-7 family, miR-21, miR-16, miR-155, miR-322 and Snord85, is regulated during the osteoblastic and osteocytic differentiation of MSCs^[86]. These miRNAs are thought to target osteogenic differentiation-, stemness-, epigenetics- and cell cycle-related

mRNAs^[86]. The effects of the mechanical stimulation of MSCs that are seeded on calcium phosphate cement have been analyzed, and it was found that a small number of immediate-early response genes that were associated with transcription were activated^[87].

MSC PROTEIN EXPRESSION

Protein expression in MSCs has been investigated in various experimental systems. Rat oligodendroglial cell maturation is promoted by MSC-derived soluble factors and induces an increase in myelin expression and a decrease in glial fibrillary acidic protein expression^[88]. In thyroid hormone-induced hypertrophy in MSC chondrogenesis, bone morphogenetic protein-4 (BMP4) is up-regulated; therefore, BMP4 signaling is suggested to be involved^[89]. These investigations may provide useful insights into the application of chondrogenic-differentiated MSCs^[90]. MSCs exhibit biocompatibility and favorable responses towards the fibronectin-gold nanocomposite film coating that is used in cardiovascular devices^[90]. MSCs on fibronectin-gold nanocomposites increase the protein expression levels of matrix metalloproteinase-9 and endothelial nitric oxide synthase^[90]. Fibronectin expression has also been linked to MSC lung adherence^[91]. In breast and prostate tumors, MSCs promote the growth and angiogenesis of tumors *via* the expression of pro-angiogenic factors associated with neovascularization, such as macrophage inflammatory protein-2, vascular endothelial growth factor, TGF- β and IL-6^[92]. In the tissue engineering of articular cartilage, chondrocytes from healthy-donor-derived MSCs exhibit similar properties to those of osteoarthritis joints^[93]. Specifically, chondrocytes from MSCs and osteoarthritis joints contain hyaline cartilage-specific type II and fibrocartilage-specific type I collagen^[93]. Differentiated MSCs have increased chitinase family glycoprotein YKL-40 protein levels, and considering that the mRNA of YKL-40 is expressed in undifferentiated MSCs, the regulation between the mRNA and protein levels would be interesting to investigate^[94]. The Fas ligand (FasL) plays an important role in regulating the determination of MSC fates into proliferation or adipogenic differentiation^[95]. Low levels of FasL induce proliferation, whereas high levels inhibit adipogenic differentiation^[95]. Adhesion and osteogenic differentiation of animal, serum-free, expanded MSCs are promoted by laminin-5 and type I collagen^[96]; therefore, these proteins may be considered for the application of the *in vitro* proliferation of MSCs in animal serum-free conditions^[96]. MSCs are committed to adipogenic differentiation under protein malnutrition conditions when PPAR- γ protein and mRNA levels increase^[97]. Furthermore, MSCs decrease the levels of TGF- β 1 in microglia/macrophages after stroke, and this is followed by a decrease in the levels of plasminogen activator inhibitor 1 in astrocytes^[98].

Upon the isolation of MSCs from bone marrow, surface antigens, such as CD10, CD73, CD140b, CD146, GD2 and CD271, can be used as MSC markers^[99]. In

addition to those antigens, pluripotency-related proteins, such as Oct4, Nanog and SSEA-4, have been identified to distinguish cellular populations in the human trabecular bone and bone marrow^[99]. Another report has shown that the transcription factor Ebf2 in adult bone marrow is useful for distinguishing between MSC or mesenchymal progenitor-like cell phenotypes^[100].

CANCER SIGNALING IN MSCS

Cancer is maintained by cancer stem cells (CSCs), which emphasizes the importance of the identification, targeting and elimination of these types of cells^[101]. The initiation of cancer is thought to occur by the activation of self-renewal mechanisms that are usually restricted to stem cells^[101]. Cancer cells showing CSC-like phenotypes may remain in the stem-cell state and tend to avoid cancer differentiation. Cancers exhibiting epithelial CSC-like phenotypes have an increased probability of migration and death, which indicates the possibility of epithelial CSC-like phenotypes as diagnosis predictors for cancer. This phenotype factor is shown to be independent from usual cancer diagnosis factors, such as patient age, cancer diameter, cancer progression, estrogen receptor status, lymphoid node status and blood vessel infiltration. A portion of these cells in cancer may be involved in initiation and infinite cancer proliferation^[101].

CD44, which is proposed to be a tumor-initiating marker for glioma sphere cultures, has been shown to be a mesenchymal signature because mesenchymal-differentiated glioma sphere cultures have radiation resistance and include a CD44 subpopulation^[102]. Recent studies demonstrate that MSCs play an important role in the formation of CSCs^[103].

It is known that subunits of the polycomb repressor complex (PRC), such as BMI1 polycomb ring finger oncogene, are associated with the epithelial-mesenchymal transition, cancer progression and stem-like expression profile^[104-106]. PRC2 is involved in the regulation of gene repression through chromatin modifications and is thought to be important in stem cells^[107-109]. A recent study in *Drosophila* has shown that a mutation in lysine 27 of histone H3 causes the same phenotype as flies, with a loss of PRC2, which indicates that the methylation of lysine 27 of histone H3 is important for PRC function^[110]. The role of chromatin regulation in CSCs will be investigated in the future.

Great efforts to understand CSCs have revealed the feature of CSCs as specific cell populations in brain, skin and intestinal tumors and the possibility of effective treatments by targeting these cells^[111-114]. The markers for CSCs have been identified and include aldehyde dehydrogenase 1 (ALDH1), CD24, CD44, CD90, CD133, Hedgehog-Gli activity and α 6-integrin in breast cancer as well as ABCB5, ALDH1, β -catenin activity, CD24, CD26, CD29, CD44, CD133, CD166 and leucine-rich repeat containing G protein-coupled receptor 5 in colon cancer^[115].

CONCLUSION

In summary, MSCs play important roles in cell life. MSCs differentiate into various cell types, and their many applications, such as for disease treatment, are being studied. Further investigation of the MSC phenotypes is needed for the development of the safe and effective application of MSCs.

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WCSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Distinction of white, beige and brown adipocytes derived from mesenchymal stem cells

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pattern distinct from that of either white or brown fat cells. The current epidemic of obesity has increased the interest in studying adipocyte formation (adipogenesis), especially in beige/brite cells. This review summarizes the developmental process of adipose tissues that originate from the mesenchymal stem cells and the features of these three different types of adipocytes.

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Key words: White adipocytes; Brown adipocytes; Beige/brite adipocytes; Mesenchymal stem cells; Adipogenesis; Thermogenesis; Browning

Core tip: Here, we summarize the characteristic differences of the white, brown and beige adipocytes derived from mesenchymal stem cells, including their anatomical location. In particular, we focus on the newly discovered brown-like adipocytes called beige/brite adipocytes. A deeper understanding of the molecular mechanism of these adipocytes may provide clues for overcoming obesity and its associated metabolic diseases.

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Abstract

Adipose tissue is a major metabolic organ, and it has been traditionally classified as either white adipose tissue (WAT) or brown adipose tissue (BAT). WAT and BAT are characterized by different anatomical locations, morphological structures, functions, and regulations. WAT and BAT are both involved in energy balance. WAT is mainly involved in the storage and mobilization of energy in the form of triglycerides, whereas BAT specializes in dissipating energy as heat during cold- or diet-induced thermogenesis. Recently, brown-like adipocytes were discovered in WAT. These brown-like adipocytes that appear in WAT are called beige or brite adipocytes. Interestingly, these beige/brite cells resemble white fat cells in the basal state, but they respond to thermogenic stimuli with increased levels of thermogenic genes and increased respiration rates. In addition, beige/brite cells have a gene expression

INTRODUCTION

Obesity is a worldwide challenge and not unique to any one country. Furthermore, obesity is closely connected to many metabolic diseases. Essentially, obesity and overweight are caused by the energy imbalance between the calories consumed and calories expended. Adipose tissue, which is composed mostly of adipocytes, is a major en-

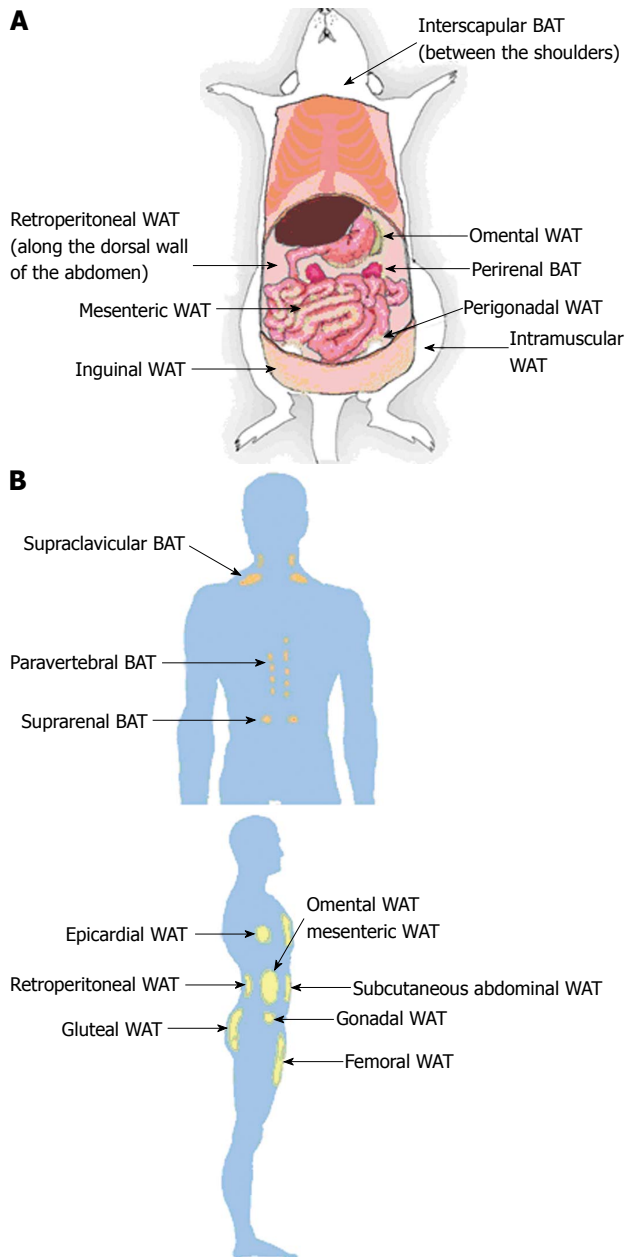


Figure 1 Locations of adipose tissue depots in a mouse (A) and an adult human (B). A: Subcutaneous (inguinal and intramuscular), visceral (mesenteric, omental, perigonadal and retroperitoneal) and brown (interscapular and perirenal) adipose tissue depots are shown in a mouse model; B: Subcutaneous (abdominal, femoral and gluteal), visceral (epicardial, gonadal, mesenteric, omental and retroperitoneal) and brown (paravertebral, supraclavicular and suprarenal) adipose tissue depots are shown in a human model. WAT: White adipose tissue; BAT: Brown adipose tissue.

doocrine organ and plays a key role in energy homeostasis. Two types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT), have been identified^[1]. In practice, obesity does not depend on body weight but depends on either the number of white adipocytes or the amount of WAT. WAT functions primarily to store excess energy in the form of triglycerides (TGs). In contrast, BAT oxidizes fuels and dissipates energy in the form of heat, which suggests that BAT plays a natural anti-obesity role. Therefore, a deeper understanding of the regulation

mechanisms of adipose tissues can potentially open the way to treating obesity-associated metabolic diseases. In this review, we describe the recent advances in studying the characteristics of white, brown, and beige/brite adipocytes (a third class of adipocytes). Additionally, we review the molecular mechanisms involved in the development of adipocytes and suggest possible future therapeutic approaches.

ANATOMICAL LOCATIONS OF ADIPOSE TISSUES

Adipose tissue depots are distinguished by their different anatomical locations. WAT is distributed throughout the body, and there are two representative types: visceral WAT (vWAT) and subcutaneous WAT (sWAT). vWAT is distributed around organs and provides protective padding. sWAT is located under the skin and provides insulation from heat or cold. vWAT, or abdominal fat, is located inside the peritoneum and is distributed around internal organs (*e.g.*, stomach, liver, intestines, and kidneys). Depending on the location, vWAT is sub-classified roughly into mesenteric, retroperitoneal, perigonadal and omental adipose tissue. Mesenteric adipose tissue resembles a web that supports the intestines, and the paired perigonadal adipose tissue is attached to the uterus and ovaries in females and the epididymis and testis in males. The paired retroperitoneal depots are found along the dorsal wall of the abdomen. Lastly, omental depots are located around the stomach and spleen and extend into the ventral abdomen (Figure 1A). The locations of sWAT differ from those of vWAT; sWAT is located inside the abdominal cavity and can be found underneath the skin as well as in the intramuscular fat that is interspersed amongst skeletal muscles. A typical example of sWAT is inguinal WAT, which is found anterior to the upper site of the hind limbs and underneath the skin. In humans, sWAT is typically distributed around the hips, thighs, and buttocks (Figure 1B).

Because beige/brite adipocyte cells were recently defined^[2], brown adipocytes are sometimes termed “classical”, “constitutive”, or “developmentally programmed” brown adipocytes to distinguish them from brown-like cells in WAT. Classical brown fat is primarily distributed around interscapular BAT (iBAT), axillary, paravertebral, and perirenal sites. The most classical brown fat depots are located in interscapular (in the upper back region) and perirenal (around the kidney) sites in rodents and large mammals. iBAT is distributed subcutaneously between the shoulders and can be easily removed. In contrast, it is difficult to selectively remove perirenal BAT from the whole pad without removing the kidney. In humans, small areas of iBAT are found in the thorax region (supraclavicular), chest and abdomen^[3]. In humans and other large mammalian species, BAT was traditionally thought to be restricted to the neonatal and early childhood periods^[3,4]. However, positron emission tomography (PET) scanning technology was recently adapted for detecting

Table 1 Differences amongst the three types of adipocytes

	Brown	White	Beige (Brown-like)
Location	Interscapular, perirenal, axillary, paravertebral	Inguinal (sWAT), mesenteric, retroperitoneal, perigonadal, omental (vWAT)	Within inguinal WAT, other sWAT?
Morphology	Multilocular/small lipid droplets	Unilocular/large lipid droplets	Unilocular large/multiple small lipid droplets
Function	Heat production	Storage of energy as triglycerides	Adaptive thermogenesis
Mitochondria	(+++)	(+)	Upon stimulation (++)
Iron content	Abundant	Low	Upon stimulation (Abundant)
Correlation with insulin resistance	Negative	Positive	Negative
UCP1	(+++)	Nearly undetectable	Upon stimulation (++)
Vascularization/ Capillaries	Abundant	Low	Cold stimulation led to increase of angiogenesis in sWAT ^[66]
α -, β -Adrenergic receptors	β 3 (+++)	β 3 (++), α 2 (+)	β 3/ α 2?
Obesity	Negative effect	Positive effect	Negative effect
Enriched markers	UCP1, Eva1, Pdk4, Ebf3, Hspb7 ^[2,9]	Ang, Resistin ^[13] , LPL, G3PDH ^[14]	Tmem26, Tbx1 ^[2] , Cited1 ^[9] , Shox2 ^[67]
Activators	Cold, thyroid hormone, thiazolidinediones, FGF21, Bmp7, Bmp8b, natriuretic peptide	HFD	Cold, thiazolidinediones, natriuretic peptide, FGF21, irisin, catecholamines, β -adrenergic receptor agonists

WAT: White adipose tissue; vWAT: Visceral WAT; sWAT: Subcutaneous WAT; UCP1: Uncoupling protein 1; FGF21: Fibroblast growth factor-21; HFD: High fat diet.

metabolically active sites for oncology diagnosis; this application is based on the uptake of radiolabeled non-metabolizable glucose derivatives. The results obtained from a scanning experiment using PET to analyze BAT clearly demonstrated that active BAT is present in adult humans at discrete anatomical sites, especially in the upper trunk, such as cervical, supraclavicular, paravertebral, pericardial, and to some extent, mediastinal and mesenteric areas^[5-8] (Figure 1B).

Recently, a new type of brown-like adipocyte was discovered that shows distinct gene expression patterns from those of white or brown adipocytes. These novel brown-like cells that reside within WAT, especially inguinal WAT, were termed beige/brite adipocytes or inducible brown adipocytes^[2]. Adult human neck fat depots are composed of classical BAT, and these depots have the molecular features of classical BAT. However, unexpectedly and interestingly, some studies analyzed the gene expressed in the BAT areas of neonate and adult humans and found beige/brite cell-selective genes^[9]. In contrast, Cypess *et al.*^[10] identified and more precisely analyzed the anatomical sites of adult human BAT around neck fat depots. The researchers isolated samples of neck fat from superficial and deep depots and then compared the gene expression patterns. The results showed that human superficial neck fat had an expression pattern similar to that of mouse sWAT; however, the expression pattern from human deep neck fat was more similar to that of mouse iBAT. Overall, these reports indicate that more extensive analysis is necessary in human BAT studies. Finding beige/brite cells, which were once roughly classified as BAT, requires us to now further distinguish BAT as either classical BAT or beige/brite adipose tissue. It is highly probable that the tissue

that was previously assumed to be BAT in some of the above mentioned studies may in fact be beige/brite adipose tissue.

FEATURES AND FUNCTIONS OF ADIPOCYTES

Traditionally, two different types of adipose tissues, WAT and BAT, have been identified in human and other mammals. These adipose tissues have different colors, morphology, metabolic functions, biochemical features, and gene expression patterns. WAT is the main storage organ of energy in the form of lipids for the organism, whereas BAT plays a role in regulating body temperature by generating heat *via* the consumption of stored energy.

WAT generally constitutes as much as 20% of the body weight of normal adult humans. The development of WAT begins *in utero* but primarily occurs after birth when specialized fat storage cells are needed to provide fuel during fasting periods. WAT is normally characterized by an ivory or yellowish color as well as unilocular/large lipid droplets. The primary function of WAT is to store excess energy as TGs to regulate energy homeostasis. Although the expression of uncoupling protein 1 (UCP1), which is known to be a unique selective marker of BAT^[11], is nearly undetectable, the isoform UCP2 has been reported to be expressed in parts of WAT^[12]. Furthermore, some genes, such as those for Adiponectin, Resistin^[13], LPL, and G3PDH^[14], are known selective markers of WAT (Table 1).

Mitochondria play an essential role in adipose tissue because mature adipocytes require a large amount of ATP to maintain processes such as lipolysis, β -oxidation of fatty acids, and fatty acid synthesis. Mature brown

adipocytes have a relatively high mitochondrial content and contain a specialized mitochondrial protein called UCP1^[15]. Lipolysis occurs during cold exposure, which activates sympathetic nervous system signaling in brown adipocytes; the resulting free fatty acids are used to generate heat using the UCP1 protein. Therefore, in comparison to white adipocytes, brown adipocytes have significantly higher levels of mitochondria that contain red-brownish iron and consequently appear brown in color. They also contain many multilocular/small lipid droplets. As mentioned above, the main function of BAT is to regulate the non-shivering thermogenesis that dissipates energy as heat in response to cold exposure^[16-18]. The thermogenic process of brown adipocytes is activated by UCP1, also known as thermogenin, in their mitochondria. The UCP1 expressed in the inner membrane of mitochondria is mainly regulated by adrenergic signaling through sympathetic innervations, and this signaling is responsible for the production of heat *via* the respiratory uncoupling reaction. UCP1 causes a proton leak across the inner membrane of mitochondria, thereby converting chemical energy into the heat. UCP1 is responsible for the main function of BAT and is a representative marker of brown adipocytes^[15,18]. Additionally, BAT is highly vascularized and innervated, which likely allows BAT to respond to sympathetic nerve activity and dissipate the generated heat throughout the body through blood vessels. In addition to UCP1, Eva1, Pdk4, Ebf3, and Hspb7 have also been reported to be BAT-specific markers^[3,4] (Table 1).

Previous evidences have supported the idea that white and brown adipocytes coexist within the same depot, which suggests that white adipocytes transdifferentiate into brown adipocytes *via* several factors that normally regulate BAT development or activity^[19-21]. However, a new type of brown-like adipocyte within WAT called beige/brite cells was recently discovered, and this transdifferentiation process is referred to as the “browning” or “briteening” of WAT. Researchers have also reported the differential expression of several genes that can be used to distinguish beige/brite adipocytes from brown adipocytes. These genes encode proteins with very distinct cellular functions, including transcription factors (*e.g.*, Tbx15), metabolism-related proteins (*e.g.*, Slc27a1), and proteins associated with inflammatory pathways (*e.g.*, CD40 and CD137)^[2,9,22]. Interestingly, beige/brite adipocytes have the characteristics of both white and brown adipocytes. They display unilocular/large lipid morphology as well as gene expression patterns similar to those of white adipocytes during basal states. However, upon cold stimulation, beige/brite adipocytes change into an “intermediate cell morphology” in which multilocular lipid droplets surround large ones; this change ultimately results in UCP1 expression and a transformation into the multilocular/small lipid morphology characteristic of brown adipocytes^[2,9,22]. Moreover, the inducible browning processes are reversible reactions. In other words, when mice were rewarmed at room temperature after

cold stimulation, the former beige/brite adipocytes reconverted into white adipocytes with decreased expression of brown-selective marker genes approximately 6 wk after the warm adaptation^[23]. This result suggests that browning and whitening are reversible processes and depend on environmental conditions.

It is unclear whether the beige/brite fat cells arise through the transdifferentiation of pre-existing white adipocytes or by *de novo* adipogenesis from a subgroup of precursor cells. Previously, several reports suggested that beige/brite adipocytes arise from pre-existing white adipocytes. Himms-Hagen *et al.*^[19] observed that mature adipocytes transform into beige/brite adipocytes without dividing, and Cinti^[24] showed that large unilocular white adipocytes convert into beige/brite adipocytes in response to cold or 3-adrenergic agonists. However, new research has recently shown conflicting results. During the writing of this paper, Wang *et al.*^[25] suggested that most beige/brite adipocytes stem from a subgroup of precursors in WAT. In that study, the researchers developed a system for inducible, permanent labeling of mature adipocytes. Although cold induced the formation of beige/brite adipocytes, the researchers observed large areas of beige/brite fat cells with multiple small lipid droplets that were not labeled in the subcutaneous white fat.

DIFFERENTIATION OF ADIPOCYTES

BAT develops and differentiates before birth because its function is to protect a newborn against cold. In contrast, the formation of WAT commences shortly after birth. Mesenchymal stem cells (MSCs), which are multipotent stem cells, become adipoblasts and subsequently differentiate into preadipocytes. Under certain types of stimulation, preadipocytes are converted into mature adipocytes in the final phase of differentiation^[26].

The initial phase of adipogenesis is characterized by the proliferation of preadipocytes. Preadipocytes progress through multiple rounds of mitosis until they reach growth arrest, the G₁ phase of the cell cycle. At this point, the preadipocytes must re-enter the cell cycle, undergo mitotic clonal expansion until they eventually exit the cell cycle, acquire the metabolic features of mature adipocytes, change their morphology, and accumulate cytoplasmic TGs^[27]. Mature adipocytes are believed to have lost the ability to divide following the completion of terminal differentiation^[28]. Thus, inducing differentiation in cells isolated from the stromal vascular fraction of adipose tissue depots requires the specific contents of a “differentiation cocktail”. The differentiation induction cocktail contains fetal bovine serum, insulin, dexamethasone (a glucocorticoid), and 3-isobutyl-1-methylxanthine (IBMX). Insulin is an adipogenesis-inducing hormone that promotes cell cycle reentry and synchronous cell division (mitotic clonal expansion). This process is dependent on the induction of two members of CCAAT/enhancer-binding protein (C/EBP) family: C/EBP-β

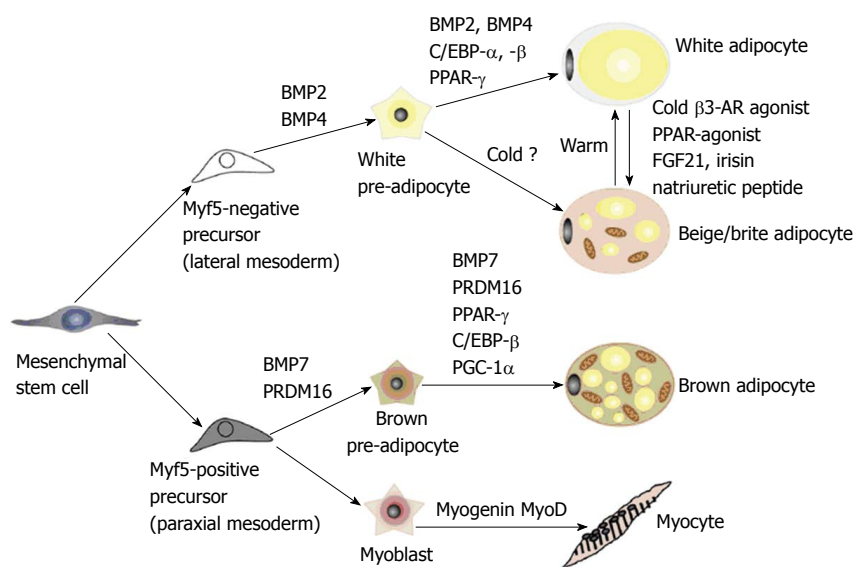


Figure 2 Differentiation into white, beige or brown adipocytes. Previously, white and brown adipocytes were thought to be derived from the same precursor cell. However, recent studies demonstrated that brown fat shares a progenitor cell (Myf5+) with skeletal muscle and not with white adipocytes. The Myf5+ precursors are induced to transform into mature brown adipocytes by bone morphogenetic protein 7 (BMP7), peroxisome proliferator-activated receptor- γ (PPAR- γ) and CCAAT/enhancer-binding proteins (C/EBPs) in cooperation with the transcriptional co-regulator PR domain-containing 16 (PRDM16) and PGC-1 α . White adipocytes can also be transformed to brown-like adipocytes, called beige/brite adipocytes, by cold exposure, a β -adrenergic agonist or a PPAR- γ agonist. AR: adrenergic receptor; FGF21: Fibroblast growth factor 21; PGC-1 α : Peroxisome proliferator activated receptor gamma coactivator 1 alpha.

and C/EBP- δ . Dexamethasone treatment is important for inducing differentiation because it activates the transcription factor C/EBP- β . IBMX is a phosphodiesterase inhibitor that increases intracellular cyclic AMP (cAMP) levels, leading to the activation of the transcription factor C/EBP- δ .

Both white and brown adipocytes originate from the mesoderm, but they are believed to be derived from different precursor cells (Figure 2). MSCs can be committed to either an adipogenic lineage of Myf5-negative cells or a myogenic lineage of Myf5-positive cells^[29,30]. Myf5 is known to be a key myogenic regulatory factor. White adipocytes are derived from the adipogenic lineage, whereas brown adipocytes are derived from the myogenic lineage. Although the adipocytes originate from different lineages, the subsequent adipogenic differentiation shares common transcriptional cascades that mainly involve peroxisome proliferator-activated receptor- γ (PPAR- γ), the dominant regulator of fat cell development, and C/EBPs^[26,28].

Differentiation into white adipocytes from progenitor cells

C/EBP family members are important for adipocyte differentiation, whereby the early induction of C/EBP- β and C/EBP- δ leads to the induction of C/EBP- α and PPAR- γ . Immediately after the induction of differentiation, the cAMP response element binding protein becomes phosphorylated and then induces the expression of C/EBP- β ^[31]. In a relatively early stage of differentiation, mitogen-activated protein kinase and GSK3 β phosphorylate C/EBP- β , which induces the dimerization of two monomers of C/EBP- β , thereby creating a DNA-binding domain. The binding of C/EBP- β to DNA allows preadipocytes to re-enter the cell cycle, that is, C/EBP- β plays a role in mitotic clonal expansion. Furthermore, the functions of C/EBP- β and C/EBP- δ may be redundant^[32]. A knockout of C/EBP- β in mice has little effect on adipose tissue accumulation, whereas C/EBP- β and C/EBP- δ double-knockout mice show considerably

reduced adipose tissue accumulation. The binding of C/EBP- β to DNA leads to increased levels of C/EBP- α and PPAR- γ , which act together as transcriptional activators^[26,33]. C/EBP- α functions to maintain PPAR- γ expression. Upon expression, PPAR- γ and C/EBP- α exert positive feedback on each other, and this stage is regarded as a key step in acquiring the adipocyte phenotype in mature adipocytes. In addition, PPAR- γ is essential for regulating gene transcription to promote and maintain the differentiated state of adipocytes (*i.e.*, lipid metabolism, glucose metabolism, and insulin sensitivity). The dominant negative form of PPAR- γ leads to de-differentiation and the loss of lipid accumulation in differentiated 3T3-L1 cells^[34]. Furthermore, the absence of C/EBP- α in mice impairs the development of WAT, but interestingly, it has no effect on BAT. Thus, some researchers have speculated that the lack of C/EBP- α can be compensated for in brown fat development by C/EBP- β ^[35].

Differentiation of into brown adipocytes from progenitor cells

In contrast to white adipocytes, brown adipocytes originate from the myogenic lineage of Myf5-positive progenitor cells. The differentiation of brown preadipocytes into brown adipocytes is controlled by transforming growth factor- β family proteins, such as bone morphogenetic protein (BMP)-7^[36] and myostatin^[37]. However, Wnt signaling is known to suppress the differentiation of the preadipocytes into brown adipocytes^[38]. C/EBP- β and PR domain containing 16 (PRDM16) have been shown to act as key transcriptional factors in the differentiation of brown adipocytes^[32,39,40]. When PRDM16 was suppressed in brown precursor cells using an shRNA system, the cells differentiated into skeletal muscle cells. Additionally, the myoblasts that ectopically expressed PRDM16 were converted into brown fat cells^[40]. PRDM16, together with C/EBP- β , operates as a critical switch factor in determining the fate of BAT from the myogenic lineage^[41]. In the Myf5-positive myogenic lineage, the PRDM16 and C/

EBP- β transcriptional complex induces the expression of PPAR- γ and peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1 α), which subsequently induces the differentiation of brown adipocytes^[42]. In particular, PGC-1 α also cooperates with PPAR- γ and PPAR- α and regulates mitochondrial biogenesis and oxidative metabolism^[43,44]. In addition, C/EBP- β has been reported to be a key transcriptional activator of UCP1 expression and the thermogenesis process^[32,41]. Interestingly, overexpression of C/EBP- β alone induces a brown fat cell-like phenotype in white adipocytes^[45].

Formation of beige/brite adipocytes (Browning)

After the completion of adipocyte differentiation, some differential processes are sometimes still observed. Interestingly, white adipose depots have the ability to switch between energy storage and expenditure. Thus, these depots can shift from a WAT phenotype to a BAT-like phenotype in terms of features such as morphology, gene expression pattern, and mitochondrial respiratory activity under some specific stimuli^[46]. As mentioned above, this induction of the brown adipocyte-like phenotype in WAT is called “browning” and the beige/brite cells of WAT are capable of this transformation. The beige/brite cells in WAT are derived from precursor cells that are different from classical brown adipocytes and are closer to the white adipocyte cell lineage^[47]. These beige/brite cells show a white adipocyte-like phenotype, including large lipid droplets and the lack of UCP1 expression, under basal conditions. However, in response to certain stimuli (cold exposure^[21] or β 3-adrenergic activators^[19]), beige/brite cells transform into cells having BAT-like characteristics, such as multilocular/small lipid droplets and UCP1 expression.

Recently, in an *in vivo* lineage-tracing study using transgenic mice^[23], brown and beige adipocytes were either transiently or permanently labeled, thereby allowing the tracing of current and past UCP1-expressing cells. After the first cold stimulation, the beige/brite adipocytes expressed both the permanent and transient labels in inguinal WAT. Additionally, when returned to warm conditions, the former beige/brite adipocytes were permanently retained but lost the transient label. The second round of cold stimulation resulted in the re-browning of the whitened former beige/brite adipocytes, as well as the formation of new beige/brite adipocytes within inguinal white fat depots. This experiment strongly suggests that inter-conversion between white and beige/brite adipocytes is possible. Considering these results, we speculate that beige/brite cells can regulate the adaptive thermogenesis against cold in sWAT because the primary function of BAT is non-shivering thermogenesis. In general, classical BAT protects an organism from decreasing temperatures during the neonatal period when the organism is not yet sufficiently capable of adapting to a change in environment; in adults, classical BAT is still present and increases energy expenditure in response to cold or an excess energy state. We think that the classical BAT has

already been set up to control energy homeostasis and is thus a fixed mechanism. Meanwhile, beige/brite cells provide a more flexible means to regulate body temperature and energy balance.

Several factors that can lead to WAT browning have been reported. One of strong inducer of beige/brite cells is cold exposure. Chronic cold exposure induces remarkable changes in metabolism as well as gene expression. In addition, it stimulates the differentiation of precursors into beige/brite adipocytes within one week of exposure^[23]. Although recent report assumed that a cool temperature (27–33 °C, *in vitro* cells) can directly activate the thermogenic gene process in a cell-autonomous manner in sWAT but not in classical BAT, the detailed mechanism is not yet clear. Traditionally, it has been accepted that thermogenic activity is regulated by a canonical β -adrenergic receptor pathway *via* the sympathetic nervous system. Catecholamines, such as norepinephrine, activate β -adrenergic receptor (there are three subtypes, 1, 2, and 3, in humans, but mainly 3 and 1 are involved) that are coupled to a G-protein and increase the intracellular cAMP level. In a subsequent process, this signal leads to fatty acid mobilization and induces the UCP1 expression in mitochondria related to non-shivering thermogenesis. Thus, catecholamines or β -adrenergic receptor agonists mimic the majority of thermogenic effects, as demonstrated using CL316243^[48–51]. Other agents, such as the PPAR- γ activator thiazolidinediones, can also promote WAT browning^[52]. In addition, multiple novel nonadrenergic soluble molecules that are capable of inducing BAT activity and WAT browning have been identified^[53]. Although some of these molecules act indirectly by modulating sympathetic activation and the subsequent noradrenergic pathways, several agents [*e.g.*, fibroblast growth factor-21 (FGF21) and the cardiac peptides (ANP/BNP)] appear to have direct effects on brown adipocytes and the browning process^[54–56]. Recently, the Spiegelman group identified irisin^[57], a novel hormonal factor that converts white fat into the more thermogenic beige fat. Irisin is secreted and released from muscle during exercise and appears to affect the browning process in WAT but not classical BAT activation (Figure 3). Other stimuli are able to enhance the recruitment of beige cells; these stimuli include prostaglandins, which are locally generated by cyclooxygenase-2-mediated production, Bmp8b, the transcription factor FOXC2, and cyclic guanosine monophosphate^[24,58–60]. A recent study suggested that the overexpression of BMP-4 promotes the browning of WAT^[61].

THERAPEUTIC POTENTIAL

sWAT and BAT have intrinsic beneficial metabolic properties, whereas vWAT is the main cause of insulin resistance and type II diabetes mellitus. Obesity and its related metabolic diseases are worldwide challenges. Many strategies to address the problems have been attempted, but there are still no clear solutions. Recently, however, the rediscovery of BAT in human adults led to many

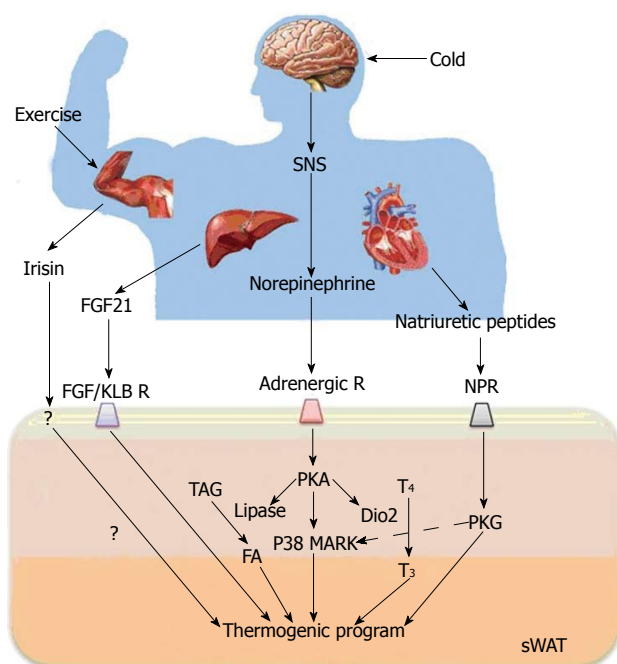


Figure 3 Key regulators of the browning process and their action mechanisms. Browning is induced by sympathetic nervous system (SNS)-independent or SNS-dependent signals. These signals sometimes synergistically or competitively influence the activation of browning of subcutaneous white adipose tissue (sWAT). Irisin is a newly discovered myokine and is released by skeletal muscle during exercise. Irisin induces the browning process of sWAT. Fibroblast growth factor-21 (FGF21), a hormonal factor from the liver, directly activates the thermogenic process via interaction with the FGF receptor/ β -Klotho (KLB) complex. The norepinephrine secreted by the SNS in response to thermogenic stimuli induces the activation of adrenergic receptor(s). The adrenergic receptor-mediated signal increases the level of intracellular cAMP and activates cAMP-dependent protein kinase A (PKA). Subsequently, PKA activates p38 MAP kinase (p38 MAPK) and 5'-deiodinase 2 (Dio2), which catalyzes the conversion of thyroxine (T4) into the active form 3,5,3'-tri-iodothyronine (T3). Then, it ultimately induces the gene process for thermogenic activation. Natriuretic peptides (NPs) originating from the heart activate the thermogenic process through binding to the NP receptor, activation of protein kinase G (PKG) and the subsequent activation of p38 MAPK and NPR. NPR: Natriuretic peptides receptor; TAG: Triacylglycerol; FA: Fatty acid.

investigations of BAT for anti-obesity treatments. Some of the experimental evidence suggests that BAT could be a new therapeutic tool as well as a precise regulator of energy homeostasis. People who have adapted to cold environments show some resistance to the development of diabetes, possibly due to the maintenance of a larger amounts of BAT^[62]. In addition, the extent of human BAT activity in patients is inversely associated with obesity, age and type II diabetes^[63]. In mouse experiments, the mouse strains with higher thermogenic gene expression in WAT depots tended to be more resistant to obesity and insulin resistance than those with lower levels^[64]. Based on these results, many molecules (such as irisin^[57], FGF21^[55], and natriuretic peptides^[65]) that induce BAT activation or WAT browning have been studied as potential drugs. Of course, these molecules also create side effects; nevertheless, these molecules may be an important key to address many challenges if the side effects can be mitigated.

CONCLUSION

WAT is an important endocrine organ that maintains body homeostasis by storing excess energy and secreting hormones. However, the excessive accumulation of fat in the organ causes obesity and obesity-associated metabolic disorders. Thus, developing treatments for obesity is important for maintaining public health. Interestingly, a potential solution to the problem of obesity-associated diseases has been found in brown fat, a type of adipose tissue that dissipates energy through a thermogenesis process. Previous studies showed that activated BAT is inversely correlated with BMI^[65], adipose tissue mass and insulin resistance. Thus, BAT is one of the best targets for creating strategies to treat obesity and obesity-associated diseases. However, the transdifferentiation of white adipocytes into brown adipocytes is difficult because each type of adipose tissue is derived from a different progenitor lineage. The recent discovery of beige/brite adipocytes within WAT that are derived from the same lineage provides the possibility to overcome this challenge. Moreover, beige/brite cells are distributed throughout the human body, and they are highly activated in response to a variety of factors, including endogenous hormones. Therefore, WAT browning as well as BAT activation may contribute to an important strategy for treating obesity. A deeper understanding of the biological mechanisms that regulate the conversion within adipocytes will help in developing browning-inducing strategies for suppressing obesity.

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Enhancing the efficacy of mesenchymal stem cell therapy

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INTRODUCTION

Human bone marrow mesenchymal stem cells (MSCs) are currently being investigated in clinical trials for immune, cardiovascular, neurodegenerative, gastrointestinal, bone/cartilage and blood disorders (<http://clinicaltrials.gov>). The clinical utility of MSCs is in part due to their lack of significant immunogenicity, permitting safe allogeneic cell transplantation without the need for immunosuppression. However, these clinical trials have thus far demonstrated moderate and at times inconsistent benefits, indicating an urgent need to optimize the therapeutic platform and enhance stem cell potency^[1-3]. Along this line, parallel preclinical studies have identified several potentially useful and logistically feasible strategies that may be employed to achieve more robust clinical efficacy of MSC therapy. On the other hand, risk factors associated with MSC therapy cannot be overlooked because long-term safety data remain lacking and unanticipated side effects may appear much later. Potential risks related to disease transmission and activation of latent viruses in allogeneic cell transplantation also highlight the importance of continued surveillance post MSC therapy. Thus, future success of MSC therapy will lie in rational optimization of therapeutic strategies in conjunction with an adequate assessment of benefit and risk factors.

TROPHIC ACTION OF MSCS

While early preclinical MSC studies suggested therapeutic mechanisms mediated by MSC trans-differentiation or fusion, these mechanisms do not occur in sufficiently

Abstract

Mesenchymal stem cell (MSC) therapy is entering a challenging phase after completion of many preclinical and clinical trials. Among the major hurdles encountered in MSC therapy are inconsistent stem cell potency, poor cell engraftment and survival, and age/disease-related host tissue impairment. The recognition that MSCs primarily mediate therapeutic benefits through paracrine mechanisms independent of cell differentiation provides a promising framework for enhancing stem cell potency and therapeutic benefits. Several MSC priming approaches are highlighted, which will likely allow us to harness the full potential of adult stem cells for their future routine clinical use.

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Key words: Mesenchymal stem cell; Therapy; Clinical trial; Paracrine

high frequency to account for the observed functional improvement after stem cell administration. Current evidence indicates that although MSCs exhibit prominent multi-lineage differentiation potential, this cellular feature bears little relevance to their therapeutic effects. Instead, production of multiple paracrine factors by MSCs provides the underlying regenerative mechanism^[4-6]. Therapeutically, the MSC-derived soluble mediators, which include many cytokines and growth factors, are functionally redundant and synergistic, contributing to cytoprotection, angiogenesis, tissue repair, normalization of extracellular matrix (ECM) and alleviation of inflammation. Preclinical studies have indeed highlighted the central role of MSC-derived interleukin (IL)-6-type cytokines, vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) in the treatment for heart failure and multiple sclerosis^[6-8]. MSCs also interact with cells of both the innate and adaptive immune systems, leading to functionally relevant immunomodulation^[9]. Of note, MSCs are widely present *in vivo* and their perivascular origin in multiple organs have been demonstrated^[5,10]. This apparent *in vivo* “drug store” function of MSCs constitutes the primary therapeutic underpinning of MSC therapy.

“COMPETENCE FACTORS” IN MSC THERAPY

Current clinical trial data do not yet support routine use of MSC therapy for the prevention and treatment of organ dysfunction or tissue degeneration. Robust cell therapy is likely dictated by at least two key competence factors affecting both the transplanted stem cells and the treated host tissue. This view necessitates a complete understanding of the cell-tissue crosstalk mechanism and the adoption of an integrative approach in maximizing therapeutic efficacy regardless of the organ system being targeted. Since the mechanisms of action of MSCs in tissue regeneration are likely multifaceted, cell competency can be dictated by the abilities of the injected MSCs to migrate, engraft, survive, differentiate and produce functional paracrine mediators. Tissue competency reflects the ability of the host tissues to favorably respond to the injected MSCs and MSC-derived paracrine factors, resulting in activation of the endogenous regenerative machinery^[11]. While the exogenous repair mechanism is imparted by the implanted MSCs and is often short-lived, the endogenous repair mechanism conferred by the host stem/progenitor cell niches can exert a powerful and long-lasting regenerative benefit. Integration of the exogenous and endogenous repair mechanisms in clinical trial design will prove instrumental in transitioning toward future routine clinical use of adult stem cells. In considering the strategies for boosting the competency factors in MSC therapy, we will focus primarily on non-genetically based methods because genetically modified MSCs will likely pose some concerns and safety issues for clinical application. Given that MSC therapy

is being used to target a wide spectrum of diseases in diverse patient populations, the logistical aspects of MSC therapy will also be considered.

SOURCE OF COMPETENT MSCS

MSCs from different donors may exhibit different degrees of competence due to varying factors such as gender, disease status and age^[12,13]. Limited information indicates that female stem cells may possess a more pronounced regenerative potential than male stem cells^[14], which is in line with the finding that female patients typically exhibit certain cardioprotective phenomenon from acute myocardial infarction and better outcome after the incidence compared to male patients^[15]. Although the gender influence is thought to be mediated through differential sex hormone receptor signaling, a recent study shows that female rodent MSCs produce a higher level of VEGF than male rodent MSC in response to hypoxia^[13]. Given the critical role of paracrine factors in MSC therapy, additional study is warranted to determine whether female MSCs are indeed more robust in production of multiple paracrine factors and should be selected for the use of allogeneic MSCs.

Aside from the gender effect, studies have further revealed disease- and age-associated functional impairment of various types of adult stem cells^[16,17]. While the basal hematopoietic capacity is maintained throughout life, the ability of hematopoietic stem cells (HSCs) to respond to stress and differentiation cues appears to decrease with age^[18,19]. The use of autologous MSCs is not always desirable or feasible because patients can exhibit declined stem cell quality and/or quantity^[20-22]. For instance, diabetes can negatively impact MSCs by reducing angiogenic capacity and therapeutic potential^[23]. Certain disease-causing genotypes may preclude therapeutic use of autologous MSCs due to the inherent genetic defects^[24,25]. Even chemotherapy can induce MSC damage and reduce cell yields in patients with hematological malignancy^[26]. Thus, the use of allogeneic MSCs from healthy donors is gaining acceptance. The use of allogeneic MSCs isolated from healthy donors offers a major advantage because these adult stem cells can be thoroughly tested and formulated into off-the-shelf medicine in advance. MSCs are particularly well suited for this application due to their immune privileged status.

CELL DOSE AND THERAPEUTIC POTENCY

Lessons learned from HSC therapy following myeloablation have revealed that administration of sufficient HSCs promotes faster cell recovery and reduces hospitalizations^[27]. Clinical trials of stem cell therapy for regenerative repair have also demonstrated the importance of administering a sufficient cell dose^[28,29]. Using the hamster heart failure model, we evaluated the relation-

Table 1 Therapeutic benefits in relation to the number of administered mesenchymal stem cells

Cell number/ animal	Cell number /kg	Cardiac repair	Cell number /70-kg human
0.01 × 10 ⁶	0.1 × 10 ⁶	- (no)	7 × 10 ⁶
0.1 × 10 ⁶	1 × 10 ⁶	+ (weak)	70 × 10 ⁶
1 × 10 ⁶	10 × 10 ⁶	++ (moderate)	700 × 10 ⁶
4 × 10 ⁶	40 × 10 ⁶	+++ (robust)	2800 × 10 ⁶

ship between the injected MSC doses [(0.1-40) × 10⁶ cells/kg body weight] and cardiac therapeutic benefits as determined by echocardiography, morphometry, gene expression analysis and histochemistry^[30,31]. The series of pharmacodynamic studies established the minimal cell dose, *i.e.*, about 1 × 10⁶ cells/kg (Table 1), which is necessary for achieving quantifiable but weak benefits for the failing hamster heart. The studies also revealed that the most prominent therapeutic benefits were achieved by about 40 × 10⁶ cells/kg, which however approximates 2.8 billion cells per 70-kg human! Notably, published clinical trials of MSC therapy have largely relied on injections of about 1 × 10⁶ cells/kg^[32-36], which appears suboptimal based on our cell dose study. Since the effective treatment dose is influenced by the body size, bio-distribution of the MSC-induced paracrine factors in the human body is likely much less efficient than in the small rodent. Given the large body weight difference between rodents and humans, obtaining sufficient MSCs necessary for mounting a prominent therapeutic response in humans constitutes a daunting challenge. In particular, obtaining sufficient MSCs to achieve maximum clinical benefits may not be economically viable as elaborated in Table 1.

CONUNDRUM OF *EX VIVO* MSC EXPANSION

Normal mitotic somatic cells gradually cease division after continuous expansion in culture and enter a state referred to as replicative aging or senescence, exhibiting a Hayflick limit of 50 population doublings^[37,38]. Embryonic stem cells (ESCs) however proliferate indefinitely in culture, which correlates with their high telomerase activity and long telomeres^[39]. MSCs constitute a minor population of the nucleated cells (0.01%-0.001%) in the adult human bone marrow. Unlike hematopoietic stem cell transplantation, which is a well-established therapeutic regimen for hematological disorders^[40], it is necessary to amplify MSCs in culture to generate sufficient cells required for therapeutic applications. This *ex vivo* cell amplification step unavoidably creates many issues that can confound MSC therapeutics. Long-term *in vitro* passaging alters bone marrow and adipose MSCs^[41]. Prolonged culturing of MSCs from several species causes senescence and prominent changes in gene expression^[42,43]. Down-regulation of genes involved in DNA repair during MSC

senescence^[44] can potentially cause genomic instability. Our study of porcine MSCs shows that late-passage MSCs exhibit significantly reduced expression of many paracrine factors compared to early-passage MSCs (Figure 1). Since cellular aging is a rapid and continuous process in culture, the use of *ex vivo* amplified MSCs, even those derived from early-passages, can generate inconsistent therapeutic effects.

TLR3 SIGNALING AND MSC COMPETENCE

Our MSC and growth factor therapy for hamster heart failure have revealed several major factors critical for tissue repair such as IL-6-type cytokines, VEGF and HGF^[6,7,30,31,45]. We show that MSC therapy increases the levels of paracrine factors present in the serum and multiple organs, suggesting a systemic distribution mode for the soluble mediators at least in the rodent. We further sought to engineer an MSC phenotype exhibiting enhanced expression of paracrine factors, aiming to lower the effective treatment cell dose. We turned our attention to the pattern recognition receptor (PRR) pathway of the innate immune system, which is capable of overproducing many immunomodulatory cytokines, most notably IL-6, upon activation^[46,47]. Distinct immune cell PRRs initiate the cytokine cascade through interacting with a variety of molecular patterns conserved among microbial pathogens. The Toll-like receptor (TLR) pathway is the best characterized PRR system and engagement of TLRs stimulates production of many immunomodulatory cytokines from antigen-presenting cells. TLR3 in particular recognizes double-stranded (ds) RNA, and is activated by the dsRNA mimetic polyinosinic-polycytidylic acid or poly(I:C)^[48,49]. MSCs also express several members of the TLR family^[50], including TLR3, which is an endolysosomal receptor protein.

We initially treated MSCs with three different concentrations of poly(I:C) for 24 h to examine the downstream effect on expression of trophic factors^[31]. Gene expression assays revealed a prominent induction of IL-6 and IL-6-type cytokines by 0.8-20 g/mL poly(I:C). For instance, a 10 fold increase in IL-6 mRNA and 40 fold increase in secreted IL-6 were observed. A less than 2 fold induction of IL-11 mRNA and ~4 fold induction of secreted IL-11 were also observed. Leukemia inhibitory factor (LIF), another member of the IL-6-type cytokines, was also induced. SDF1, VEGF and HGF, all of which are activated by IL-6/JAK/STAT3 signaling, were significantly induced by poly(I:C). Interestingly, the anti-inflammatory cytokine IL-10 was significantly induced. The inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were only induced by the highest poly(I:C) concentration (20 g/mL). This finding prompted us to adopt an MSC-boosting protocol based on 4 g/mL poly(I:C) for 24 h, which induced IL-6, IL-10, IL-11, LIF, VEGF, SDF1 and HGF without

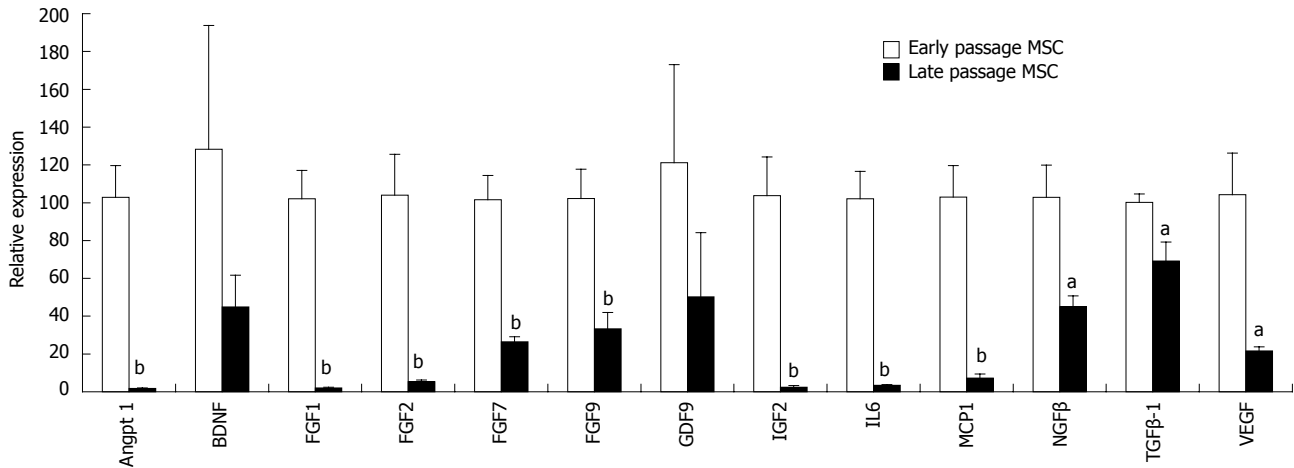


Figure 1 *Ex vivo* expansion of mesenchymal stem cells reduces expression of growth factor/cytokine genes. Porcine mesenchymal stem cells (MSCs) were expanded as described^[42]. Threshold cycle (C_T) for the illustrated genes was determined by real-time reverse transcription polymerase chain reaction. Early and late passage MSCs received less than 5 and more than 10 trypsin passages, respectively. ^a $P < 0.05$, ^b $P < 0.01$ vs early passage MSCs.

induction of the inflammatory cytokines. Longer treatment of MSCs with poly(I:C), *e.g.*, 2 d, was found to be cytotoxic.

Upon testing the potency of the PRR-primed MSCs using the hamster heart failure model, we found that the super MSCs reduced the effective therapeutic cell dose by 40 fold (Table 1) through actively recruiting cardiac progenitor cells and decreasing myocardial inflammation, culminating in a 50% reduction in myocardial fibrosis, a 40% reduction in apoptosis and a 50% increase in ventricular function. This pioneering study of engaging the MSC PRR axis for reducing cell dose requirement in heart failure therapy was recently featured in an AJP editorial^[51]. Although the function of immune cell PRRs has been well established, their role in stem cell function is just beginning to be unraveled. Prolonged TLR activation of the immune system is invariably associated with chronic inflammation. Interestingly, Cole *et al.*^[52] demonstrated an unexpected beneficial role for TLR3 in the arterial wall upon systemic administration of poly(I:C). Further, Packard *et al.*^[53] found poly(I:C) administration to be protective against cerebral ischemia-reperfusion injury. Since MSCs are widely present *in vivo* and their perivascular origin in multiple human organs appears certain^[5,10], it is possible that these prophylactic benefits of poly(I:C) may be mediated through its trophic stimulatory effect on the endogenous MSC niches.

INFLUENCE OF OTHER PRR SYSTEMS

Recognition of various pathogen-associated molecular patterns by immune PRRs leads to transcriptional activation of distinct gene targets, and sets forth a diverse array of pathways that determine the magnitude, duration, and type of the host inflammatory response. Immune cell TLR2 and TLR4 are major PRRs responding to bacterial invasion, and their activation leads to increased

IL-6 and a host of other cytokines similar to the antiviral response mediated by dsRNA-sensing PRRs^[46]. Given the prominent roles of IL-6 in stem cell maintenance and cardiac regeneration^[6,54-56], transient low-dose priming of MSC TLR2/4 may also represent a physiologically significant mechanism for tissue repair. It has indeed been shown recently that TLR2/6-dependent stimulation of MSCs promotes angiogenesis *in vitro* and *in vivo* in bone tissue engineering^[57]. TLR2 forms functional heterodimers with TLR1 and TLR6, and is activated by peptidoglycan. Immune TLR4 upon activation by lipopolysaccharide (LPS) causes elevated levels of IL-6, IL-8, IL-10, IL-12, IL-15, TNF α , IL-1 and TGF β . Potential effects of TLR2 and TLR4 engagement on MSC paracrine profiles can therefore be tested by treating cells with low-dose peptidoglycan and LPS (1-20 g/mL each). However, since TLR2 and TLR4 are also known to be involved in tissue inflammation triggered by ischemia/reperfusion injury^[58], it is unclear whether transient low-dose priming of MSC TLR2/4 may favorably impact the failing heart as demonstrated for MSC TLR3.

Unlike TLR3, TLR2 and TLR4 are present on the plasma membrane, recruiting the adaptor protein MyD88 for signal transduction. Since TLR activation in the absence of MyD88 generally results in delayed kinetics^[59], the difference in the paracrine cascades can be expected to influence MSC therapeutics. Notably, MSCs have been found to be differentially primed by TLR4 and TLR3 ligands to adopt a pro-inflammatory (MSC1) and anti-inflammatory (MSC2) status, respectively^[60]. The MSC1 and MSC2 phenotypes were further found to attenuate and promote tumor growth/metastasis, respectively^[61]. These studies thus indicate that the cytokine secretion profile of MSCs plays a decisive role in dictating the therapeutic potency and treatment outcome, and warrants special consideration in the design of stem cell therapy.

HYPOXIA AND MSC COMPETENCE

Rapid loss of the implanted MSCs has been frequently observed and may be caused in part by hypoxic stress, which triggers apoptosis^[62-64]. The bone marrow environment contains oxygen tensions ranging from 1% to 7%. However, most *in vitro* cell culture work is performed at a pO₂ level of 142 mmHg or 20% O₂, which is much higher than that of the *in vivo* environment^[65]. The implanted MSCs are expected to experience reduced oxygen levels as they attempt to establish contacts with the ECM environment. Preconditioning MSCs by brief hypoxia prior to cell administration may thus allow the cells to better adapt to the lower pO₂ tissue environment and promote cell engraftment. Typically, MSCs are cultured in normoxia (95% air and 5% CO₂) as control and in hypoxia (1% oxygen, 5% CO₂, and 94% nitrogen) for 2 d. Assay of cultured MSCs for cell surface phosphatidylserine, which is a sensitive method for detecting early apoptosis, can be used to determine whether an increase in MSC apoptosis after hypoxic exposure may be induced.

In addition to induction of many angiogenic growth factors, hypoxia is known to induce SDF-1 and its cognate receptor CXCR4^[66,67]. Indeed, low oxygen has been shown to increase expression of CXCR4 and CX3CR1 and promote MSC engraftment^[68,69]. A hypoxia-regulated heme oxygenase-1 vector modification of MSCs was found to enhance the tolerance of engrafted MSCs to hypoxic injury and improves their viability in ischemic hearts^[70]. Note however that although hypoxia promoted MSC proliferation *in vitro*, it unexpectedly attenuated MSC osteogenic potential^[71], suggesting that the utility of hypoxia preconditioning may be application specific. Additional relevant preconditioning strategies intended to enhance MSC survival have been based on the use of unique compounds such as prolyl hydroxylase inhibitor^[62], lysophosphatidic acid^[72], HMG-CoA reductase inhibitor^[73,74], eNOS enhancer^[75] and sphingosine-1-phosphate^[76]. Whether this pharmacological approach may also reduce the effective MSC dose as observed for TLR3-activated MSCs remains to be determined. These pharmacological strategies may also find their application in tackling the issues of host tissue deficiency related to aging and disease (see below). This is because the function and competence of the endogenous host tissue progenitor cell niche also dictates the therapeutic outcome.

CYTOKINE PRECONDITIONING AND MSC COMPETENCE

Rapid loss of the injected cells is perceived as a major hurdle in stem cell therapy^[63,64,77] and may be caused in part by inadequate ECM engagement. Expression of chemokines and their receptors is known to be regulated by cytokines and this phenomenon has been explored to facilitate MSC engraftment after cell implantation^[78,79].

Intervention through the use of growth factors and/or cytokines is appealing because the trophic factor network is typically marked by cross-talk mechanisms enabling mutual induction of gene expression. Priming MSCs with a cocktail of growth factors and cytokines has indeed been found to enhance the cardiac therapeutic efficacy^[80]. In this study, a cocktail of growth factors containing 50 ng/mL FGF-2, 2 ng/mL IGF-1 and 10 ng/mL BMP-2 was used for MSC pretreatment and its effect on the viability under hypoxia and paracrine profiles of MSCs were evaluated. The growth factor pretreatment was found to enhance expression of cardiac transcription factors and promote cell viability under hypoxia. Transplantation of the pretreated MSCs resulted in smaller infarct size and better cardiac function than transplantation of untreated MSCs. This cytokine preconditioning approach is particularly relevant because MSCs are adherent cells and depend on adequate ECM engagement for growth and survival. Anoikis is initiated when trypsinized MSCs are forced into suspension for injections^[81]. Along this line, plasminogen activator inhibitor-1 (PAI-1) has been found to promote anoikis, and PAI-1 null MSCs exhibit enhanced *in vivo* survival after implantation^[64].

Many cytokines are known to exhibit cell adhesion-promoting activities including HGF, IGF-1, SDF-1, TGF- β and VEGF and interestingly these trophic factors are also produced by MSCs, suggesting that MSCs can be regulated by diverse autocrine mechanisms. These cytokines act in part by affecting the integrin and matrix metalloproteinase (MMP) systems. In particular, EGF can promote activation of MMP-2 and cell migration^[82]. TGF-1 can stimulate MMP-9-mediated cell migration^[83]. SDF-1 can increase V 3 integrin expression, cell migration, and therapeutic potentials of EPCs^[84,85]. We also demonstrated that human MSCs overexpressing VEGF exhibited significantly enhanced cardiac repair capacity^[7]. Since no cell retention and survival enhancement strategies have translated to the clinic, strategies aimed at promoting long-term maintenance of the injected cells are worth pursuing, which may ultimately lead to the production of more potent stem cells that can be delivered in lesser quantity.

HOST TISSUE COMPETENCE

Host tissue competence can greatly influence the outcome of MSC therapy because it is increasingly been recognized that aging and disease can adversely affect the tissue milieu into which MSCs are introduced^[86]. The parabiosis study exposing old mice to factors present in young mouse serum^[87] indicates that the age-related decline of muscle satellite cell activity is modulated by systemic factors that change with age. This is because stem cell activity is profoundly influenced by the supporting ECM and cells in the immediate vicinity^[88]. The presence of ECM breakdown products and the extra lamina

caused by the deposition of collagens in aged muscle tissue can potentially interfere with paracrine signaling. Aged muscle for instance exhibits increased Wnt signaling and fibrosis^[89,90], which can impinge unfavorably on the functional paracrine cascade initiated by the implanted MSCs. Importantly, although the intrinsic regenerative potential of aged muscle appears to be largely intact, critical factors such as the Notch ligand Delta required for regeneration appear limiting^[87,91,92].

Increasing age has been found to be associated with adverse prognosis in the setting of ischemic injury, coronary angioplasty, and cardiac surgery^[93-97]. Although the adult heart contains resident cardiac stem cells capable of supporting limited myocardial regeneration^[98], age-associated fibrotic remodeling and senescence of cardiac stem cells lead to contractile dysfunction and gradual loss of cardiomyocytes^[99,100], and the aged heart exhibits significant structural deteriorations including fibrosis and poor angiogenic capacity^[101,102]. Thus, the aged heart is more refractory to regenerative therapy^[103,104]. The harmful host tissue milieu present in the aged tissue may interfere with the trophic actions of MSCs. Several tissue proteases such as elastase, cathepsin and dipeptidylpeptidase (DPP) are known to cleave and inactivate cytokines. Elevated activities of these proteases in the aged tissue may destabilize the trophic factors induced by MSC therapy, rendering the therapy ineffective. Therapeutic efficacy may thus be improved by optimizing tissue retention and stability of the delivered proteins^[105-107]. For instance, administration of Diprotin A, a pharmacologic inhibitor of DPP, enhanced the stability of SDF-1, which increased myocardial homing of CXCR4⁺ progenitor cells and function^[108,109]. Thus, a potential strategy to boost the trophic response of the older tissue is to inject non-toxic protease inhibitor(s) into the host tissue prior to MSC administration. This tissue preconditioning strategy is aimed at promoting trophic factor stability by attenuating abnormally elevated local or systemic protease activities.

The bone marrow compartment harbors many populations of primitive progenitor/stem cells that are mobilized by various chemokines. Of note, a lack of bone marrow support for cardiac repair in aged animals has been documented^[110], indicating that the MSC-initiated healing process may be compromised by the impaired tissue cross-talk mechanism, leading to a greater susceptibility of the old heart to ischemic injury and an inefficient response to protective interventions. IL-6 deficiency, for instance, affects bone marrow stromal precursor cells, resulting in defective hematopoietic support^[54]. This host tissue impairment represents a significant hurdle to regenerative medicine because most preclinical therapeutic studies are based on the use of young animals, but stem cell therapy typically targets the elderly. Development of suitable preconditioning strategies targeting MSCs and aged host tissue is thus expected to lead to more efficacious regenerative treatment regimens.

A RATIONAL DESIGN OF MSC ADMINISTRATION ROUTE

Routes of drug administration are major considerations in pharmacokinetic and pharmacodynamics studies and applications. The choices are however fairly limited for cell-based medicine as cell viability needs to be preserved. Since diseased tissue is often associated with ischemia, inflammation, and fibrosis, which can impair cell survival, therapeutic delivery of stem cells to areas away from the damaged tissue offers an advantage. Intravenously (*iv*) infused MSCs are currently being adopted for clinical trials of neurodegenerative and heart diseases^[36,111], highlighting the significance of formulating a minimally invasive stem cell delivery approach for patient care. Although *iv* MSCs are largely distributed to the lungs, this systemic cell delivery method appears feasible with MSCs because their therapeutic benefits are largely mediated by paracrine mechanisms independent of stemness^[5,6]. Thus, intracoronary infusion of MSCs for heart therapy, which retained only 1%-2% of the infused cells in the porcine myocardium, was found to result in significant functional improvement in the hibernating myocardium^[112,113].

The recognition that IL-6 and IL-6-type cytokines are abundantly produced by MSCs^[6,55] and that skeletal muscle actively induces IL-6 during exercise^[56,114] prompted us to pioneer an intramuscular (*im*) MSC delivery route for cardiac repair^[6,30,115]. This *im* MSC therapeutic strategy is coupled to the inherent ability of skeletal muscle to produce beneficial trophic factors in response to exercise and injury^[116-118], and therefore represents an integrative physiological approach. The skeletal muscle is capable of regeneration after injury, and this ability is coupled to its production of many cardioprotective factors such as VEGF and HGF, which have been used in preclinical or clinical trials for cardiovascular therapy^[119,120]. Although *im* MSCs are trapped in the local musculature, their trophic actions promote increased growth factor levels in the quadriceps, liver, and brain, suggesting a possible global physiological effect^[6,30]. We further demonstrated that blocking JAK/STAT3 signaling abrogated the therapeutic effects of MSCs, indicating the functional relevance of MSC IL-6-type cytokines in initiating the paracrine cascade^[6].

As depicted in Figure 2, MSC-derived IL-6 and IL-6-type cytokines activate the injected muscle through JAK/STAT3 signaling, inducing downstream trophic factor genes such as VEGF, HGF, SDF-1 and IGFs. These factors mediate mobilization of bone marrow progenitor cells, cardioprotective signaling and activation of cardiac progenitor cells, resulting in decreased myocardial fibrosis and inflammation and increased cardiac regeneration and function. Notably, *im* MSCs also induce Suppressor of Cytokine Signaling 3 (SOCS3), which functions in a negative feedback loop to terminate cytokine signaling^[6]. Since excessive and prolonged IL-6 activity can cause tissue inflammation, induction

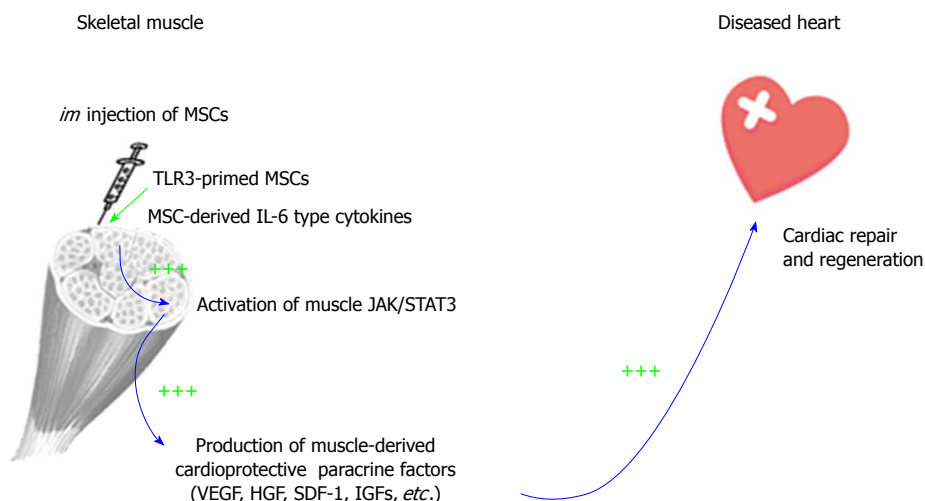


Figure 2 Intramuscular administration of mesenchymal stem cells mediates a paracrine mechanism of distal organ repair. The paracrine cascade initiated by mesenchymal stem cells (MSCs) is illustrated by blue arrows. TLR3 priming by poly (I:C) generates a super MSC phenotype through amplification of paracrine factors, which enhances MSC potency for cardiac repair (indicated by triple green plus signs). Supporting data have been published^[16,30,31,45,126].

of SOCS3 by *im* MSCs reduces the risk of this adverse reaction. The induced paracrine factors further enhance the expression of myocardial growth factors, activating the pro-survival signaling pathways in the diseased heart. Given that exercise is known to increase production of several beneficial trophic factors from the contracting skeletal muscle^[121-123], preventing coronary artery disease and cognitive decline^[124,125], our findings illustrate an *im* MSC-mediated cardioprotective paracrine mechanism mimicking the trophic action of exercise.

CONCLUSION

MSC therapy is entering a new era shifting the focus from initial feasibility study to optimization of therapeutic regimen and enhancement of treatment potency. Since tissue degeneration is often complex in nature and likely entails a therapeutic intervention strategy targeting multiple pathogenic mechanisms, the multiple paracrine factors released by MSCs and the injected host tissue acting in synergy are well suited as a regenerative medicine. Complete identification and understanding of these trophic factors can eventually lead to the development of cell-free trophic factor cocktails ideal for the treatment of tissue injury and degeneration, which may eliminate the concern associated with potential MSC transformation. Major challenges exist, however, regarding suboptimal stem cell potency and age/disease-related host tissue impairment, which may dampen enthusiasm for translational application of stem cells in general. The strategies outlined in this review offer a testable platform to launch innovative clinical trials based on rational design of MSC therapy.

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WCSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

"Ins" and "Outs" of mesenchymal stem cell osteogenesis in regenerative medicine

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etc.) that may provide peculiarities to self-renewal, differentiation, and physiologic function that may differ from non-transformed native cells. Tissue engineering approaches to use hMSCs to repair bone defects utilize the growth of hMSCs on three-dimensional scaffolds that can either be a base on which hMSCs can attach and grow or as a means of sequestering growth factors to assist in the chemoattraction and differentiation of native hMSCs. The use of whole native extracellular matrix (ECM) produced by hMSCs, rather than individual ECM components, appear to be advantageous in not only being utilized as a three-dimensional attachment base but also in appropriate orientation of cells and their differentiation through the growth factors that native ECM harbor or in simulating growth factor motifs. The origin of native ECM, whether from hMSCs from young or old individuals is a critical factor in "rejuvenating" hMSCs from older individuals grown on ECM from younger individuals.

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Abstract

Repair and regeneration of bone requires mesenchymal stem cells that by self-renewal, are able to generate a critical mass of cells with the ability to differentiate into osteoblasts that can produce bone protein matrix (osteoid) and enable its mineralization. The number of human mesenchymal stem cells (hMSCs) diminishes with age and *ex vivo* replication of hMSCs has limited potential. While propagating hMSCs under hypoxic conditions may maintain their ability to self-renew, the strategy of using human telomerase reverse transcriptase (hTERT) to allow for hMSCs to prolong their replicative lifespan is an attractive means of ensuring a critical mass of cells with the potential to differentiate into various mesodermal structural tissues including bone. However, this strategy must be tempered by the oncogenic potential of TERT-transformed cells, or their ability to enhance already established cancers, the unknown differentiating potential of high population doubling hMSCs and the source of hMSCs (*e.g.*, bone marrow, adipose-derived, muscle-derived, umbilical cord blood,

Key words: Mesenchymal stem cell; Telomerase reverse transcriptase; Extracellular matrix; Osteogenesis; Regenerative medicine; Tissue engineering; Proliferation; Differentiation

Core tip: When human telomerase reverse transcriptase (hTERT) transformed human mesenchymal stem cells (hMSCs) are used to prolong replicative potential and osteogenic differentiation, consideration should be given to using lower population doubling hTERT-transformed hMSCs to avoid potential oncogenesis. An inducible hTERT system may also avoid oncogenic transformation. Demonstration of *in vivo* bone forming capacity of hTERT-transformed cells should be used as standard in determining osteogenic differentiation of such cells rather than *in vitro* culture mineralization; the CD146 marker may be a suggested surface marker for hTERT-transformed hMSCs that may have the capacity to form bone *in vivo*. Native ECM from early population

doubling hMSCs or hMSCs from a younger source may be best when seeking to extend the proliferative and differentiating potential of hMSCs from either young or older sources.

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INTRODUCTION

The regeneration of mesodermal and neural crest-derived structural or connective tissues such as bone, cartilage, muscle and tendon continues to be a widely pursued for the reason that such structural tissues are generally homogeneous with either a predominantly single cell type or limited number of cells that contribute to the make-up of the tissue and that precursors to the mature cell types can be found in adult tissues. These precursor cells are generally multipotent, in that they can differentiate into a variety of connective tissue phenotypes. These precursor cells are generally referred to as adult mesenchymal stem cells (MSCs) or bone marrow stromal cells and can be found in the bone marrow but also as similar multipotent cells in specific tissues as well as circulating cells in blood.

Tissue engineering seeks to replace tissues that are either lost by traumatic events or by disease through the use of specific cell types that can recapitulate the lost or diseased tissue, and generally used in combination with a three-dimensional structural scaffold, and in many instances in combination with various growth factors, cytokines, and hormones or other biological molecules to assist in either the creation of a critical mass of needed cells or to assist in differentiating these cells to the required tissue type.

Because generating a critical mass of cells used in the regenerative process is a key to successful tissue engineering followed by differentiating those cells into the specific cell type comprising the tissue, stem cells have been the preferred starting cell type in many tissue engineering trials. This minireview will focus only on human adult bone marrow MSCs (herein assumed to be synonymous with bone marrow stromal cells) as much as possible and the telomerase strategy of inducing self-renewal of these cells to create a critical cell mass. Secondly, the minireview will examine the strategy of using extracellular matrix as a native scaffold upon which mesenchymal stem cells can self-renew and differentiate into bone.

MESENCHYMAL STEM CELL SELF-RENEWAL

The ability to self-renew is a hallmark of any stem cell^[1].

Self-renewal is simply defined as the ability of the resulting daughter cells, after mitotic division of the original mother cell, to retain the ability to generate a variety of differentiated cell types identical to that of the ability of the mother cell to differentiate in to those same cell types, and for a daughter cell to be able to generate daughter cells that also maintain the ability to differentiate into the same variety of cell types as the original “grandmother” and mother cells^[2]. The maintenance of self-renewal and pluripotency of stem cells occurs in the stem cell niche, where stem cells are able to receive cues from the stroma and other cell types either by direct contact or by secreted soluble factors within this microenvironmental niche^[3,4].

Adult MSCs also share the ability to self-renew. This potential to self-replicate and to differentiate into connective tissue phenotypes has led to the exploration to utilize MSCs in the repair of injured tissues^[5,6]. While the bone marrow has been a common site to harvest MSCs, other cell types similar to bone marrow-derived MSCs can also be found in other sites. Adipose-derived stem cells, satellite cells in muscle, and pericytes around blood vessels and umbilical cord blood cells also may share multipotent characteristics for differentiation into connective tissue phenotypes under specific conditions which include selective differentiation media and growth factors^[7-10]. In a comparison of MSCs from bone marrow, adipose tissue, and cord blood, Rebelatto *et al*^[11] (2008) reported that isolation rate of MSCs from umbilical cord blood was only a third that of bone marrow-derived and adipose-derived MSCs. The initial growth rate of bone marrow-derived and adipose-derived MSCs was much higher than that of umbilical cord blood MSCs. However, others have shown that the proliferation of umbilical cord tissue-derived MSCs show higher population doublings and shorter doubling times compared to adipose-derived MSCs although adipose-derived MSC had higher numbers of colony-forming units compared to MSCs from umbilical cord tissue^[12]. Surface marker expression of CD34 (cluster of differentiation molecule in family of sialomucin proteins) was significantly higher in adipose-derived MSCs compared to that of bone marrow-derived MSCs. Interestingly, CD117 (tyrosine-protein kinase Kit) was found to be positive in about 98% of adipose-derived MSCs but positive in only 52% and 39% of bone marrow-derived and umbilical cord blood-derived MSCs. Additionally, while osteogenic and chondrogenic differentiation was similar in MSCs from all three sources, umbilical cord blood-derived MSCs showed a lesser propensity for adipogenic differentiation. Others have also noted differences in marker expression between bone marrow-derived and adipose-derived MSCs. For instance, CD106 (vascular cell adhesion molecule-1) is expressed in bone marrow-derived MSCs but its expression in adipose-derived MSCs is either low or non-existent while CD49d (integrin $\alpha 4$ subunit) is expressed in adipose-derived MSCs but not in bone-marrow-derived MSCs^[13]. Culture conditions such as the use of fetal bovine serum, human serum, or serum-free medium have been shown to influence

not only the expression of surface markers for adipose-derived MSCs [e.g., CD117, CD166 (activated leucocyte cell adhesion molecule)] and bone marrow-derived MSCs but also in differentiation potential of such MSCs. As an example, fetal bovine serum has a stronger influence on osteogenic differentiation of adipose-derived MSCs than it does on adipogenic differentiation while allogeneic human serum and serum-free conditions have greater propensity to drive adipose-derived MSCs towards adipogenic differentiation than towards either osteogenic or chondrogenic lineages^[14]. Thus while adipose tissue and perhaps umbilical cord tissue sources may provide ample sources for MSCs compared to that of bone marrow and umbilical cord blood, differences in some specific surface markers for MSCs, proliferative potential, and differentiation potential *in vitro* occur based on the source of starting material to isolate MSCs, tissue culture supplements and conditions, and even human individual heterogeneity. Whether non-bone marrow-derived MSCs favor differentiation into specific connective tissue types or even non-mesodermal cell types as in the case of umbilical cord blood MSCs and adipose-derived MSCs in an *in vivo* environment is still a ripe area of investigation^[13-15].

Age of the organism is a determinant of the number of bone marrow MSCs present as well as *in vitro* tissue culture conditions that are critical for MSCs to retain their ability to self-renew yet demonstrate plasticity in their ability to differentiate into various mesodermal tissues^[16]. The number of cells from human bone marrow that are MSCs as determined by colony forming unit-fibroblastic (CFU-f) assay are less than 0.1% of total bone marrow mononuclear cells, thus demonstrating a minimal number of hMSCs that can be used in bone regeneration^[17]. The numbers of CFU-f and the capacity of CFU-fs that can differentiate into osteoblasts further decrease as a function of age of the bone marrow donor up to age 40; after age 40, there does not appear to be any further diminishing of CFU-fs that can differentiate into osteoblasts^[18]. It was suggested that hMSCs have decreased proliferative capacity as a function of age^[19]. Thus hMSCs from young individuals ages 18-29 years achieved an average population doubling level of 41 whereas hMSCs from older individuals ages 66-81 years achieved an average population doubling level of 24 with about a 55% lower population doubling rate than in hMSCs from the younger individuals. However, no difference in *in vivo* bone formation was noted as a function of donor age with early passage cells from either age group. Thus, once placed in primary culture, hMSCs have a limited lifespan (average 20 to 40 population doublings, but the number of population doublings may differ depending on growth medium or any added growth factors)^[19-21] under environmental conditions normally used for *in vitro* cell culture (humidified 5% CO₂ and 95% air (21% O₂) and when grown on tissue culture plastic. hMSCs grown in such conditions attain the Hayflick limit where cell division ceases, and the usual hMSC size becomes larger and the usual spindle shape of normal hMSCs becomes more polygonal or

with a variety of shapes and sizes, at times with multi-nucleation, and overall with less cell density per culture than cells undergoing cell division^[22]. As the number of population doublings for such cells is limited practically in primary culture, slower cell division and finally lack of cell division ensues and the above morphological changes are noted, and the expression of senescence-associated β -galactosidase, and p16, markers of cellular senescence, are increased^[23]. However, it has been shown that if environmental conditions simulate the MSC niche in the bone marrow, specifically low oxygen tension, that self-renewal of hMSCs can be prolonged. D'Ippolito *et al*^[24] (2004) developed a multilineage inducible MSC model from human cadaveric vertebral body marrow (MIAMI cells) and propagated them in 3% O₂/5% CO₂/92% N₂. They reported that more than 50 cell doublings beyond the Hayflick limit for primary cells could be achieved from hMSCs from at least 3 of 12 donors and at least 30 population doublings could be achieved from all of their donors. In a follow-up communication, they reported that MIAMI cells grown in 3% O₂ doubled more quickly than those grown at 21% O₂ and maintained the embryonic transcription factors OCT-4, REX-1, and hTERT and had suppressed osteoblastic differentiation when exposed to osteogenic differentiation medium. At higher O₂ concentrations of 21%, these embryonic transcription factors were lost and osteogenic differentiation was enhanced^[25]. The mechanism by which hypoxia regulates stem cell self-renewal appears to be *via* hypoxia inducible factor-1 α (HIF-1 α). Low oxygen concentrations stabilize HIF-1 α by inhibiting its degradation by the proteasome. Mazumdar *et al*^[26] (2010) reported that hypoxia induced canonical Wnt/ β -catenin signaling and increased transcription of Lef/Tcf genes which have hypoxia response elements in their promoter regions that bind HIF-1 α . Canonical Wnt/ β -catenin signaling thus can induce increased cell proliferation.

HTERT TRANSFORMATION OF HMSCS- THE "IN'S" FOR SELF-RENEWAL

In lieu of special resources needed to grow hMSCs in a hypoxic environment to maintain a proliferative state, a self-renewal strategy, engineering of hMSCs to over express telomerase has been an alternative means to maintain a longer proliferative lifespan of such cells. Telomerase, which is a multi-subunit ribonucleoprotein found in the cell nucleus and perhaps closely associated with nucleoli, allows for the addition of non-coding telomere DNA at the 3' end of linear chromosomes^[27-29]. Maintenance of telomere length by the addition of TTAGGG repeats onto the ends of telomeres allows for cells to continue to divide^[30]. Telomerase is expressed in human embryonic cells and in fetal, newborn, and adult testes and ovaries but not in mature spermatozoa or oocytes. Moreover, expression of telomerase disappears in human somatic cells in the neonatal period and later in life^[31]. Thus lacking telomerase, telomeres shorten with

each cell division leading to replicative senescence once cells reach a critical shortened telomere length. Specifically, with respect to MSCs, a number of laboratories have reported that hMSCs from bone marrow do not express telomerase activity or have activity below detectable levels by telomeric repeat amplification protocol (TRAP) assay when hMSCs are asynchronously dividing^[20,32-34]. However, human telomerase reverse transcriptase (hTERT) expression and telomerase activity could be detected when cells were synchronized to S-phase^[34]. Others have found that telomere length in hMSCs is short upon initial isolation and tend to further shorten with cell passage *in vitro* and appear to correlate with low to undetectable levels of hTERT^[35]. Thus theoretically, maintaining telomerase expression should prevent replicative senescence. Additionally, the decrease in telomere length correlates with CFU-f numbers suggesting that telomere length and telomerase activity could also be related to the ability of hMSCs to differentiate along various cell lineages including the osteogenic lineage^[35]. Gronthos *et al*^[36] (2003) reported that expression of hTERT in human bone marrow-derived MSCs not only increased proliferative capacity by up-regulating G1 to S phase transition cell cycle genes but also increased the expression of osteogenic genes for cbfa-1, osterix, and osteocalcin and induced bone formation earlier and to a much larger degree in an *in vivo* ectopic bone formation assay of hTERT-transformed hMSCs. Saeed *et al*^[37] (2011) demonstrated that in telomerase-deficient mice (*Terc*^{-/-}), there was delayed ossification in occipital bone, sternum, vertebrae, and metatarsals. Overall bone volume was decreased compared to wild type controls, and trabecular bone parameters showed decreased trabecular thickness and increased trabecular spacing^[37]. Additionally, bone formation rate was decreased which correlated with decreased osteoblast surface per bone surface, and osteoclast surface per bone surface was increased. The proliferative ability of bone marrow-derived MSCs from *Terc*^{-/-} mice was diminished compared to wild type mice, and there was increased β -galactosidase staining of *Terc*^{-/-} cells suggesting a more senescent phenotype of MSCs. There was up-regulation of pro-inflammatory genes (*e.g.*, IL-1 receptor type 2, toll-like receptor 6, leukotriene B4 receptor 1, tumor necrosis factor, *etc.*) indicative of osteoclastic activity as well as a decrease of osteoblast-specific bone markers. Thus both decreased bone formation and increased bone resorption as a result of an inflammatory microenvironment were found in this telomerase deficient model.

The critical components of human telomerase include the hTERT catalytic subunit and the RNA subunit, telomerase RNA (hTR), that provides a template for the synthesis of the DNA repeats at the ends of chromosomes. However, generally only hTERT is sufficient to maintain telomere length when transfected into various cell types although integration of ectopic hTERT alone to extend cell replicative ability may be dependent on integration site, availability of other proteins associated

with telomeres, or cell specificity^[32]. Thus a number of studies have demonstrated the feasibility of using hTERT in hMSCs to allow for prolonged replicative lifespan as well as capability of differentiating hTERT-transformed hMSCs towards the osteogenic lineage^[38-42]. The strategy used to transform hMSCs to over express the hTERT gene is generally a retroviral vector approach that uses green fluorescent protein expression as a positive selection marker to enable sorting of positively transformed cells by fluorescence activated cell sorting^[41]. An alternative approach to select transformed cells is an antibiotic resistance strategy^[42]. A technique to control hTERT expression in transfected hMSCs on demand utilizes the tetracycline inducible approach (Tet-On) so that proliferative and differentiation ability can be assessed at selected population doublings although “leakiness” of hTERT even in the Tet-off state could be a limitation^[40]. hTERT-transformed hMSCs have been reported to undergo at least 70 population doubling levels^[42] but upwards of 120 to 400 population doubling levels have been reported depending on the length of time in culture, plating density of cells, and subcultured clonal populations^[32,39-41]. The interesting aspect of hTERT-transformed hMSCs is that they are able to maintain their proliferative ability while being induced to differentiate along osteogenic, but also adipogenic, and chondrogenic lineages. Thus hTERT-transformed cells are different from non-transformed hMSCs and mesenchymal (stromal) cells from other species that are able to differentiate into osteoblasts where it is observed that as osteogenic differentiation proceeds, the proliferative ability of the cells diminishes^[43,44].

Three important criteria must be met when hMSCs are transformed by hTERT expression to achieve a critical mass of cells *via* self-renewal that would be necessary to populate fabricated scaffolds for tissue engineering. Firstly, markers of hMSCs should be maintained after hTERT transformation that would suggest maintenance of multipotency of the cells to undergo differentiation into various mesenchymal cell lineages. Secondly, it is important that hTERT transformation of hMSCs does not lead to malignant transformation either in the pluripotent state or in differentiated cell types. Thirdly, it is critical that hTERT expressing hMSCs will be able to specifically differentiate along the osteogenic lineage and to form bone which is the tissue of interest in this minireview.

Surface markers have been traditionally used to identify hMSCs. The International Society for Cellular Therapy set minimal criteria for positive markers to define hMSCs which are > 95% expression of CD105 (endoglin), CD73 (ecto-5'-nucleotidase), CD90 (Thy-1) and < 2% expression of hematopoietic stem cell markers, CD45 (protein tyrosine phosphatase, receptor type, C), CD34 (sialomucin family adhesion factor), CD14 (monocyte differentiation antigen/lipoglycan receptor) or CD11b (integrin alpha M), CD79 α (immunoglobulin associated alpha) or CD19 (B-lymphocyte antigen), and HLA-DR^[45]. Other markers used to identify hMSCs include STRO-1, CD146 (melanoma cell adhesion molecule/MUC18), CD49a

(integrin alpha subunit), CD271 (low-affinity nerve growth factor receptor), CD63 (lysosome-associated membrane protein-3), found on only on marrow-derived hMSCs and CD166 (activated leucocyte cell adhesion molecule)^[6,16,46-49]. Interestingly, stage-specific embryonic antigen-4 (SSEA-4), found on human embryonic stem cells, was identified as a marker for both mouse and human bone marrow-derived MSCs that had the ability to differentiate into both adipogenic and osteogenic lineages^[50]. Most recently CD44 was identified as a negative marker in freshly isolated although acquisition of the CD44 marker may be a function of *in vitro* cell culture of hMSCs^[51].

Telomerase expression and activity has been found in a majority of human tumors thus suggesting that hTERT expression in human cells could potentially lead to uncontrolled cell proliferation^[52]. However, it has also been suggested that the immortalization induced by hTERT may only in part be due to maintaining telomere length and stabilization and that non-canonical functions of hTERT such as the up-regulation of NF- κ B transcription by TERT binding to the p65 subunit of NF- κ B as well as activating the Wnt/ β -catenin pathway and its target genes, MYC and CCND1 (Cyclin D1), which are regulators of oncogenic targets, and the ability of NF- κ B to inhibit apoptosis, may be more important in promoting tumorigenesis^[53]. The loss of expression of p16^{INK4a}, the protein transcript of the *CDKN2A* gene, in addition to loss of p53 tumor suppressor function, and resistance to growth inhibition by transforming growth factor- β (TGF- β), are among other observations found in the acquisition of oncogenic potential in TERT transformed cells^[54].

Specifically in hMSCs that are transformed with hTERT, there is still the potential of such cells to express tumorigenic properties. Yamaoka *et al*^[55] (2011), constructed hTERT transformed bone marrow hMSCs and found that teratocarcinoma formation could occur when such transformed cells were implanted in immune deficient mice. However, the cells that these investigators transformed with hTERT had first been selected due to their ability to be maintain a proliferative state in the presence of fibroblast growth factor-2 (FGF-2) (> 100 population doubling levels) compared to hMSCs not cultured with FGF-2 that could proliferate to only 20 population doubling levels. As telomerase activity was absent in these FGF-2 maintained clones but had maintained long telomere length, an alternative lengthening of telomeres (ALT) pathway induced by FGF-2 in combination with TERT immortalization could have accounted for the malignant transformation. Serakinci *et al*^[56] (2004) also reported that hMSCs transformed with hTERT could exhibit neoplastic characteristics as shown by loss of contact inhibition and development of mesenchymal tumors after implantation of cells in immunodeficient mice. Loss of p16^{INK4a} and hypermethylation of DBCCR1 (deleted in bladder cancer chromosomal region candidate 1), a cell-cycle associated gene, were observed. Interestingly,

tumors were generated only in high population doubling level hTERT-transformed hMSCs and not in relatively lower population doubling level hTERT-transformed hMSCs. Similarly, Abdallah *et al*^[39] (2005) reported that mesodermal type tumors formed from hTERT transformed hMSCs that had a short population doubling time and accelerated growth, but no tumors developed in hTERT transformed hMSC clones with longer population doubling times that were slower growing. Thus the potential for neoplastic change may be associated with loss of proliferative control as evidenced by cell cycle gene alterations with continued proliferation.

Nevertheless, others have reported that hTERT-transformed hMSCs did not exhibit changes associated with neoplasia even at higher population doubling levels (up to 275)^[32,41,57]. However, whether or not potential oncogenic development occurs in hTERT-transformed hMSCs, functional changes in hMSC parameters need to be considered. Baumer *et al*^[58] (2011) reported that hTERT-transformed human coronary artery endothelial cells demonstrated changes in an *in vitro* co-culture angiogenesis assay where TERT-transformed human coronary artery endothelial cells co-cultured with human fibroblasts and treated with vascular endothelial growth factor (VEGF) did not form tubular networks indicative of angiogenesis; non-TERT-transformed endothelial cells in co-culture with fibroblasts and treated with VEGF were able to form tubular networks. Moreover, hTERT-transformed endothelial cells responded differently to exogenous tumor necrosis factor- α (TNF- α) compared to non-hTERT transformed cells where vascular cell adhesion molecule-1 (VCAM-1) expression was lower, and endothelial barrier function as measured by transepithelial resistance was lost in hTERT-transformed cells. Since hMSCs are immunomodulatory cells that can affect the function of immune hematopoietically derived cells (lymphocytes, monocytes, *etc.*) in an inflammatory environment, there needs to be further investigation if hTERT transformation of hMSCs do not affect these immunomodulating properties of normal hMSCs or have altered function in differentiation or on angiogenesis when interacting with other cell types in a microenvironmental setting.

Perhaps the most prudent approach to ensure that hTERT transformed hMSCs would be useful for bone repair after induction of osteogenic differentiation would be to use inducible vectors for hTERT expression that can then be regulated both temporally and spatially to avert problems with continuous cell proliferation that could result in oncogenic transformation of hTERT-transformed hMSCs^[40].

One other caveat involving the potential enhancement of carcinogenesis may be specific to adipose-derived stem cells (stromal cells) and endothelial cells from white adipose tissue that is independent of hTERT transformation. Zhang *et al*^[59] (2009) reported that the stromal/vascular fraction of white adipose tissue that have proliferative and multipotent differentiative capacity

as well as pericyte-like characteristics can home to human breast and prostate carcinoma cell lines, Kaposi's sarcoma endothelial cell line, and a mouse lung carcinoma cell line implanted in xenograft and allograft mouse models. These stromal/vascular cells engrafted into the tumors and enhanced cancer progression in part through stimulating angiogenesis in the tumors but also perhaps through immunosuppressive effects of the adipose-derived mesenchymal cells found in the stromal/vascular fraction. In follow-up studies, these investigators showed that the increase in the number of adipose-derived stromal (mesenchymal) cells found in obesity could be recruited to mouse and human breast cancer and mouse ovarian cancer models and stimulate tumor growth by increasing tumor vascularity and by differentiating into adipocytes and stimulating proliferation of tumor cells^[60]. In human studies, it was reported that there was increased frequency of mesenchymal stromal (CD34^{bright}CD45⁻CD31⁻) cells (also harboring the pericyte marker, NG2) and CD34^{bright} leucocytes (CD45^{bright}CD34^{bright}) in obese patients (BMI > 30) with colorectal cancer compared to obese non-cancerous subjects^[61,62]. Lean patients with colorectal cancer also had a higher frequency of mesenchymal stromal cells and CD34^{bright} leucocytes compared to lean, non-cancerous controls. However, there was a significant increase in MSCs in obese colorectal cancer patients compared to lean colorectal cancer patients. Thus mobilization of MSCs and CD34^{bright} leucocytes may potentially be markers of colorectal cancer but that there may be a higher frequency of CD34⁺ MSCs (adipose stromal cells) released into circulation even in non-cancerous obese patients suggesting that adipose tissue contributes to MSC mobilization.

OSTEOGENIC DIFFERENTIATION OF HTERT-TRANSFORMED HMSCS

Differentiation of hMSCs along the osteogenic lineage has been demonstrated using both *in vitro* and *in vivo* techniques. Induction of *in vitro* osteogenic differentiation in hMSCs include addition of dexamethasone, ascorbate, and a source of phosphate, mainly β -glycerophosphate to a culture medium base (generally Dulbecco's modified Eagle's medium) containing 10% bovine serum. However, recently it was reported that hMSCs from bone marrow may not require the addition of dexamethasone and ascorbate to form bone *in vivo* although bone marrow-derived hMSCs respond to dexamethasone and ascorbate with increased proliferation *in vitro*^[63]. Osteogenic marker expression by mRNA and protein is usually assessed over the course of *in vitro* cell culture. Early markers of osteogenesis include core binding factor 1 [cbfa1 or runx2 (Runt-related transcription factor 2)] which is found in chondro-osseous precursor cells, osterix which appears in committed osteogenic cells, and collagen type I. Intermediate markers of osteogenesis include alkaline phosphatase and osteopontin and bone sialoprotein and osteocalcin (usually induced in hMSCs by 1.25 dihy-

droxyvitamin D3) are generally used as later markers of terminally differentiated osteoblasts. Determination of mineralization of culture *in vitro* is also critical in assessing terminal differentiation along the osteogenic lineage. This is usually accomplished by staining cell cultures using alizarin red or von Kossa stains which bind to calcium and/or eluting these stains for semi-quantitation of calcium spectrophotometrically. It is also suggested that to distinguish amorphous calcium-phosphate precipitation in cultures from hydroxyapatite [$\text{Ca}_{10}\text{P}_8(\text{OH})_2$], X-ray diffraction, nuclear magnetic resonance, or other technique be used to compare the calcium-phosphate complexes in *in vitro* cell cultures with standard hydroxyapatite patterns by these techniques. Additionally, negative markers for other mesodermal cell types that can be differentiated from hMSCs should be assessed. These are usually markers for the adipogenic lineage [adipsin, peroxisome proliferator-activated receptor gamma (PPAR- γ), adiponectin], the chondrogenic lineage (sox9, collagen type II, collagen type X, aggrecan), tenogenic lineage (scleraxis)^[64], and myogenic lineage Pax3, Pax7 (myogenic precursors), MyoD and myogenin (skeletal muscle), α -smooth muscle actin, vascular endothelial (VE) cadherin (smooth muscle). Essentially, similar techniques to demonstrate osteogenic differentiation have been used for hTERT-transformed hMSCs.

In vivo osteogenesis of hMSCs, whether or not transformed with hTERT, is usually accomplished by ectopic bone ossicle formation assay. In this assay, hMSCs are usually mixed with hydroxyapatite and/or treated with various bone morphogenetic proteins (BMPs) and are implanted into subcutaneous pockets in either immunocompromised rodents (e.g., nude mice; NOD/SCID mice)^[32,39,65,66] or into immune competent rodents^[41]. Assessment for bone formation is done by microCT and/or histology to identify trabecular bone formation and the expression of the above bone marker genes and proteins in tissue sections. hMSCs have been shown to create a locally immunosuppressive microenvironment and are able to avoid allo-recognition^[67] perhaps in rodent species although it is unknown if the same holds true for transplantation of hMSCs into human recipients or if there are any consequences of immunogenicity of hMSCs once they are differentiated into specific lineages in a human recipient^[68].

It is highly important that the both *in vitro* and *in vivo* confirmation of hydroxyapatite or bone formation be done especially in hTERT-transformed hMSCs. It is possible that not all hTERT-transformed hMSCs will be able to form bone *in vivo*. Larsen *et al.*^[69] (2010) established subclones from hTERT transformed hMSCs at a relatively early population doubling level (PDL 77) and from a later PDL 233. They found that both subclones retained surface markers for hMSCs (CD63, CD73, CD105, and CD166) as well as expressed osteoblast markers, alkaline phosphatase, collagen type I, and osteocalcin upon induction with osteogenic medium. Both clones also formed mineralized matrix *in vitro* as assessed

by alizarin red staining. However, the PDL 77 clone was able to form bone in an *in vivo* ectopic bone formation assay while the PDL 233 clone did not form bone. Interestingly, these investigators reported that CD146 was highly expressed in the hTERT-transformed hMSC clone that could form bone *in vivo* while CD146 was minimally expressed in the hTERT-transformed clone that did not form bone *in vivo*. Thus the criteria for *in vivo* bone formation and expression of CD146 should be helpful in assessing hTERT-transformed hMSCs that may be useful for potential bone repair or regenerative therapy, and sole dependence on osteogenic markers and *in vitro*, two-dimensional cell culture mineralization assays may be insufficient. Also observed in additional hTERT-transformed hMSC clones that formed bone *in vivo* was the increased number of extracellular matrix genes expressed as well as the increased number of Sp3 binding sites in the promoter regions of these expressed genes compared to that of hTERT-transformed hMSC clones that did not form bone *in vivo*. Sp3 is a transcription factor necessary for bone development and ossification.

In attempts to seed hTERT-transformed hMSCs in areas requiring their presence for tissue repair, strategies such as intracardiac or intravenous injection of hMSCs expressing a fluorescent marker (*e.g.*, green fluorescent protein) have been used to identify sites where such injected hMSCs populate as well as to assess the longevity of transplanted hMSCs in the desired regions. Bentzon *et al*^[70] (2005) reported that hTERT-transformed hMSCs injected intracardiac or intravenously into NOD/SCID mice were trapped mainly in microvasculature of the lungs, kidneys and heart. It was also found that only a small fraction of the injected telomerized hMSCs survived or were retained possibly due to protracted trans-endothelial migration. Thus direct engraftment of hTERT-transformed hMSCs may be a better approach to healing bone.

In addition to cells, such as MSCs, that have the potential to self-replicate and differentiate into the cell type of choice, tissue engineering in regenerative medicine strategies generally combine the cellular component with various growth and differentiation factors that can promote differentiation of undifferentiated precursor cells and with the employment of a structural framework on which either such cells and/or growth and differentiation factors can be assembled. The use of three-dimensional culture platforms may simulate the natural three-dimensional *in vivo* tissue architecture and provide advantages over that of assessing hMSC growth and differentiation on tissue culture plastic in a two-dimensional format^[71,72]. Two dimensional cultures may only yield woven type bone (random orientation of collagen fibrils) and not allow for the formation of lamellar bone, the final desired bone product, and microenvironments that may develop in a three-dimensional framework that could affect cell-cell and cell-matrix interactions cannot fully develop in a two dimensional culture system.

For *in vivo* uses, three-dimensional platforms or scaffold

needs need to be biocompatible, potentially biodegradable, have sufficient porosity to allow great surface area for cell attachment, and in general be non-immunogenic. The more rigid platforms or scaffolds composed of material such as hydroxyapatite or other calcium-phosphate bases which are osteoinductive and can induce ectopic bone formation. Titanium has been used to grow hMSCs that can then be differentiated along the osteogenic lineage with or without BMP stimulation prior to direct surgical implantation into bone defects in translational models of bone repair^[73-75]. Biological scaffolds that are composed of polymer blends such as poly(l-lactide-co-glycolide) (PLGA) are biocompatible and can be degraded by the body have also been used as a base on which hMSCs can be grown and differentiated^[76]. Polymer blends have also been used in combination with inorganic hydroxyapatite crystals or naturally occurring proteins such as collagen to construct composite scaffolds that improve mechanical and osteoinductive properties of the scaffolds have also been designed^[77]. Hydrogels have also been used as scaffold material due to biocompatibility; natural hydrogels are derived from collagen or gelatin, while synthetic hydrogels can be made from poly(ethylene glycol). While natural hydrogels are excellent for cell adhesion and biodegradation, immunogenic reactions may be a concern if the hydrogels are derived from animal-derived extracellular matrix (ECM) protein. Synthetic hydrogels have the advantage of creating scaffolds *in situ* using photopolymerization and also are non-immunogenic^[78]. Hydrogels as well as polymer blends with or without ceramic material (*e.g.*, hydroxyapatite) have also been useful in serving as reservoirs for bioactive molecules such as growth factors^[77-79]. Thus scaffolds impregnated with various growth factors or composed in part of ECM-derived short peptides, modified heparin, chondroitin sulfate or hyaluronic acid to tether growth factors such as the BMPs, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), TGF- β , FGF-2 have been useful in the differentiation of transplanted hMSCs and/or the chemotaxis of native MSCs useful in bone repair^[75,77-82].

Stromal-derived factor 1 (SDF-1), a chemokine, has also been impregnated in scaffolds to serve as a chemotactic factor for bone marrow-derived MSCs^[83-85]. It has also been shown that human cord blood-derived MSCs as well as human adipose tissue-derived MSCs (the stromal/vascular fraction of adipose tissue) express CXC receptor 4 (CXCR4), the receptor for SDF-1, and are induced to migrate in response to SDF-1^[86,87]. Human bone marrow-derived MSCs have also been shown to migrate to bone marrow stroma in a CXCR4-dependent manner^[88]. Bone-marrow-derived MSCs can also express SDF-1 and serve to maintain hematopoietic stem cells in a quiescent state in the bone marrow^[89,90]. However, under conditions of inflammation with the release of pro-inflammatory cytokines such as TNF- α and IL-1 β and hypoxia that can be found in tissue injury and the early phases of wound repair, bone marrow-derived MSCs as well as MSCs from adipose tissue or other local sources could migrate to the

wound location *via* the SDF-1/CXCR4 axis to participate in the repair or regeneration of mesenchymal tissues (e.g., bone)^[91-94]. Potential sources of SDF-1 that could potentially be involved in local MSC migration and homing to disrupted bone may be endothelium, local osteoblasts, platelets involved in initial wound hemostasis, and perios-teal cells^[87,95-97]. VEGF has been used to stimulate angiogenesis that would allow for improved blood supply to repairing tissues; use of VEGF incorporated into natural hydrogels or injected directly into scaffolds and in combination with BMPs and MSCs attached to scaffolds have been tested to improve bone healing^[81,98]. In the absence of seeding MSCs onto scaffolds, delivery of SDF-1 *via* implantable infusion pump to poly- ϵ -caprolactone scaffolds preceded by delivery of VEGF to the scaffolds and followed by BMP-6 to induce osteogenic differentiation was able to induce mature mineralized bone formation^[99]. Tasso *et al*^[100] (2009) also reported that in a mouse model of ectopic bone formation, donor murine bone marrow MSCs loaded onto hydroxyapatite scaffolds were needed in the early development of ectopic bone (up to one week after implantation) to recruit host osteoprogenitor cells, but native (host) osteoprogenitor cells actually contributed the most to the bone formation *via* endochondral ossification. Thus native MSCs can be induced to populate scaffolds using SDF-1 and osteogenically differentiate to form vascularized bone. MSCs harboring viral vectors (adeno-associated virus or lentivirus) to over express growth factors and chemoattractants and attached to various types of scaffolds have been used as an alternative strategy to increase local concentrations of bioactive molecules such as BMP-2, BMP-7, VEGF, and CXCR4, the transmembrane G-protein coupled receptor for SDF-1-induced chemoattraction, to enhance osteogenic marker expression^[101-106]. Finally, other chemokines may also play roles in migration of MSCs. Chemokines of the α family (CXC chemokines) as well as the β family (CC chemokines) have been reported to stimulate migration of MSC from both bone marrow and omental adipose tissue^[92,107,108]. Interestingly, under pro-inflammatory conditions as is found in the initial phase of wound healing, priming with TNF- α enhances the expression of these chemokines such as CXCL8 (interleukin 8), CCL5/RANTES (regulated on activation, normal T cell expressed and secreted), CCL22 (macrophage-derived chemokine) which are then able to stimulate MSC migration^[94,95]. Additionally, CXC chemokines with the glu-leu-arg motif in the N-terminus of CXC chemokines are also angiogenic and thus may play a role in new blood vessel formation during wound repair during bone regeneration^[109].

Three dimensional spheroid cultures consisting of high density cell aggregates in agarose or alginate have also been used to traditionally differentiate chondrocytes from hMSCs^[110,111]. Burns *et al*^[112] (2010) used a variation of this method by using caroxymethylcellulose in their high density cell preparation to form spheroids of hTERT transformed hMSC cells. When combined with hydroxyapatite/ β -tricalcium phosphate scaffolds,

induced with osteogenic medium, and implanted into immunodeficient mice in an *in vivo* ectopic bone formation assay, lamellar bone formation was observed in scaffold concavities in addition to the expression of usual osteoblastic markers of cbfa1, alkaline phosphatase, osteonectin, osteopontin, collagen type I and osteocalcin. CD146 expression which had been high in hMSCs was lost as osteogenic differentiation proceeded. Interestingly, transcriptional co-activator with PDZ binding motif (TAZ)^[113], a cbfa1 binding transcription co-activator that allows for commitment to the osteogenic lineage while inhibiting adipogenic differentiation of hMSCs was also induced in the hTERT transformed hMSC spheroids. Stimulated expression of other extracellular matrix proteins such as biglycan, lumican, elastin, periostin, microfibrillar-associated proteins (MFAP2 and MFAP5), tetranectin and decorin also occurred suggesting correlation between these extracellular matrix protein and osteogenesis.

EXTRACELLULAR MATRIX AND HMSCS- THE "OUTS" FOR OSTEOGENESIS

The use of extracellular matrix (ECM) components to enhance either rigid type scaffolds or hydrogel scaffolds or to serve as scaffolds themselves has become more popular in tissue engineering. For instance, collagen type I in the form of gels or sponges or as a protein coating of hydroxyapatite platforms has been useful in providing an attachment for cells in addition to being able to deliver growth factors such as TGF- β , BMPs, or VEGF^[114]. ECM contains proteoglycans which are comprised in part of heparin sulfate that can bind many types of growth factors such as FGFs and VEGF and degradation of ECM by matrix metalloproteases can release these growth factors to subsequently bind to their receptors on specific cells^[115]. Other ECM proteins such as laminin and tenascin have epidermal growth factor (EGF)-like motifs that could potentially bind to EGF receptors on cells and then initiate an EGF signaling cascade through tyrosine kinase activation resulting in cell proliferation and/or differentiation^[116]. The binding of cells to naturally occurring proteins such as collagen occur *via* integrins, comprised of α and β subunits and binding cell membranes to ECM proteins with the arginine-glycine-aspartic acid (RGD) or leucine-valine-aspartic acid (LVD) (consensus sequence L/I (isoleucine)-D/E (glutamic acid)-V (valine)/S(serine)/T(threonine)-P (proline)/S) domains^[117,118]. The short cytoplasmic domains of integrins interact with various cytoskeletal elements such as talin and kindlin to initiate inside-out signaling through integrin-linked kinase that is involved in activating integrins to bind to ECM components^[119,120]. Outside-in signaling occurs with the interaction of specific sequences of ECM proteins and activated integrins to activate focal adhesion kinase to allow in part for functions such as cell spreading and migration but also activating other signaling pathways enabling cell proliferation, and survival^[121].

Thus scaffolds composed of native ECM proteins such as collagen have been applied as one strategy to expand hMSCs *ex vivo* and to promote osteogenic differentiation and to enhance bone repair^[17,122,123]. Bone marrow-derived hMSCs, express various integrins such as $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$; however, the β_1 integrin subunit was found to be most responsible for hMSCs to adhere to collagen, laminin and fibronectin and be involved in proliferation of hMSCs and for their differentiation into osteoblasts^[124]. However, pre-coating scaffolds with a specific protein such as collagen type I or MatrigelTM (BD Biosciences) composed of collagen type IV, entactin, and laminin, may not yield the natural three dimensional environment, nor account for all appropriate ECM proteins that interact with hMSCs *in vivo*, nor retain the natural elasticity or stiffness required for proper self-renewal or tissue-specific differentiation. Degree of stiffness or elasticity of support structures or ECM has been shown to be important in part to be a determinant of stem cell differentiation. In reference to MSCs, softer substrates favor adipocyte or chondrogenic differentiation while stiffer substrates direct osteogenic differentiation. Intermediate stiffness can assist in directing myogenesis from MSCs^[125-128]. ECM or bioengineered support structure stiffness or elasticity can be sensed by cells through the organization of stress fibers composed of actin microfilaments and myosin. Specifically, non-muscle myosin II isoforms, II A, II B, and II C appear to be involved in the MSC's ability to sense matrix stiffness through their interaction with cortical actin that is linked to focal adhesions. Increased matrix stiffness is associated with increased activity of non-muscle myosin II. The increased non-muscle myosin II also correlates with specific lineage determination of MSCs^[129]. Interestingly, ECM stiffness that can set the stage for specific lineage differentiation *via* expression or repression of specific genes is transduced to nuclear chromatin *via* lamin-A^[130]. Cytoskeletal stresses and tension increase with increasing ECM stiffness and the degree of lamin-A expression and phosphorylation is inversely related to ECM stiffness. Thus osteogenic differentiation of MSCs is correlated with increased lamin-A levels and decreased lamin-A phosphorylation when MSCs are grown on a stiff ECM. It would follow that lamin-A would act in a manner to maintain nuclear rigidity or stiffness which could translate into epigenetic regulation of chromatin thus enabling transcription of osteogenic genes and repression of genes specific with other mesenchymal lineages through lamin A-associated domains which contain repressive heterochromatin.

Thus the use of cell-free preparations of secreted ECM proteins produced by MSCs or cells of the desired differentiated type (*i.e.*, osteoblasts) may perhaps allow for better osteogenic differentiation of MSCs in a native three dimensional microenvironment similar to the MSC niche found in bone marrow. Chen *et al*^[131] (2007) prepared ECM from mouse MSCs that supported self-renewal of mouse MSCs when cultured on this native

ECM and the proliferative ability of the MSCs grown on native ECM was greater than MSCs grown on fibronectin or collagen type I individually. Differentiation of mouse MSCs into both adipogenic (in response to rosiglitazone) and osteogenic lineages (in the presence of exogenous BMP-2) was also enhanced in cells cultured on native ECM compared to tissue culture plastic or culture plastic coated with fibronectin alone or with collagen type I alone. However, mouse MSCs had a delay in osteogenic differentiation when grown on native ECM in the absence of exogenous BMP-2, and it was suggested that the native ECM components such as collagen and biglycan bind BMP-2, making it less available to MSCs to allow for earlier osteogenic differentiation.

hMSCs can also be used to generate native ECM that supports self-replication of hMSCs, and the degree of enhanced proliferation of hMSCs was found to be greater than that of hMSCs grown on tissue culture plastic, or fibronectin or collagen type I independently^[132]. It was also found that SSEA-4, a marker for bone marrow-derived hMSCs, was maintained at a high level throughout the culture period on native ECM and interestingly, telomerase activity was stable and reactive oxygen species was low on ECM-grown hMSCs compared to hMSCs grown on plastic, fibronectin, or collagen type I. *In vivo* bone formation was also significantly higher in hMSCs grown on native ECM compared to those grown on plastic. Thus native ECM from hMSCs can better support self-renewal and osteogenic differentiation compared to single ECM components or a two dimensional culture platform (plastic).

It has been shown that ECM from human foreskin young fibroblasts (< 20-30 population doublings) supported the proliferation of old fibroblasts (> 68 population doublings) so that the proliferative rate of the old fibroblasts approached that of young cells grown on ECM from young cells^[133]. Additionally, telomere length was restored in old fibroblasts grown on ECM from young cells by a telomerase independent mechanism and reduced reactive oxygen species similar to young cells was also found. Interestingly, SIRT 1, a gene for the NAD-dependent histone deacetylase, sirtuin 1, which was downregulated during senescence was increased when old fibroblasts were grown on ECM from young cells. This suggests that epigenetic mechanism(s) may play a role the mechanism of how young ECM can restore the proliferative ability of old fibroblasts. SIRT 1 can be directly activated by lamin A^[134], which is critically involved in the process of information flow from ECM to the nucleus to perhaps determine chromatin configuration and thus confer epigenetic regulation on gene expression or repression. Thus the potential role of epigenetics in ECM rejuvenation of old fibroblast cells is an area of interesting investigation.

With regards to MSCs, the composition of ECM from young (low passage) adipose-derived MSCs compared to that of old (higher passage) MSCs is different. For instance, while collagen type I is increased in young

MSCs, laminin, fibronectin, vimentin, keratin, and lamin A/C are decreased in old MSCs. When old MSCs are seeded onto ECM from young MSCs, the pluripotency markers of Oct4, Sox2, and Nanog are increased and growth factors such as TGF β are also upregulated^[135]. The ECM component, biglycan, has been shown to increase canonical Wnt/ β -catenin signaling. Wnt signaling is a critical morphogen in osteoprogenitor development. Bone marrow MSCs from mice deficient in biglycan were less proficient in Wnt-induced mineral deposition in culture, did not respond to exogenous Wnt3a, and made significantly less trabecular bone when used in an *in vivo* ectopic bone formation assay^[136]. Thus one could speculate that ECM from young MSCs may have more biglycan than ECM from old MSCs and thus young ECM would be able to enhance Wnt signaling to enhance both proliferation of osteoprogenitors and potentially more bone formation. However, the exact mechanism of how biglycan can regulate either canonical or non-canonical Wnt signaling is unclear.

In another interesting study, Sun *et al*^[137] (2011) reported the differential effect of ECM from mouse bone marrow stromal cells derived from young (3 mo) versus old mice (18 mo). Replicative ability was restored in MSCs from old mice cultured on ECM from young mice, similar to that of the replicative ability of young mice grown on ECM from young mice. However, the replicative ability of MSCs from either young or old mice was significantly less when cultured on ECM from old mice. Telomerase levels were also increased in MSCs from young and old mice cultured on ECM from young mice compared to that of MSCs cultured on tissue culture plastic or on ECM from old animals. Examination of bone forming ability using an *in vivo* assay where MSCs from young or old mice pre-cultured on ECM from young or old mice demonstrated that MSCs from old mice pre-cultured on ECM from young mice had increased cancellous bone formation compared to MSCs from young or old mice pre-cultured on tissue culture plastic. Culture of MSCs from either young or old mice on ECM from old mice demonstrated less bone formation. In trying to dissect the differential effect of ECM from old versus young mice, these investigators found that ECM from old mice contained more mineral phosphate and less collagen although the total amount of ECM produced by young or old cells was the same. Furthermore, reactive oxygen species levels were higher in MSCs grown on ECM from old mice but were reduced in MSCs grown on ECM from young mice; there was also an inverse correlation of the number of colony forming units-osteoblast and the level of reactive oxygen species. How ECM from old mice is incapable of handling reactive oxygen species and how this may relate to changes in ECM composition (lower collagen and proteoglycans) remains unknown.

In a recent communication, Prewitz *et al*^[138] (2013) used early passage bone marrow-derived hMSCs to generate native ECM but used either osteogenic medium to allow the hMSCs to differentiate towards the osteogenic

lineage or ascorbic acid alone in the growth medium to allow the hMSCs under these conditions to generate an “enriched” ECM. These generated ECMs were then tethered to tissue culture plastic using poly(octadecene-*alt*-maleic anhydride). These investigators reported that ascorbic acid-stimulated native ECM contained twice as much collagen and sulfated glycosaminoglycans compared to native ECM generated using osteogenic medium although the spectrum of ECM protein were the same. Release of hepatocyte growth factor, FGF, VEGF, and interleukin-8 was also higher from ascorbic acid-stimulated ECM. Nevertheless, both types of ECM supported higher population doublings of hMSCs grown on these surfaces compared to hMSCs grown on either plasma-treated tissue culture plastic, fibronectin or MatrigelTM. Both ascorbic acid and osteogenic-induced ECM also stimulated more osteogenic differentiation as well as adipogenic differentiation although the ascorbic acid-induced ECM yielded better osteogenic and adipogenic differentiation than osteogenic-induced ECM. Finally, both ascorbic acid-induced and osteogenic-induced ECM were able to support the engraftment of hematopoietic stem and progenitor cells, similar to a hematopoietic stem cell niche. Hence, bolstering native ECM by stimulation its production from hMSCs with either ascorbic acid or osteogenic medium could potentially be a useful strategy in rejuvenating old hMSCs.

Thus whether the total or individual amounts of native ECM, the breadth of composition of native ECM, the geometry of ECM organization, or the ability of ECM to sequester growth factors, retain growth factor-like motifs (*e.g.*, similar to the EGF-like repeats found on laminin and tenascin), or regulate other morphogens such as Wnt signaling that can potentially regulate MSC proliferation and differentiation are important factors in explaining the mechanism(s) of how young ECM can rejuvenate old MSCs are salient areas for future investigation.

CLINICAL UTILITY OF MESENCHYMAL STEM CELLS IN ORTHOPAEDIC CONDITIONS

MSCs from various sources in combination with specific growth factors and/or scaffold material potentially lend themselves to a variety of clinical orthopaedic conditions involving bone and cartilage. There are a number of clinical trials and case reports using MSCs to repair critical sized defects caused by trauma or infection as well as replacing chronically degenerated tissue such as articular cartilage and intervertebral discs. There are a number of excellent and comprehensive published reviews on the subject of orthopaedic applications for MSC therapy^[139-143] and which are listed in Table 1. Two clinical trials and two other case reports using MSCs in human orthopaedic conditions are also included in Table 2. The clinical trial to treat knee osteoarthritis enrolled 25 pa-

Table 1 Reviews of mesenchymal stem cell use in human orthopaedic conditions

Ref.	Reviewed Orthopaedic conditions treated	MSC source	Additional repair components
Shenag <i>et al</i> ^[139] , <i>Stem Cell Int</i> , 2010	Osteonecrosis humerus, femoral head; Fracture non-union; Cartilage defect; Osteogenesis imperfecta; Critical size defect limbs; Calvarial defect	Autologous or allogeneic bone marrow; Fetal liver; Adipose	Ceramic scaffolds; Collagen gels
Rastegar <i>et al</i> ^[140] , <i>World J Stem Cells</i> , 2010	Critical size defect in long bones; Articular cartilage of knee; Osteogenesis imperfecta; Hypophosphatasia	Autologous bone marrow; Allogeneic bone marrow; Fetal liver	
Zhang <i>et al</i> ^[141] , <i>Biomaterials</i> , 2012	Segmental bone defects of limbs; Distraction osteogenesis; Tibial osteotomy; Posterior spinal fusion; Maxilla defects; Sinus augmentation; Osteogenesis imperfecta; Articular cartilage repair; Osteoarthritis	Autologous bone marrow; Allogeneic bone marrow; Fetal liver	Hydroxyapatite scaffolds; autologous platelet rich plasma, allogeneic bone chips or bone grafts; β -tricalcium phosphate scaffolds
Veronesi <i>et al</i> ^[143] , <i>Stem Cell Dev</i> , 2013	Osteoarthritis of knee, hip, elbow, ankle; medial femoral condyle, patellar, patella-femoral joint lesions; osteochondral lesions talar dome and femoral condyle	Autologous bone marrow	Hyaluronate; collagen type 1 sheet; platelet rich plasma; periosteal patch; collagen powder
Kim <i>et al</i> ^[142] , <i>Korean J Int Med</i> , 2013	Osteogenesis imperfecta; Cartilage defects	Allogeneic bone marrow, fetal liver	

MSC: Mesenchymal stem cell.

Table 2 Clinical trials and case reports using mesenchymal stem cells in human orthopaedic conditions

Orthopaedic condition	MSC source	Technique	Patients/controls	Study length	Outcome	Ref.
Osteoarthritis-Knee	Adipose	Autologous MSCs with platelet rich plasma	25/25 retrospective controls	12 mo	Study group significantly higher degrees of improvement from pre-treatment levels in pain and activity	[144]
Intervertebral Disc Degeneration	Bone marrow	MSC injection into nucleus pulposus	10/self-controls - pre- and post procedure	12 mo	Pain, disability, quality of life, disc water content improved	[145]
Maxillary Reconstruction	Adipose	Vascular flap with ADCs, β -tricalcium phosphate, BMP-2	1 case	12 mo	Regeneration of normal bone	[146]
Mandibular Reconstruction	Bone marrow	BMP-2, collagen sponges + bone marrow MSCs + allogeneic bone chips	5 cases	22 mo	Bone regeneration in 2/4 cases using MSCs; failure overall in 2 of 5 cases	[147]

MSC: Mesenchymal stem cell; BMP: Bone morphogenetic protein.

tients. Infrapatellar fat pad-derived MSCs and platelet rich plasma were injected into knee joints after arthroscopic debridement, excision of degenerative material/osteophytes, or synovectomy^[144]. Comparison was made to retrospective age- sex- and follow-up period matched controls who had received only platelet rich plasma injections with arthroscopic debridement. Various scales used in knee symptoms (visual analog pain scale, Lysholm knee scoring scale, Tegner activity level scale) showed that the initial or pre-treatment scores of the study group were significantly poorer compared to controls but by the last follow-up visit (12 mo) after MSC therapy, the study group showed significantly higher degrees of improvement from pre-treatment levels in all of the assessment scales measured compared to that of the retrospective control group. Orozco *et al*^[145] (2011) injected autologous bone marrow-derived MSCs that were expanded under Good Manufacturing Practice conditions into the nucleus pulposus in 10 patients. These patients apparently served as their own controls and pain (visual analog scale), disability (Oswestry Disability Index), and quality of life (SF-36) were improved over the 12 mo trial. Water content of the diseased discs also improved by 12 mo after

treatment. Two other communications consisting of case reports are also entered into Table 2. One report used autologous adipose-derived stem cells expanded in vitro and combined with β -tricalcium phosphate scaffolding material harboring rhBMP-2 placed in a muscle flap and used to repair a maxillary bone defect^[146]. The other was a series of 5 cases using collagen sponges impregnated with rhBMP-2 with or without autologous bone marrow cells and allogeneic cancellous bone (4 cases) and one case using only rhBMP-2 adsorbed onto collagen sponges to reconstruct mandibular bone defects. Although not stated, it was presumed that bone marrow MSCs were the bone marrow cells referred to in three of the cases, two of which were successful in healing the bone defects^[147].

CLOSING THOUGHTS (A WORKING MODEL)

In summary, MSCs have promising utility in resolving orthopaedic problems although there is a need for more prospective randomized controlled trials. At this point it is still unclear if MSCs from various sources (bone mar-

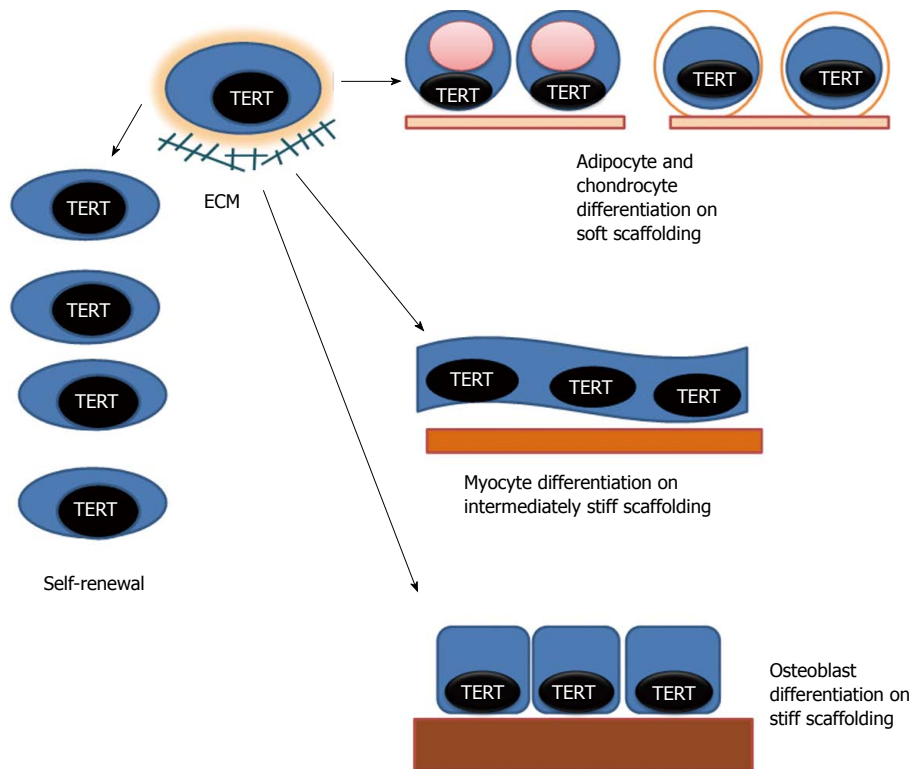


Figure 1 Model of human telomerase reverse transcriptase-transformed mesenchymal stem cell self-renewal and differentiation. Human telomerase reverse transcriptase (hTERT) can be expressed by transfection in human mesenchymal stem cells (hMSCs) from various sources to enhance self-renewal. hTERT transformed cells can be induced to differentiate along multiple mesenchymal lineages. Stiffness of support structures and/or extracellular matrix (ECM) upon which hMSCs are situated is important in differentiated lineage determination. Softer or less stiff support structures/ECM (lightest colored and thinnest bar under the cells) support adipogenic or chondrogenic lineages. Intermediate stiffness (medium colored and thicker bar) can direct myogenesis. Stiffer substrates (darkest colored and thickest bar) can support osteogenic differentiation. Native ECM made from hMSCs from younger hosts may also enhance self-renewal and the differentiative capacity of hMSCs from older sources, and may be superior to singular or limited number of defined ECM components in promoting self-renewal and specific lineage differentiation.

row, adipose, cord blood, cord tissue, muscle, *etc.*) would all be useful in orthopaedic repair and regeneration in general and bone in particular. It does appear that MSCs from either bone marrow or adipose tissue are quite similar in their capacity to serve in bone repair and regeneration. However, work still needs to be done regarding ideal scaffolding material and whether addition of MSCs or growth factors, angiogenic factors, and/or chemotactic factors onto scaffolds alone or in combination with MSCs would be the best strategy for bone repair and regeneration in the human situation.

With specific reference to MSC self-renewal and differentiation into osteogenic tissue, addition of hTERT to MSCs would seem to assist in increasing population doublings and decreasing population doubling times to enhance a critical mass of MSCs (Figure 1). However, there is still debate over initiation of tumorigenesis associated with TERT transformation of MSCs and the potential of MSCs (TERT transformed or not) to enhance the growth of already established tumors. Differentiation of TERT-transformed MSCs into osteogenic cells appear to be kept intact although whether exceeding a certain level of population doublings can lead to a decrease or change in differentiation capacity must still be considered. The use of native ECM from young MSCs appears to enhance the proliferative and differentiative capacity of MSCs

and the stiffness of the ECM appears to steer MSCs to differentiate along specific lineages, with osteogenic differentiation being assisted on a stiffer ECM (Figure 1). Thus TERT expression that can be regulated in a time and stage of differentiation manner may be an ideal strategy to both enhance a critical mass of MSCs necessary for bone repair and regeneration but to try to limit the potential of malignant transformation.

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WCSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Progress of mesenchymal stem cell therapy for neural and retinal diseases

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Abstract

Complex circuitry and limited regenerative power make central nervous system (CNS) disorders the most challenging and difficult for functional repair. With elusive disease mechanisms, traditional surgical and medical interventions merely slow down the progression of the neurodegenerative diseases. However, the number of neurons still diminishes in many patients. Recently, stem cell therapy has been proposed as a viable option. Mesenchymal stem cells (MSCs), a widely-studied human adult stem cell population, have been discovered for more than 20 years. MSCs have been found all over the body and can be conveniently obtained from different accessible tissues: bone marrow, blood, and adipose and dental tissue. MSCs have high proliferative and differentiation abilities, providing an inexhaustible source of neurons and glia for cell replacement therapy.

Moreover, MSCs also show neuroprotective effects without any genetic modification or reprogramming. In addition, the extraordinary immunomodulatory properties of MSCs enable autologous and heterologous transplantation. These qualities heighten the clinical applicability of MSCs when dealing with the pathologies of CNS disorders. Here, we summarize the latest progress of MSC experimental research as well as human clinical trials for neural and retinal diseases. This review article will focus on multiple sclerosis, spinal cord injury, autism, glaucoma, retinitis pigmentosa and age-related macular degeneration.

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Key words: Mesenchymal stem cells; Stem cell therapy; Central nervous system; Retina; Clinical trial

Core tip: Central nervous system (CNS) disorders are the most challenging and difficult for functional repair. Neurons are still diminishing in many patients despite surgical and medical interventions. Stem cell therapy has been proposed as a viable option. Mesenchymal stem cell (MSC) is a widely-studied human adult stem cell population. MSCs can be conveniently obtained from different accessible tissues. MSCs have high proliferative and differentiation abilities, providing an inexhaustible source of neurons and glia. MSCs also show neuroprotective effects and possess extraordinary immunomodulatory properties. These qualities heighten the clinical applicability of MSCs when dealing with the pathologies of CNS disorders.

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STEM CELL THERAPY AND MESENCHYMAL STEM CELLS

Stem cells are undifferentiated cells defined by their ability to self-renew and differentiate into mature cells. Stem cells are attractive because they are highly proliferative, implying that an inexhaustible number of mature cells can be generated from a given stem cell source. On this basis, cell replacement therapy has been proposed in recent years as a viable alternative for various pathologies. Cell replacement therapy hypothesizes that new retinal cells could be generated from stem cells so as to substitute the damaged cells in the diseased retina. This theory is mainly established from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). In addition to cell replacement function, stem cells could have another protective effect, the paracrine effect. The paracrine effects of stem cells are believed to modulate the micro-environment of the diseased tissues so as to protect the injured cells, promote survival and activate any available endogenous repair mechanisms. This latter observation applies mainly to the transplantation of adult stem cells.

Adult stem cells are defined as the stem cells found in fully developed tissues. The function of adult stem cells is the maintenance of adult tissue specificity by homeostatic cell replacement and tissue regeneration^[1]. Adult stem cells are presumed quiescent within adult tissues, but divide infrequently to maintain their own niche by generating a stem cell clone and a transiently-amplifying cell. The transiently-amplifying cells will undergo a limited number of cell divisions before terminal differentiation into mature functional tissue cells. The existence of adult stem cells has been reported in multiple organs; these include: brain, heart, skin, intestine, testis, muscle and blood, among others.

Mesenchymal stem cells (MSCs), also called marrow stromal cells, are an adult stem cell population of stromal progenitor cells of mesodermal origin^[2]. MSCs were originally identified in the bone marrow, representing 0.001%-0.01% of the bone marrow population. MSCs can also be found in other systems all over the body, such as adipose tissue, liver, umbilical cord, central nervous system (CNS) and dental tissues^[3]. According to the International Society of Cellular Therapy^[4], the minimal criteria to define MSCs are: (1) grown in adherence to plastic surface of dishes when maintained in standard culture conditions; (2) positive expression of cytospecific cell surface markers (CD105, CD90 and CD73) and negative expression of other cell surface markers (CD45, CD34, CD14 and CD11b); and (3) capacity to differentiate into mesenchymal lineages, under appropriate *in vitro* conditions. In addition to the expression of the three cell surface markers, MSCs also express CD29, CD44, CD146 and STRO-1^[5].

The function of MSCs is to differentiate into osteocytes, chondrocytes, myoblasts and adipocytes^[6,7]. An increasing number of studies, however, report that MSCs are capable of giving rise to cells of an entirely distinct

lineage, including neuron-like cells. MSCs are not only able to differentiate into neurons for cell replacement therapy, they also exert paracrine effects by modulating the plasticity of damaged host tissues, secreting neurotrophic and survival-promoting growth factors, restoring synaptic transmitter release, integrating into existing neural and synaptic networks, and re-establishing functional afferent and efferent connections^[8]. These paracrine activities have not been reported in ESCs or iPSCs. Moreover, MSCs possess strong immunosuppressive properties and inhibit the release of pro-inflammatory cytokines^[9]. This allows autologous, as well as, allogeneic transplantation of MSCs without the need of pharmacological immunosuppression. Furthermore, MSCs can be transplanted directly without genetic modification or pre-treatments, and are able to migrate to the tissue injury sites^[10]. In addition, there is no teratoma formation concern after transplantation^[11], and no moral objection or ethical controversies involved in their attainment^[12]. These advantageous properties, as well as the expansion potential of MSCs initiate the idea of clinical applications of MSCs to treat different human diseases, especially CNS disorders. Currently, over 100 MSC clinical trials for different diseases have been listed by the United States National Institutes of Health trial database (www.clinicaltrials.gov), indicating that MSC therapy is a popular trend for the field of regenerative medicine in the years to come.

This review article provides an update on the progress of MSC experimental research as well as human clinical trials for neural and retinal diseases with emphasis on multiple sclerosis, spinal cord injury, autism, glaucoma, retinitis pigmentosa and age-related macular degeneration.

MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is an immune-mediated neurodegenerative disorder of the CNS, affecting over 1.3 million people worldwide. The histopathological hallmark of MS is the formation of an inflammatory plaque, which originates from a breach in the integrity of the blood-brain barrier^[13]. The histologic features of lesions in MS include: lymphocyte infiltration, loss of oligodendrocytes, demyelination, and widespread axonal damage^[14]. Myelin-reactive T cells, which secrete interferon- and interleukins, have been suggested to be responsible for the inflammatory demyelination seen in MS^[15]. Currently, there are three treatment options approved by the Food and Drug Administration (FDA) for MS: administration of interferon beta, glatiramer acetate, or mitoxantrone^[16]. However, there is still no medical cure for MS.

Experimental autoimmune encephalomyelitis (EAE), the best known and most commonly used model for MS, mechanistically defines the immune processes responsible for the clinical manifestations and development of MS^[17]. This animal model provides insight for the application of immunotherapy to treat MS^[18]. MSCs have been pro-

posed as a treatment for autoimmune diseases, including MS, because of their immunosuppressive properties and neural repair function^[19]. Transplantation of human MSCs into animals with ongoing EAE results in rapid and sustained functional recovery due to a reduced number of inflammatory myelin-specific Th1 cells and astrocytes as well as an increased number of inflammatory-inhibiting Th2 cells, oligodendrocytes and neurons^[20]. This functional benefit is a critical stepping-stone towards effective MSC therapies in MS patients.

Among all of the CNS disorders, MS has the highest number of registered clinical trials. Altogether there are 14 registered clinical trials for MS (Table 1), and two of them have been published. The study from Israel is a phase-1/2 open safety clinical trial to evaluate the feasibility, safety and immunological effects of intrathecal and intravenous administration of autologous MSCs in 15 MS patients (NCT00781872; <http://clinicaltrials.gov/>)^[21]. No major adverse effects have been reported in this study, and the mean Expanded Disability Status Scale (EDSS) improved from 6.7 to 5.9 (EDSS steps 1.0 to 4.5: MS patients are fully ambulatory, whereas EDSS steps 5.0 to 9.5: MS patients are impaired to ambulation). Moreover, magnetic resonance imaging visualized the MSCs in the occipital horns of the ventricles, indicating migration of the cells. In addition, the proportion of CD4+/CD25+ regulatory T cells increased, whereas the proliferative responses of lymphocytes decreased. The mesenchymal stem cells in the multiple sclerosis trial (MSCIMS) originated in the United Kingdom, is an open-label phase 2a proof-of-concept study of autologous MSCs in secondary progressive MS (NCT00395200; <http://clinicaltrials.gov/>)^[22,23]. In this study, 10 patients received intravenous infusion of autologous bone marrow-derived MSCs (1.6×10^6 cells per kg body weight). The “sentinel lesion approach” assessing the anterior visual pathway was used to measure the efficacy of treatment. Results show that treatment improved visual acuity, visual evoked response latency, and increased the optic nerve area of the recipients. No serious adverse events were identified. For other clinical trials, mainly autologous MSCs have been used, although one study from China uses umbilical cord MSCs (NCT01364246; <http://clinicaltrials.gov/>). Interestingly, an open-label phase I clinical trial from New York was designed to evaluate autologous MSC-derived neural progenitor cells in progressive MS patients (NCT01933802; <http://clinicaltrials.gov/>) even though neural stem cells from EAE animals mainly develop astrocytes rather than oligodendrocytes, or oligodendrocyte precursor cells and neurons^[20].

SPINAL CORD INJURY

Spinal cord injury (SCI) is the most devastating and traumatic disorder among CNS conditions^[24]. The worldwide frequency of SCI is about 40 cases per million individuals^[25]. SCI can be caused by traffic accidents, violent assaults, falls, sport and other traumatic events. Depending on the injury location, extent, phases and time frames,

SCI therapeutic strategies can vary greatly^[26]. Most SCI patients are in the chronic phase, characterized by ongoing demyelination, local inflammation and apoptosis, decreased number of activated macrophages, and formation of glial scar and pseudocysts^[27]. The present standard treatment for SCI patients is surgical intervention, high doses of methylprednisolone, and symptomatic therapy followed by rehabilitation^[28]. New neuroregenerative strategies will be focused on neuroprotection and axonal regeneration in a permissive environment.

Cellular therapy aims to reconstruct the spinal cord through cellular replacement, glial scar remodeling, axonal guidance, and filling of formed syringomyelia^[29]. *In vivo* administration of MSCs in different SCI animal models showed functional recovery including: increased motor activity and sensation in the paralyzed limbs, reduced cavity formation in the spinal cord, and axonal sprouting through the glial scar^[30,31]. The objective of MSC application is to ameliorate the consequences of secondary injury by preserving the host nerve cells, rather than replacing them^[32].

Comparable to MS studies, there are 11 registered clinical trials using MSCs for SCI treatment (Table 1), among which two studies (one from Egypt and one from South Korea) have been completed. The Korean study investigated the safety of single intravenous infusion of autologous adipose tissue-derived MSCs (4×10^8 cells) in 8 male patients with chronic SCI (NCT01274975; <http://clinicaltrials.gov/>)^[33]. No adverse events were observed. Although one patient showed improvement in the American Spinal Injury Association (ASIA) scale from grade A (No sensory or motor function is preserved in sacral segments S4-S5) to grade C (Motor function is preserved below the neurologic level, and most key muscles below the neurologic level have muscle grade less than 3) and three patients showed motor score improvement, this phase I clinical trial might not have the statistical power to conclude on the efficacy of treatment effect with adipose tissue-derived MSCs on SCI. The study conducted in Egypt (NCT00816803; <http://clinicaltrials.gov/>), is a Phase-1/2 clinical trial applying bone marrow-derived MSCs at the injury site of chronic SCI patients. However, no results of this study have been released. Finally, there are two Phase-3 clinical trials taking place in China (NCT01873547; <http://clinicaltrials.gov/>) and Korea (NCT01676441; <http://clinicaltrials.gov/>). The study in China plans to use umbilical cord MSCs to treat 100 chronic SCI patients compared to the rehabilitation-only group and no stem cell and rehabilitation group, whereas the study in Korea was designed to transplant bone marrow-derived MSCs to treat 32 chronic SCI patients. For other ongoing clinical trials in SCI, the approaches are mainly intrathecal transplantation of bone marrow-derived MSCs and adipose tissue-derived MSCs in chronic SCI patients.

AUTISM

Autism belongs to a spectrum of heterogeneous neuro-

Table 1 Registered clinical trials on mesenchymal stem cells for neural diseases

Identifier	Country	Status	Study	Phase of trial	Estimated number of patients	Estimated trial end	Disease
NCT01377870	Iran	Recruiting	Evaluation of autologous mesenchymal stem cell transplantation (effects and side effects) in multiple sclerosis	Phase 1/2	30	2013	Multiple sclerosis
NCT01895439	Jordan	Recruiting	Safety and efficacy study of autologous bone marrow mesenchymal stem cells in multiple sclerosis	Phase 1/2	30	2014	Multiple sclerosis
NCT01883661	India	Not yet recruiting	Safety and efficacy of MSCs in MS	Phase 1/2	15	2015	Multiple sclerosis
NCT00395200	United Kingdom	Completed	MSCIMS	Phase 1/2	10	2010	Multiple sclerosis
NCT01854957	Italy	Recruiting	MESEMS	Phase 1/2	20	2014	Multiple sclerosis
NCT01730547	Sweden	Recruiting	Mesenchymal stem cells for multiple sclerosis	Phase 1/2	15	2015	Multiple sclerosis
NCT01364246	China	Recruiting	Safety and efficacy of umbilical cord mesenchymal stem cell therapy for patients with progressive multiple sclerosis and neuromyelitis optica	Phase 1/2	20	2014	Multiple sclerosis
NCT01056471	Spain	Unknown	Autologous mesenchymal stem cells from adipose tissue in patients with secondary progressive multiple sclerosis (CMM/EM/2008)	Phase 1/2	30	2012	Multiple sclerosis
NCT01228266	Spain	Active, not recruiting	Mesenchymal stem cell transplantation in MS (CMM-EM)	Phase 2	16	2013	Multiple sclerosis
NCT00813969	United States	Active, not recruiting	Autologous MSC transplantation in MS	Phase 1	24	2014	Multiple sclerosis
NCT01933802	United States	Not yet recruiting	Intrathecal administration of autologous MSC-NP in patients with multiple sclerosis	Phase 1	20	2016	Multiple sclerosis
NCT01606215	United Kingdom	Recruiting	STREAMS	Phase 1/2	13	2015	Multiple sclerosis
NCT01745783	Spain	Recruiting	Mesenchymal cells from autologous bone marrow, administered intravenously in patients diagnosed with multiple sclerosis	Phase 1/2	30	2014	Multiple sclerosis
NCT00781872	Israel	Unknown	MSCs for the treatment of MS	Phase 1/2	20	2009	Multiple sclerosis
NCT01694927	Chile	Enrolling by invitation	Autologous mesenchymal stem cells in spinal cord injury (SCI) patients (MSC-SCI)	Phase 2	30	2014	Spinal cord injury
NCT01446640	China	Recruiting	Mesenchymal stem cells transplantation to patients with spinal cord injury (MSC)	Phase 1/2	20	2014	Spinal cord injury
NCT01676441	South Korea	Recruiting	Safety and efficacy of autologous mesenchymal stem cells in chronic spinal cord injury	Phase 2/3	32	2014	Spinal cord injury
NCT01769872	South Korea	Recruiting	Safety and effect of adipose tissue derived mesenchymal stem cell implantation in patients with spinal cord injury	Phase 1/2	15	2014	Spinal cord injury
NCT01162915	United States	Active, not recruiting	Transfer of bone marrow derived stem cells for the treatment of spinal cord injury	Phase 1	10	2013	Spinal cord injury
NCT01274975	South Korea	Completed	Autologous adipose derived mscs transplantation in patient with spinal cord injury	Phase 1	8	2010	Spinal cord injury
NCT01624779	South Korea	Recruiting	Intrathecal transplantation of autologous adipose tissue derived msc in the patients with spinal cord injury	Phase 1	15	2013	Spinal cord injury
NCT01393977	China	Unknown	Difference between rehabilitation therapy and stem cells transplantation in patients with spinal cord injury in China	Phase 2	60	2012	Spinal cord injury
NCT01873547	China	Recruiting	Different efficacy between rehabilitation therapy and stem cells transplantation in patients with SCI in China (SCI-III)	Phase 3	300	2014	Spinal cord injury
NCT01325103	Brazil	Unknown	Autologous bone marrow stem cell transplantation in patients with spinal cord injury	Phase 1	20	2013	Spinal cord injury
NCT00816803	Egypt	Completed	Cell transplant in spinal cord injury patients	Phase 1/2	80	2008	Spinal cord injury
NCT01343511	China	Completed	Safety and efficacy of stem cell therapy in patients with autism	Phase 1/2	37	2011	Autism

Information obtained from <http://clinicaltrials.gov/>. MSCs: Mesenchymal stem cells; MS: Multiple sclerosis; MSCIMS: Mesenchymal Stem Cells in Multiple Sclerosis; MESEMS: MEsenchymal StEm Cells for Multiple Sclerosis; MSC-NP: Mesenchymal Stem Cell-derived Neural Progenitors; STREAMS: Stem Cells in Rapidly Evolving Active Multiple Sclerosis.

developmental disorders^[34]. It is characterized by abnormalities in social interaction, impaired verbal and nonverbal communication, and repetitive, obsessive behavior^[35]. According to the Centers for Disease Control, the prevalence of autism hovers around 60 in every 10000 children^[36]. Even though there is no defined gold standard approach, current interventions for autism can be divided into behavioral, nutritional and pharmacological^[37]. Medical interventions aim to ameliorate the neuropsychiatric disorders associated with autism. The medications include selective serotonin reuptake inhibitors (SSRI's), typical and atypical anti-psychotic drugs, psycho-stimulants, α -2 agonists, β blockers, lithium, anti-convulsant mood stabilizers and anti-depressants^[38-40]. Unfortunately, autism is still not treatable.

The pathogenic mechanism of autism is not clearly understood and remains elusive. Nevertheless, two pathologies are commonly found within the autism patients: the first observation is an impaired central nervous system circulation and hypoperfusion to the brain, whereas the second observation is systemic T cell and B cell abnormalities as well as active neuroinflammatory processes in the brain^[41]. Based on the immunomodulatory properties of MSCs, therapies employing MSCs have been proposed to target the immune deregulation observed in autism. Basically, it is believed that MSCs are able to inhibit the release of pro-inflammatory cytokines and have strong immunosuppressive activity^[42]. This not only allows for autologous transplantation, but also heterologous transplantation without the requirement of pharmacological immunosuppression^[43].

Currently, there is only one registered human clinical trial using MSCs to treat autism (NCT01343511; <http://www.clinicaltrials.gov/>; Table 1). This study aimed to test the safety and efficacy of human umbilical cord MSCs and human cord blood mononuclear cell transplantation in Chinese patients with autism^[44]. Outcomes from this study assuaged the safety concerns in using MSCs and mononuclear cells for transplantation in autism patients, and no severe adverse effects were observed. In addition, results also showed that combined transplantation of MSCs and mononuclear cells (combination group) had better therapeutic effects than transplantation of mononuclear cells alone (CBMNC group) in terms of the Childhood Autism Rating Scale (CARS) total score (combination group: 28.00 ± 6.18 ; CBMNC group: 37.14 ± 10.15 ; CARS total score > 30 means the child is considered to be autistic), Clinical Global Impression (CGI) scale (combination group: 88% much improved or higher; CBMNC group: 49% much improved or higher) and the Aberrant Behavior Checklist (ABC) total score (combination group: 36.78 ± 16.95 ; CBMNC group: 58.36 ± 31.73 ; a high score indicates greater severity while a low score indicates a milder degree of difficulty).

GLAUCOMA

Glaucoma is a group of chronic, degenerative optic

neuropathies. It is characterized by a slow progressive degeneration of retinal ganglion cells (RGCs) and their axons, which results in visual field defects^[45]. Glaucoma is the leading cause of irreversible blindness, affecting more than 60 million people worldwide^[46]. Traditional and current treatments for glaucoma are based on surgical or medical interventions to slow disease progression and limit visual loss^[47]. However, in many patients, the numbers of RGCs still diminish, and glaucoma cannot be completely cured.

The molecular basis of glaucoma is complex. The pathophysiological mechanisms leading to RGC degeneration in glaucoma include a complex interaction between primary axonal injury, neurotrophic factor deprivation, ischemia, oxidative stress, mitochondrial dysfunction and inflammation^[48]. New therapies aim to supplement neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF)^[49]. However, repeated injections are needed to achieve an observable effect^[50]. To avoid multiple injections, cell-based delivery of neurotrophic factors was proposed. A phase-I clinical trial for glaucoma (NCT01408472; <http://clinicaltrials.gov/>) using genetically modified CNTF-secreting retinal pigment epithelial cells (NT-501 CNTF implant) has already been launched—the outcomes have not been reported yet. Since MSCs can produce neurotrophic factors, including BDNF, CNTF, GDNF and basic fibroblast growth factor (bFGF), without the requirement of genetic modification, MSC transplantation has been suggested as a potential reservoir for neurotrophic factor secretion^[51]. Bone marrow-derived MSC transplantation increases RGC survival in a model of transient ischemia followed by reperfusion^[52], and reduces RGC loss in ocular hypertension models^[53,54]. Similarly, transplantation of human umbilical cord blood MSCs promotes RGC survival in an optic nerve crush model even after 7 d of injury^[55]. In addition, intracranial human umbilical cord blood MSC transplantation at the site of optic tract transaction also protects RGCs and induces axonal regeneration^[56]. The neuroprotective effect of MSCs on RGC survival has clearly been proven, and the first clinical trial using bone marrow-derived MSCs on glaucoma in Florida (Stem Cell Ophthalmology Treatment Study (SCOTS)) has just started in August 2013 (NCT01920867; <http://clinicaltrials.gov/>; Table 2). This study will be complete in 2017.

RETINITIS PIGMENTOSA AND AGE-RELATED MACULAR DEGENERATION

Retinitis pigmentosa (RP) is characterized by a classic pattern of difficulties in dark adaptation and night blindness in adolescence, loss of mid-peripheral visual field in young adulthood and central vision later in life. These are due to the severe attenuation of rod and cone photoreceptors^[57]. RP is one of the hereditary degenerative diseases, affecting 1 in 4000 individuals. Age-related

Table 2 Registered clinical trials on mesenchymal stem cells for retinal diseases

Identifier	Country	Status	Study	Phase of trial	Estimated number of patients	Estimated trial end	Disease
NCT01531348	Thailand	Enrolling by invitation	Feasibility and safety of adult human bone marrow-derived mesenchymal stem cells by intravitreal injection in patients with retinitis pigmentosa	Phase 1	10	2014	Retinitis pigmentosa
NCT01914913	India	Not yet recruiting	Clinical study to evaluate safety and efficacy of stem cell therapy in retinitis pigmentosa	Phase 1/2	15	2015	Retinitis pigmentosa
NCT01920867	United States	Recruiting	Stem cell ophthalmology treatment study		300	2017	Glaucoma, retinitis pigmentosa, age-related macular degeneration

Information obtained from <http://clinicaltrials.gov/>.

macular degeneration (AMD) is the leading cause of irreversible blindness in people aged 50 years or above in the developed world^[58]. It influences the central portion of the retina (the macula). Early AMD is characterized by drusen (pale yellowish lesions), or by hyperpigmentation and hypopigmentation of retinal pigment epithelium in the macula. Late AMD is divided into the “non-exudative” and “exudative” forms. The non-exudative form (geographic atrophy) starts with a sharply demarcated round or oval hypopigmented spot in which large choroidal vessels are visible, whereas the exudative form, characterized by choroidal neovascularization, is the detachment of the neuroretina or RPE from Bruch’s membrane by serous or hemorrhagic fluid^[59,60].

Both RP and AMD involve photoreceptor cell death. MSC research studies targeting this common pathology can be divided into two categories: first, cell replacement-based studies aim to generate photoreceptor cells from different sources of MSCs. MSCs from the trabecular meshwork as well as the conjunctiva have been used to produce photoreceptor-like cells *in vitro*^[61,62]. Interestingly, subretinal injection of MSCs has also been reported to induce differentiation into photoreceptor cells in a sodium iodate-induced retinal degeneration rat model^[63]. Second, studies based on paracrine effects hypothesize that MSCs can secrete neurotrophic factors to protect against photoreceptor degeneration in different animal models. Transplantation of bone marrow-derived MSCs can rescue photoreceptor cells of the dystrophic retina in the rhodopsin knockout mouse model^[64]. Moreover, intravenous injection of bone marrow-derived MSCs rescue photoreceptor cells as well as visual function in the Royal College of Surgeons rat model^[65]. For AMD, beside photoreceptor cell loss, retinal pigment epithelial (RPE) cells are also affected. Adipose tissue-derived MSCs can be induced to an RPE phenotype^[66]. In addition, adipose tissue-derived MSCs rescue mitomycin C-treated RPE cell lines (ARPE19) from death in culture^[67]. Furthermore, subretinal injected MSCs adopt RPE morphology and preserve the retinal layer integrity in the sodium iodate-induced retinal degeneration rat model^[68].

To date, there are three ongoing registered clinical trials using MSCs on RP (Table 2). The first clinical trial

aims to determine the feasibility and safety of human adult bone marrow-derived MSCs by intravitreal injection in patients with RP in Thailand (NCT01531348; <http://clinicaltrials.gov/>). The second clinical trial is the Stem Cell Ophthalmology Treatment Study (SCOTS) in Florida (NCT01920867; <http://clinicaltrials.gov/>) proposed to use autologous bone marrow-derived MSCs by different means of injection (retrobulbar, subtenon, intravitreal, intraocular, subretinal and intravenous). The third clinical trial is a Phase-1/2 open labeled study done in India to evaluate the safety and efficacy of bone marrow-derived MSCs in RP (NCT01914913; <http://clinicaltrials.gov/>). For AMD, there is only one registered clinical trial using bone marrow-derived MSCs (Table 2), the Stem Cell Ophthalmology Treatment Study (SCOTS) in Florida (NCT01920867; <http://clinicaltrials.gov/>). Results from these studies have not been reported yet.

CONCLUSION

MSCs have been discovered for more than 20 years^[69], and have been found all over the body. MSCs can be conveniently obtained from different accessible tissues: bone marrow, blood, and adipose and dental tissue. They can also be easily expanded in standard culture conditions. In addition to the above mentioned characteristics, MSCs demonstrate neuroprotective effects, immunomodulatory properties and self-migratory activity, making them an attractive therapeutic tool. In recent years, MSC research has already begun the transition from preclinical experiments to human clinical trials. There are currently more than 60 MSC clinical trials dealing with CNS disorders and three clinical trials on retinal diseases. Although transient rash, self-limiting bacterial infections or fever might occur in some patients after MSC transplantation, serious adverse events have never been observed. This can foresee that MSC transplantation will become routine clinical practice for disease treatment in the near future. However, there are critical challenges still needed to be conquered before MSC therapy can be adopted in daily clinical practice. These include: (1) poor MSC retention *in vivo*; (2) poor MSC engraftment, viability and function *in vivo*; (3) unclear mechanisms of action; and (4) lack

of standardized trials^[70]. Moreover, few studies showed the contradictory results of MSC immunomodulatory properties. This might be explained by the heterogeneous MSC population. TLR4-primed human MSCs (MSC1) mostly secrete pro-inflammatory cytokines (IL-6, IL-8) while TLR3-primed human MSCs (MSC2) express mostly immunosuppressive mediators (IL-10, IDO, TSG-6)^[71]. Addition of fewer MSCs (10-1000) would lead to a less consistent suppression or a marked lymphocyte proliferation in culture, whereas addition of 10000-40000 MSCs have an inhibitory effect^[72]. Besides, there are uncertainties that must be answered. What is the optimal cell number for transplantation? Which MSC types are optimal for regenerative medicine? When is the optimal stage to receive MSC therapy? Which transplantation route is suitable for each individual CNS disorder? Further research is needed to understand the mechanisms elicited by stem cells in regenerating damaged tissues after transplantation.

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WCSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Mesenchymal stem cells in the treatment of spinal cord injuries: A review

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Abstract

With technological advances in basic research, the intricate mechanism of secondary delayed spinal cord injury (SCI) continues to unravel at a rapid pace. However, despite our deeper understanding of the molecular changes occurring after initial insult to the spinal cord, the cure for paralysis remains elusive. Current treatment of SCI is limited to early administration of high dose steroids to mitigate the harmful effect of cord edema that occurs after SCI and to reduce the cascade of secondary delayed SCI. Recent evident-based clinical studies have cast doubt on the clinical benefit of steroids in SCI and intense focus on stem cell-based therapy has yielded some encouraging results. An array of mesenchymal stem cells (MSCs) from various sources with novel and promising strategies are being developed to improve function after SCI. In this review, we briefly discuss the pathophysiology of spinal cord injuries and char-

acteristics and the potential sources of MSCs that can be used in the treatment of SCI. We will discuss the progress of MSCs application in research, focusing on the neuroprotective properties of MSCs. Finally, we will discuss the results from preclinical and clinical trials involving stem cell-based therapy in SCI.

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Key words: Spinal cord injury; Mesenchymal stem cells; Bone marrow stromal cells; Umbilical cord derived mesenchymal stem cells; Adipose tissue derived mesenchymal stem cells

Core tip: Despite our deeper understanding of the molecular changes that occurs after the spinal cord injury (SCI), the cure for paralysis remains elusive. In this review, the pathophysiology of SCI and characteristics and potential sources of mesenchymal stem cells (MSCs) that can be used in the treatment of SCI were discussed. We also discussed the progress of application of MSCs in research focusing on the neuroprotective properties of MSCs. Finally, we discussed the results from preclinical and clinical trials involving stem cell-based therapy in SCI.

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INTRODUCTION

Traumatic spinal cord injury (SCI) continues to be a devastating injury to affected individuals and their families and exacts an enormous financial, psychological and emotional cost to them and to society. Despite years of

research, the cure for paralysis remains elusive and current treatment is limited to early administration of high dose steroids and acute surgical intervention to minimize cord edema and the subsequent cascade of secondary delayed injury^[1-3]. Recent advances in neurosciences and regenerative medicine have drawn attention to novel research methodologies for the treatment of SCI. In this review, we present our current understanding of spinal cord injury pathophysiology and the application of mesenchymal stem cells (MSCs) in the treatment of SCI. This review will be more useful for basic and clinical investigators in academia, industry and regulatory agencies as well as allied health professionals who are involved in stem cell research.

Direct mechanical damage to the spinal cord usually results in either partial or total loss of neural functions such as sensory perception and mobility^[4]. The prevalence of people with SCI who are alive in the United States in 2013 is estimated to be approximately 273000^[5]. According to census data, motor vehicle accidents (36.5%), falls (28.5%), and acts of violence (14.3%) are the most frequent causes of SCI since 2010. The average age at injury is 42.6 years and 80.7% of spinal cord injuries occur in males. Among those injured since 2010, 67.0% are Caucasian, 24.4% African American, 0.8% Native American and 2.1% Asian. The most frequent neurologic category at discharge of persons reported to the database since 2010 is incomplete tetraplegia (40.6%), followed by incomplete paraplegia (18.7%), complete paraplegia (18.0%) and complete tetraplegia (11.6%). Less than 1% of SCI patients experienced complete neurologic recovery by the time of hospital discharge. Over the last 20 years, the percentage of SCI patients with incomplete tetraplegia spinal cord injury has increased while the more devastating complete paraplegia and complete tetraplegia numbers have decreased^[5]. Whether complete or incomplete injury, SCI is a devastating condition that not only paralyzes the affected individuals but also exacts tremendous emotional, social and financial burdens^[6]. These patients also face increased risks of cardiovascular complications, deep vein thrombosis, osteoporosis, pressure ulcers, autonomic dysreflexia and neuropathic pain^[3]. The limitation of any clinical treatment success is most likely due to the complex mechanisms of SCI and the relative inability of the human body to repair or regenerate neurons in the spinal cord^[7].

PATHOPHYSIOLOGICAL FEATURES AFTER SCI

The pathophysiological processes that underlie SCI comprise the primary and secondary phases of injury^[1,8]. Initial physical trauma to the spinal cord includes traction injury, compression forces and direct mechanical disruption of neural elements. Immediate microvascular injuries with central gray hemorrhage and dis-

ruption of cellular membrane and blood-spinal cord barrier are followed by edema, ischemia, release of cytotoxic chemicals from inflammatory pathways and electrolyte shifts. Subsequently, a secondary injury cascade is triggered that compounds the initial mechanical injury with necrosis and apoptosis that are injurious to surviving neighboring neurons, further reducing the chance of recovery of penumbra neurons and rendering any functional recovery almost hopeless^[3,8]. Pathophysiological processes that occur in the secondary injury phase are responsible for exacerbating the initial damage and creating an inhibitory milieu that is hostile to endogenous efforts of repair, regeneration and remyelination. These secondary processes include inflammation, ischemia, lipid peroxidation, production of free radicals, disruption of ion channels, axonal demyelination, glial scar formation, necrosis and programmed cell death^[3]. The post-trauma inflammatory response plays a critical role in the secondary phase after SCI through modulation of a series of complex cellular and molecular interactions^[9]. After SCI, the blood-spinal cord barrier is disrupted due to hemorrhage and local inflammation^[10]. The activation and recruitment of peripheral and resident inflammatory cells including microglial cells, astrocytes, monocytes, T-lymphocytes, and neutrophils promotes the development of secondary damage following SCI^[11]. This secondary injury can be subdivided into the acute-phase (2 h-2 d), the sub-acute phase (days-weeks), and the chronic phase (months-years), each with distinct different pathophysiological processes^[12-14]. These changes include edema, ischemia, hemorrhage, reactive oxygen species (ROS) production and lipid peroxidation, glutamate-mediated excitotoxicity, ionic dysregulation, blood-spinal-cord barrier permeability, inflammation, demyelination, neuronal cell death, neurogenic shock, macrophage infiltration, microglial activity, astrocyte activity and scar formation, initiation of neovascularization, Wallerian degeneration, glial scar maturation, cyst and syrinx formation, cavity formation and schwannosis. The end of spontaneous post-SCI changes is identified as a pathophysiological phenomenon with solid glial scar formation, syrinx formation, and neuronal apoptosis^[15]. However, endogenous repair and regenerative mechanisms do occur during the secondary phase of injury to minimize the extent of the lesion (through astrogliosis), reorganize blood supply through angiogenesis, clear cellular debris, and reunite and remodel damaged neural circuits, and as such, offer exploitable targets for therapeutic intervention^[3], the most promising of which is stem cell-based therapy^[16].

MSC THERAPY AFTER SCI

An array of new and promising strategies is being developed to improve function after SCI. At present, two main therapeutic strategies, cell-based and gene-based therapies

are being investigated to repair the injured mammalian spinal cord. At this time it appears that neither strategy by itself is efficacious, whereas a combinatory strategy appears to be more promising. The targeting of an array of deleterious processes within the tissue after SCI will require a multi-factorial intervention, multi-phasic polytherapy such as the combination of cell- and gene-based approaches^[17]. This review focuses only on stem cell-based therapy. Cell-based therapy faces numerous challenges including selection of a SCI model, timing and mode of cell implantation, location of cellular injection and their subsequent migration, survival, transdifferentiation, immune incompatibility and rejection, and tracking of implanted cells^[17]. Cellular therapies for SCI repair may involve modification or recruitment of endogenous cells *in vivo*, harvest and/or alteration *ex vivo* of endogenous cells that are subsequently implanted as autogeneic graft or transplanted into the injured organism as allogeneic or xenogeneic grafts. Transplanted stem cells promote neural regeneration and rescue impaired neural function after SCI by paracrine/permissive neurotrophic molecules at the lesion site to enhance the regenerative capacity thereby providing a scaffold for the regeneration of axons and replacing lost neurons and neural cells^[17]. Mesenchymal stem cells have recently been advocated as a promising source for cellular repair after central nervous system (CNS) injury^[15]. MSCs, also known as marrow stromal cells^[18] or mesenchymal progenitor cells^[19] are self-renewing, multipotent progenitor cells with the capacity to differentiate into several distinct mesenchymal lineages^[20]. These cells are multipotent adult stem cells present in all tissues as part of the perivascular population. As multipotent cells, MSCs can differentiate into different mesodermal tissues ranging from bone and cartilage to cardiac muscle^[21]. Several small clinical trials have investigated the efficacy and safety of MSCs in diseases including chronic heart failure, acute myocardial infarction, hematological malignancies and graft *vs* host disease. Pre-clinical evidence suggests that MSCs exert their beneficial effects largely through immunomodulatory and paracrine mechanisms^[22].

MSCs are favored in stem cell therapy for SCI for the following reasons: (1) ease of isolation and cryopreservation^[23], (2) maintenance of viability and regenerative capacity after cryopreservation at -80 °C^[24], (3) rapid replication with high quality progenitor cells and high potential of multilineage differentiation^[25], and (4) minimal or no immunoreactivity and graft-versus-host reaction of transplanted allogeneic MSCs^[26]. MSCs were initially identified in bone marrow and later in muscle, adipose and connective tissue of human adults^[21]. Bone marrow and umbilical cord blood are rich sources of these cells, but MSC have also been isolated from fat^[27], skeletal muscle^[28], human deciduous teeth^[29], and trabecular bone^[30]. Mesenchymal stem cells are ideally suited to address many pathophysiological consequences of SCI^[3]. The major goals for the therapeutic use of stem cells is regeneration of axons, prevention of apoptosis and re-

placement of lost cells, particularly oligodendrocytes, in order to facilitate the remyelination of spared axons^[31]. In this review, we touch upon the therapeutic applications of MSCs after SCI.

BONE MARROW STROMAL CELLS

Bone marrow-derived mesenchymal stem cells (BMSC) differentiate into cells of the mesodermal lineage but also, under certain experimental conditions, into cells of the neuronal and glial lineage. Their therapeutic translation has been significantly boosted by the demonstration that MSCs display significant anti-proliferative, anti-inflammatory and anti-apoptotic features. These properties have been exploited in the effective treatment of experimental autoimmune encephalomyelitis (EAE), experimental brain ischemia and in animals undergoing brain or spinal cord injury^[32]. Several investigators have reported that MSCs possess immunosuppressive features^[33-36]. These immunosuppressive properties, in combination with the restorative functions of BMSC reduce the acute inflammatory response to SCI, minimize cavity formation, as well as diminish astrocyte and microglia/macrophage reactivity^[37-39]. BMSC transplantation in an experimental SCI model has been shown to enhance neuronal protection and cellular preservation *via* reduction in injury-induced sensitivity to mechanical trauma^[39]. It was suggested that the beneficial effects of MSCs on hindlimb sensorimotor function may, in part, be explained by their ability to attenuate astrocyte reactivity and chronic microglia/macrophage activation^[39]. These significant results demonstrated the potential of MSCs to serve as attenuators of the immune response. It was proposed that as attenuators, MSCs could potentially serve in an immunoregulatory capacity in disorders in which chronic activation of immune cells, such as reactive astrocytes and activated microglia/macrophages play a role. Studies by Hofstetter *et al.*^[40], indicated that transplanted MSC attenuates acute inflammation and promotes functional recovery following SCI. Ohta *et al.*^[41], suggested that BMSCs reduced post-SCI cavity formation and improved behavioral function by releasing trophic factors into the cerebrospinal fluid (CSF) or by direct interaction with host spinal tissues. Infusion of transplants through CSF provides no additional traumatic injury to the damaged spinal cord and BMSCs might be administered by lumbar puncture to the patients. Lumbar puncture can be done without severe invasion, so BMSCs can be repeatedly administered to maintain their effects. This study has demonstrated for the first time that the transplantation of BMSCs through CSF can promote the behavioral recovery and tissue repair of the injured spinal cord in rats, thus providing a road map for the clinical autograft of BMSCs without severe surgical infliction to human patients^[41]. In another study, human mesenchymal stem cells (hMSCs) isolated from adult bone marrow were found to infiltrate primarily into the ventrolateral white matter tracts, spreading to adjacent segments

rosto-caudal to the injury epicenter, and facilitate recovery from SCI by remyelinating spared white matter tracts and/or by enhancing axonal growth^[42]. In our laboratory, we used mesenchymal stem cells from rat bone marrow to evaluate the therapeutic potential after SCI in rats^[43]. We observed that caspase-3 mediated apoptosis after SCI on both neurons and oligodendrocytes was significantly downregulated by BMSC. Treatment with BMSC had a positive effect on behavioral outcome and better structural integrity preservation as seen in histopathological analysis. BMSC secrete protective factors that prevent neuronal apoptosis through stimulation of endogenous survival signaling pathways, namely PI3K/Akt and the MAPK/ERK1/2-cascade. Overall, these findings demonstrate that BMSC trigger endogenous survival signaling pathways in neurons that mediate protection against apoptotic insults. Moreover, the interaction between stressed neurons and BMSC further amplifies the observed neuroprotective effect^[44].

Lu *et al.*^[45], investigated the nature of the chronic scar and its ability to block axon growth by testing the hypothesis that chronically injured spinal cord axons can regenerate through the gliotic scar in the presence of local growth-stimulating factors. BMSC, genetically modified to secrete neurotrophin-3 (NT-3) were injected into the lesion site of rats with cervical SCI^[45]. It was observed that a modest number of axons penetrated through the chronic scar that contained a mixture of inhibitory and growth stimulating factors. Furthermore, robust axonal growth can be induced by the local provision of neurotrophic factors without resecting the chronic scar. In another study, Urdžíková *et al.*^[46], have shown that treatment with different cell populations obtained from bone marrow (MSCs, BMCs and the endogenous mobilization of bone marrow cells) has a beneficial effect on behavioral and histological outcomes after SCI. However, it is not clear whether the injection of MSCs, BMCs or granulocyte-colony stimulating factor (G-CSF) treatment induces functional and morphological improvement through the same mechanisms of action. Transplanted MSCs mollify the inflammatory response in the acute setting and reduce the inhibitory effects of scar tissue in the subacute/chronic setting to provide a permissive environment for axonal extension. In addition, grafted cells may provide a source of growth factors to enhance axonal elongation across spinal cord lesions^[47]. Down-regulation of TNF- α expression in macrophages/microglia was observed at an early stage after SCI in rats transplanted with a gelatin sponge (GS) scaffold impregnated with rat bone marrow-derived mesenchymal stem cells at the site of injury^[48]. It was also shown that 3D gelatin sponge scaffolds allowed MSCs to adhere, survive and proliferate and also for fibronectin to deposit. *In vivo* transplantation experiments demonstrated that these scaffolds were biocompatible and MSCs seeded to the scaffolds played an important role in attenuating inflammation, promoting angiogenesis, and reducing cavity formation. Novikova *et al.*^[49], observed that differentiated BMSC provided neuroprotection for

axotomized rubrospinal neurons and increased the density of rubrospinal axons in the dorsolateral funiculus rostral to the injury site. They suggested that BMSC induced along the Schwann cell lineage increased expression of trophic factors and have neuroprotective and growth-promoting effects after SCI^[49]. Cizkova *et al.*^[50], standardized the intrathecal (IT) catheter delivery of rat MSCs after SCI in adult rats. Based on these results, it was suggested that repetitive IT transplantation, which imposes a minimal burden on the animals, may improve behavioral function when the dose, timing, and targeted IT delivery of MSCs towards the lesion cavity was optimized. Kang *et al.*^[51], indicated that therapeutic rat BMSCs in a poly (D,L-lactide-co-glycolide)/small intestinal submucosa scaffold induced nerve regeneration in a complete spinal cord transection model and demonstrated that functional recovery further depended on defect length.

Park *et al.*^[52] evaluated the therapeutic efficacy of combining autologous BMSC transplantation with granulocyte macrophage-colony stimulating factor (GM-CSF) by subcutaneous administration directly into the spinal cord lesion site of six patients with complete SCI. At the 6-mo and 18-mo follow-up periods, four of the six patients showed neurological improvements by two ASIA (American Spinal Injury Association) grade (from ASIA A to ASIA C), while another improved from ASIA A to ASIA B^[52]. Moreover, BMSC transplantation together with GM-CSF was not associated with increased morbidity or mortality. In another clinical trial, the safety of autologous bone marrow cell implantation was tested in twenty patients^[53]. Motor-evoked potential, somatosensory-evoked potential, magnetic resonance imaging, and ASIA scores were measured in a clinical follow-up. This study demonstrated that BMSC transplantation is a relatively safe procedure, and BMSC-mediated repair can lead to modest improvements in some injured patients. It is also anticipated that a Phase II clinical trial designed to test the efficacy will be initiated in the near future. In a study conducted by Deng *et al.*^[54], implantation of BMSC elicited *de novo* neurogenesis, and functional recovery in a non-human primate SCI model with rhesus monkeys achieved Tarlov grades 2-3 and nearly normal sensory responses three months after transplantation. Zurita *et al.*^[55], observed progressive functional recovery three months after SCI in paraplegic pigs injected with autologous BMSC in autologous plasma into lesion zone and adjacent subarachnoid space. Intramedullary post-traumatic cavities were filled by a neoformed tissue containing several axons, together with BMSC, that expressed neuronal or glial markers. Furthermore, in the treated animals, electrophysiological studies showed recovery of the previously abolished somatosensory-evoked potentials. Despite promising data, further research is needed to establish whether bone marrow cell treatments can serve as a safe and efficacious autologous source for the treatment of SCI^[47]. However, the use of BMSC in SCI does present certain issues-migration beyond the injection site (for intraspinally delivered cells) is limited and

Table 1 Overview of effects of bone marrow stromal cells after spinal cord injury

Source of MSC	Main pathological features improved/repared	Limitations/recommendations/conclusions	Ref.
Human	Axonal growth, partial recovery of function	Differences in donor or lot-lot efficacy of MSC	Neuhuber <i>et al</i> ^[37] , 2005
Human	Axonal growth, significant behavioral recovery	Survival of BMSC grafts for longer duration	Himes <i>et al</i> ^[38] , 2006
Human	Significant motor improvements in human patients	Autologous bone marrow cell transplantation with GM-CSF administration has no serious complications. More comprehensive multicenter clinical studies are recommended	Park <i>et al</i> ^[52] , 2005
Human	Homing of MSC, functional recovery	Mechanisms of engraftment, homing, long-term safety	Cizkova <i>et al</i> ^[42] , 2006
Rhesus monkey	<i>De novo</i> neurogenesis and functional recovery in rhesus monkeys	Synergetic effects of MSC implantation and locally delivered neurotrophic factors in rhesus SCI models	Deng <i>et al</i> ^[54] , 2006
Pig	Improvement in somatosensory-evoked potentials, functional recovery in pigs	Possible utility of BMSC transplantation in humans suffering from chronic paraplegia	Zurita <i>et al</i> ^[55] , 2008
Rat	No allodynia, anti-inflammatory, increase in white matter volume and decrease in cyst size, sensorimotor enhancements	Survival of MSC	Abrams <i>et al</i> ^[39] , 2009
Rat	MSC form bundles bridging the lesion epicenter, functional recovery	Neuron-like MSC lacked voltage-gated ion channels for generation of action potentials	Hofstetter <i>et al</i> ^[40] , 2002
Rat	Cavity reduction, functional recovery	Unknown trophic factors secreted by BMSC	Ohta <i>et al</i> ^[41] , 2004
Rat	Downregulation of apoptosis, functional recovery	Intrinsic properties of MSC, microenvironment of the injured spinal cord, host-graft interactions	Dasari <i>et al</i> ^[43] , 2007
Rat/gerbil	Activation of survival signaling pathways, neuroprotection	Neuroprotective factors released by BMSC, interactions between neurons and BMSC	Isele <i>et al</i> ^[44] , 2007
Rat	Axonal regeneration, myelination of axons	Resection of the chronic scar	Urdzíkova <i>et al</i> ^[46] , 2006
Rat	Increase in spared white matter, functional recovery	Differences in mechanism of action of MSCs or BMCs (bone marrow cells) or G-CSF in inducing functional and morphological improvement	
Rat	Reduction in inflammation, promoting angiogenesis, and reducing cavity formation	GS scaffolds may serve as a potential supporting biomaterial for wound healing after SCI	Zeng <i>et al</i> ^[48] , 2011
Rat	Extensive in-growth of serotonin-positive raphespinal axons and calcitonin gene-related peptide-positive dorsal root sensory axons, attenuation of astroglial and microglial activity	Production of trophic factors support neuronal survival and axonal regeneration	Novikova <i>et al</i> ^[49] , 2011
Rat	Functional recovery	Repetitive IT transplantation may improve behavioral function depending on optimization of dose, timing, and targeted IT delivery of MSCs	Cizkova <i>et al</i> ^[50] , 2011
Rat	Axonal regeneration, functional recovery	Feasibility of therapeutic cell delivery using 3D scaffolds, especially in complete spinal cord transection	Kang <i>et al</i> ^[51] , 2011
Rat	Partial improvement in ASIA score in human patients	Polymer hydrogels may become suitable materials for bridging cavities after SCI	Sykova <i>et al</i> ^[53] , 2006

SCI: Spinal cord injury; MSC: Marrow stromal cell; IT: Intrathecal; CSF: Cerebrospinal fluid; GS: Gelatin sponge; BMSC: Bone marrow-derived mesenchymal stem cell.

inter-donor variability in efficacy and immunomodulatory potency might be reflected in variable clinical outcome^[37], making BMSC evaluation as a therapy for SCI difficult^[3]. The pathological improvements of BMSC after SCI are summarized in Table 1.

ADIPOSE TISSUE-DERIVED MESENCHYMAL CELLS

Adipose tissue is abundant in the body and contains a stromal fraction rich in stem- progenitor cells capable of undergoing differentiation into osteogenic, chondrogenic, and adipogenic lineages^[56]. The *in vitro* as well as *in vivo* properties of adipose tissue-derived stromal cells (ADSCs) resemble those of MSCs obtained from bone marrow, and the liposuction procedure employed to harvest ADSCs is minimally invasive for the patient^[57]. Kang *et al*^[58], reported that intravenous infusion of oligodendrocyte precursor cells (OPCs) derived from rATSC autograft cells improved motor function in rat models of

SCI. Moreover, cytoplasmic extracts prepared from adipose tissue stromal cells (ATSCs) inhibit H₂O₂-mediated apoptosis of cultured spinal cord-derived neural progenitor cells (NPCs) and improved cell survival^[59]. ATSCs extracts mediated this effect by decreasing caspase-3 and c-Jun-NH₂-terminal kinase (SAPK/JNK) activity, inhibiting cytochrome c release from mitochondria and reducing Bax expression levels in cells. Direct injection of ATSCs extracts mixed with matrigel into the spinal cord immediately after SCI also resulted in less apoptotic cell death, astrogliosis and hypo-myelination and showed significant functional improvement. Zhang *et al*^[60], showed that ADSCs can differentiate into neural-like cells *in vitro* and *in vivo*. However, neural differentiated ADSCs did not result in any better functional recovery than did undifferentiated ones following SCI. Ryu *et al*^[61], evaluated the implantation of allogenic adipose-derived stem cells (ASCs) for the improvement of neurological function in a canine SCI model. Using both *in vitro* and *in vivo* injury models, Oh *et al*^[62], confirmed that hypoxic

Table 2 Overview of effects of Adipose tissue-derived mesenchymal cells after spinal cord injury

Source of MSC	Main pathological features improved/repared	Limitations/recommendations/conclusions	Ref.
Human	Functional recovery	Interaction between engrafted rATSC-OPCs and endogenous spinal cord-derived NPCs promotes host injury repair	Kang <i>et al</i> ^[58] , 2006
Human	Improvement in both the cell survival and the gene expression of the engineered NSC observed in SCI rats	Hypoxia preconditioning strategy and combined stem cell/ gene therapies can be used to augment the therapeutic efficacy at target injury sites	Oh <i>et al</i> ^[62] , 2010
Human	mNSCs transplanted into rat spinal cords with AT-MSCs showed better survival rates than mNSCs transplanted alone	Co-transplantation of mNSCs with AT-MSCs may be a more effective transplantation protocol to improve the survival of cells in the injured cord	Oh <i>et al</i> ^[63] , 2011
Human	Transplantation of 3DCM-ASCs into the injured spinal cord significantly elevated the density of vascular formations and enhanced axonal outgrowth at the lesion site, functional recovery	Transplantation of 3DCM-ASCs may be an effective stem cell therapy	Oh <i>et al</i> ^[64] , 2012
Human	No toxicity of hAdMSCs in immunodeficient mice, none of 8 male patients developed any serious adverse events related to hAdMSC transplantation in phase I clinical trial	Systemic transplantation of hAdMSCs appears to be safe and does not induce tumor development. Slow intravenous infusion of autologous hAdMSCs may be safe in SCI patients	Ra <i>et al</i> ^[66] , 2011
Human	Increase in BDNF levels, increased angiogenesis, preserved axons, decreased numbers of ED1-positive macrophages, reduced lesion cavity formation, functional recovery in rats	Compared with hBMSCs, hADSCs may be a better source of MSCs for cell therapy for acute SCI because of their relative abundance and accessibility	Zhou <i>et al</i> ^[67] , 2013
Dog	Significant improvement in nerve conduction velocity based on SEP, partial improvement in neurological functions of dogs	ASCs in spinal cord injuries might be partially due to neural differentiation of implanted stem cells	Ryu <i>et al</i> ^[61] , 2009
Dog	Anti-inflammation, anti-astrogliosis, neuronal extension, neuronal regeneration, functional recovery	The combination of Matrigel and NMSC produced beneficial effects	Park <i>et al</i> ^[65] , 2012
Rat	Reduced apoptotic cell death, astrogliosis and hypo-myelination, functional recovery	ATSC extracts may provide a powerful autoplasmic therapy for neurodegenerative conditions in humans	Kang <i>et al</i> ^[59] , 2007
Rat	Neural differentiated ADSCs did not result in better functional recovery than undifferentiated ones following SCI	<i>In vitro</i> neural transdifferentiation of ADSCs might therefore not be a necessary pre-transplantation step	Zhang <i>et al</i> ^[60] , 2009
Rat	Functional recovery	Predifferentiation of ASCs plays a beneficial role in SCI repair	Arboleda <i>et al</i> ^[57] , 2011
Rat	Axonal regeneration, remyelination, functional recovery	Adipose tissue-derived Schwann cells can support axon regeneration and enhance functional recovery	Zaminy <i>et al</i> ^[68] , 2013

OPCs: Oligodendrocyte precursor cells; NPCs: Neural progenitor cells; NSC: Neural stem cell; SCI: Spinal cord injury; MSC: Marrow stromal cell; AT: Adipose tissue; 3DCM-ASCs: Three-dimensional cell mass transplantation of adipose-derived stem cells; hAdMSCs: Human Adipose tissue-derived mesenchymal stem cells; NMSC: Neural-induced mesenchymal stem cells; ATSC: Adipose tissue stromal cell; ADSCs: Adipose tissue-derived stromal cells; BMSC: Bone marrow-derived mesenchymal stem cell.

preconditioning (HP)-treated adipose tissue-derived mesenchymal stem cells (HP-AT-MSCs) increased cell survival and enhanced the expression of marker genes in DsRed-engineered neural stem cells (NSCs-DsRed). Based on their findings, it was suggested that the co-transplantation of HP-AT-MSCs with engineered neural stem cells (NSCs) can improve both cell survival and gene expression of the engineered NSCs. This novel strategy can be used to augment the therapeutic efficacy of combined stem cell and gene modulation therapy for SCI. In another study, Oh *et al*^[63], examined the effects of co-transplanting mouse neural stem cells (mNSCs) and adipose tissue-derived mesenchymal stem cells (AT-MSCs) on mNSC viability. It was observed that mNSCs transplanted into rat spinal cords with AT-MSCs showed better survival rates than mNSCs transplanted alone, thereby suggesting that co-transplantation of mNSCs with AT-MSCs is a more effective strategy to improve the survival of transplanted stem cells into the injured spinal cord. In a more recent study, the same group investigated the effectiveness of a three-dimensional cell mass trans-

plantation of adipose-derived stem cells (3DCM-ASCs) in hind limb functional recovery by the stimulation of angiogenesis and neurogenesis^[64]. These results revealed a significantly elevated density of neovascular formations through angiogenic factors released by the 3DCM-ASCs at the lesion site, enhanced axonal outgrowth, and significant functional recovery. These findings suggest that transplantation of 3DCM-ASCs may be an effective stem cell transplantation modality for the treatment of spinal cord injuries and neural ischemia. In a similar study, Park *et al*^[65], observed that a combination of matrigel and neural-induced mesenchymal stem cells (NMSC) reduced the expression of inflammation and/or astrogliosis markers and improved hind limb function in dogs with SCI. The predifferentiation of ASCs plays a beneficial role in SCI repair by promoting the protection of denuded axons and cellular repair that was induced mainly through paracrine mechanisms^[57]. The propensity of proliferation and the potential of unchecked differentiation of stem cells raised the concern of inherent tumorigenicity and toxicity. Ra *et al*^[66], observed that systemic transplanta-

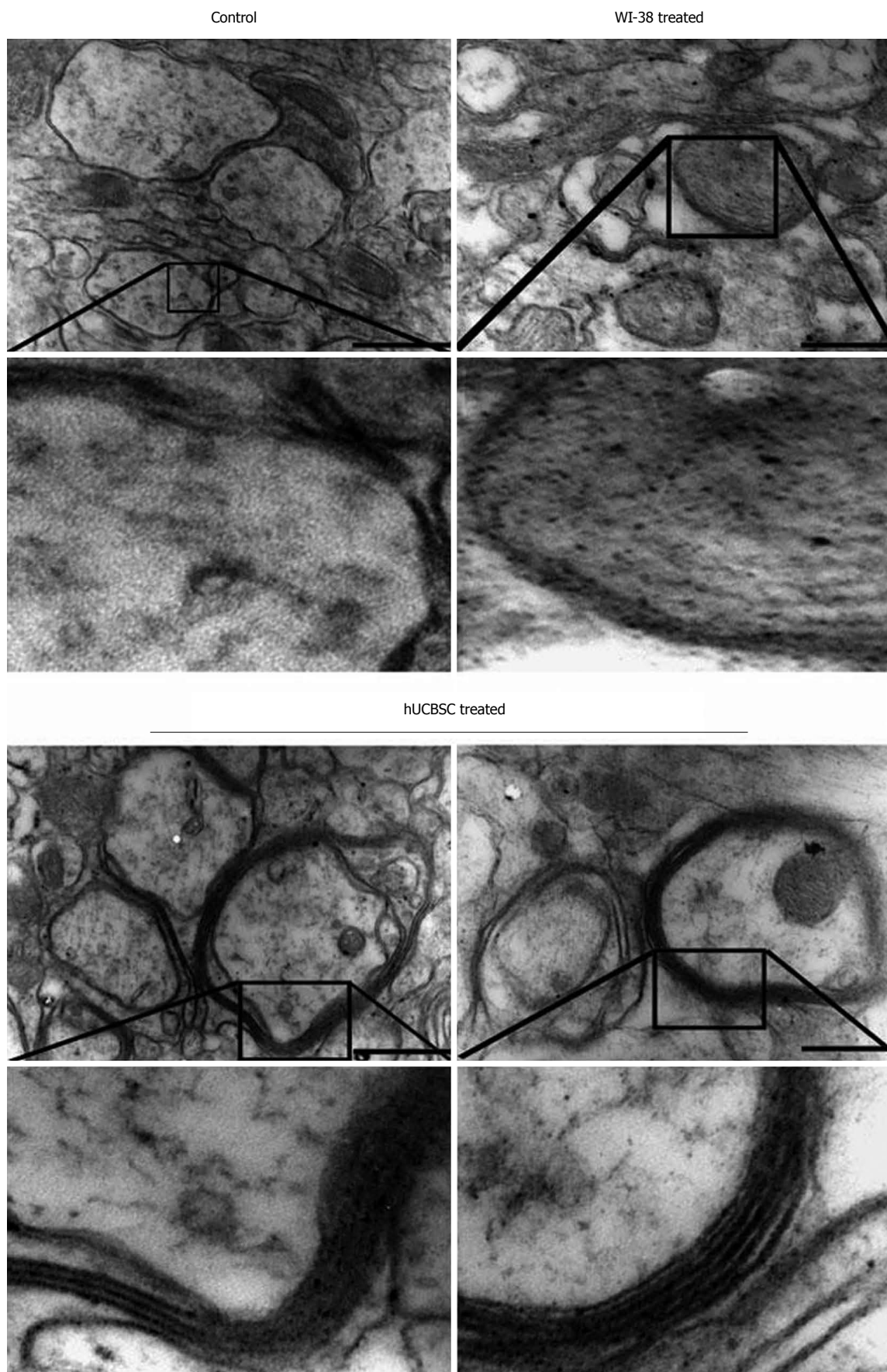


Figure 1 Transmission electron micrographs of shiverer mice brain showing thin and fragmented myelin around the axons in control and WI-38- implanted mice. In contrast, human umbilical cord blood-derived mesenchymal stem cells-treated shiverer brains showing myelin with several layers. Images are representatives of the several sections obtained from 3 different animals ($n = 3$). Scale bar = 33000. *Stem Cells Dev* 2011; **20**: 881-891.

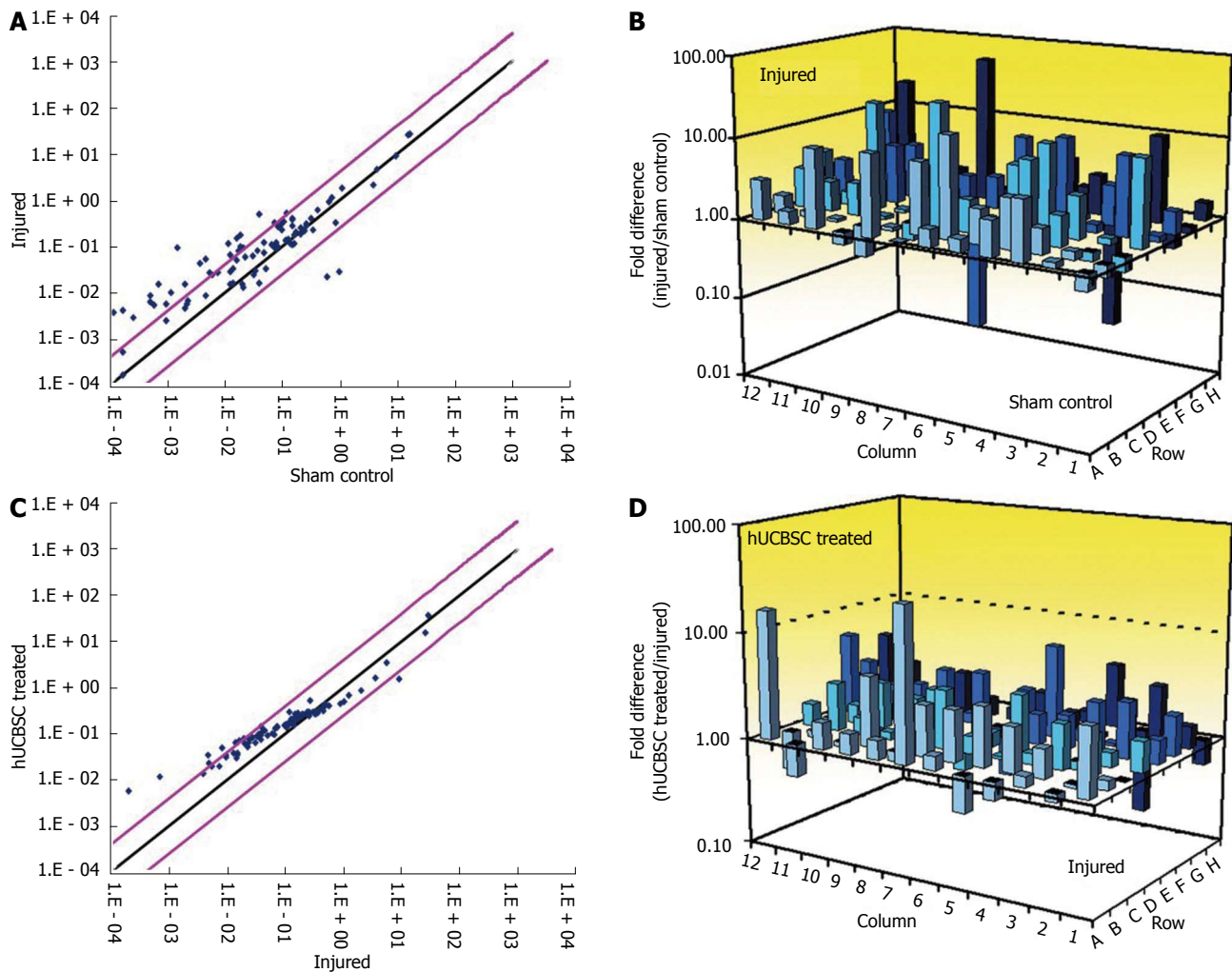


Figure 2 Microarray analysis of apoptotic genes after spinal cord injury. Total RNA was extracted from sham control, 3-wk post-spinal cord injury (SCI), and 3-wk post-SCI plus human umbilical cord blood-derived mesenchymal stem cells (hUCBSC)-treated tissues, reverse-transcribed, and the corresponding cDNA was loaded into a 96-well plate. In each group, RNA from at least three different animals was pooled together. A and C: Representative scatter plots show the validity of the experiment and the expression level of each gene in the control vs injured and injured vs hUCBSC-treated samples; B and D: These 3D profile graphs show the fold difference in expression of each gene between sham control vs injured and injured vs hUCBSC-treated samples. These experiments were performed in duplicate (hUCBSC, human umbilical cord blood-derived mesenchymal stem cells; SCI, spinal cord injury). *J Neurotrauma* 2009; **26**: 2057-2069.

tion of human Adipose tissue-derived mesenchymal stem cells (hAdMSCs) appeared to be safe and did not induce tumor development as none of the patients developed any serious adverse events related to hAdMSC transplantation during the three-month follow-up. Zhou *et al.*^[67], compared mesenchymal stromal cells from human bone marrow and adipose tissue for the treatment of spinal cord injury and suggested that hAdMSCs would be more appropriate than hBMSCs for transplantation to treat SCI. Recently, Zaminy *et al.*^[68], proved that adipose tissue-derived Schwann cells can modulate the hostile environment of the damaged spinal cord and generate a more stimulating environment to support axon regeneration and enhance functional recovery (Table 2).

HUMAN UMBILICAL CORD BLOOD-DERIVED MSCS

Human umbilical cord blood-derived mesenchymal stem

cells (hUCBSC) offer great potential for novel therapeutic approaches targeted against many CNS diseases. Previous studies have reported that hUCBSC are beneficial in reversing the deleterious effects of SCI, even when infused five days after injury^[69]. Transplanted hUCBSC differentiate into various neural cells and induce motor function improvement in SCI rat models^[70]. In our laboratory, hUCBSC transplanted in rats one week after SCI were shown to transdifferentiate into neurons and oligodendrocytes and also to downregulate Fas-mediated apoptosis^[71,72]. These transdifferentiated oligodendrocytes facilitated the secretion of neurotrophic hormones NT3 and BDNF and synthesized MBP and PLP, thereby promoting the remyelination of demyelinated axons in the injured spinal cord^[71]. We observed that hUCBSC treatment increased myelin basic protein *in vitro* in PC-12 cells, which are normally not myelinated. To further confirm the ability of transplanted hUCBSC in remyelination, we injected hUCBSC into shiverer mice brains. This study

Table 3 Changes in the expression of apoptotic genes and inhibitors after spinal cord injury and human umbilical cord blood stem cells treatment

UniGene	GenBank	Gene name	Fold change after SCI	Fold change after hUCBSC treatment
Rn. 36696	NM_022698	Bad	3.12 ± 1.34	-1.47 ± 0.14
Rn. 14598	NM_053812	Bak1	2.28 ± 0.99	1.36 ± 0.79
Rn. 13007	NM_031328	Bcl10	8.83 ± 1.91	1.51 ± 1.45
Rn. 19770	NM_133416	Bcl2a1	7.95 ± 1.98	1.79 ± 0.75
Rn. 10323	NM_031535	Bcl2l1	2.13 ± 0.85	-2.01 ± 0.89
Rn. 162782	NM_022684	Bid	2.45 ± 1.27	1.86 ± 0.99
Rn. 89639	NM_057130	Bid3	5.43 ± 1.06	2.62 ± 0.75
Rn. 38487	NM_053704	Bik	4.41 ± 0.64	3.58 ± 0.14
Rn. 92423	XM_226742	Birc1b	25.84 ± 0.85	3.01 ± 0.67
Rn. 64578	NM_023987	Birc3	10.14 ± 1.06	3.01 ± 0.78
Rn. 54471	NM_022274	Birc5	-2.84 ± 1.98	4.57 ± 1.14
Rn. 55946	NM_057138	Cflar (Flip)	3.12 ± 1.77	-1.20 ± 0.86

Results are expressed as mean ± SD. hUCBSC: Human umbilical cord blood stem cells; SCI: Spinal cord injury. Refer Dasari *et al*^[73].

clearly demonstrated that transplanted hUCBSC survived, migrated *in vivo* and myelinated genetically denuded axons in shiverer mice brains. The expression level of myelin basic protein, a major component of the myelin sheath, was significantly elevated *in vivo* and *in vitro* as revealed by Western blotting, reverse transcription polymerase chain reaction, immunohistochemistry, immunocytochemistry, and fluorescent *in situ* hybridization results. Further, transmission electron microscopic images of hUCBSC-treated shiverer mice brains showed several layers of myelin around the axons compared with a thin and fragmented layer of myelin in untreated animals (Figure 1). Moreover, the frequency of shivering was diminished one month after hUCBSC treatment. Our results strongly indicated that hUCBSC transplantation played an important role in re-myelination and could be an effective therapeutic approach for demyelinating or hypomyelinating disorders^[73]. Furthermore, apoptotic pathways mediated by caspase-3, Fas and TNF- α were downregulated by hUCBSC^[72,74]. The locomotor scale scores in hUCBSC-treated rats were significantly improved as compared to those of the control injured group. To further extend our studies, we utilized RT-PCR microarray and analyzed 84 apoptotic genes to identify the genetic modulation that occurred after traumatic SCI and after hUCBSC transplantation^[73]. We observed that the genes involved in inflammation and apoptosis were up-regulated (TNF- α , TNFR1, TNFR2, Fas, Bad, Bid, Bid3, Bik, and Bak1) in the injured rat spinal cords, whereas genes such as XIAP, which are involved in neuroprotection, were up-regulated in the hUCBSC-treated rats (Figure 2, Tables 3 and 4). Our findings from co-cultures of cortical neurons with hUCBSC and blocking of the Akt pathway by a dominant-negative Akt and Akt-inhibitor IV confirmed that the mechanism underlying hUCBSC neuroprotection involved activation of the Akt signaling pathway. These results suggested the neuroprotective potential of hUCBSC against glutamate-induced apoptosis of cultured cortical neurons^[74]. Both the *in vivo* and *in vitro* studies supported

Table 4 Changes in the expression of caspase-related and nuclear factor- κ B-related apoptotic genes after spinal cord injury

UniGene	GenBank	Gene name	Fold change after SCI	Fold change after hUCBSC treatment
Rn. 37508	NM_012762	Casp1	9.14 ± 1.70	1.27 ± 0.78
Rn. 81078	NM_130422	Casp12	2.91 ± 1.34	1.46 ± 0.68
Rn. 10562	NM_012922	Casp3	3.56 ± 0.92	1.18 ± 0.84
Rn. 88160	NM_031775	Casp6	3.34 ± 1.06	1.46 ± 0.79
Rn. 53995	NM_022260	Casp7	2.81 ± 1.27	2.81 ± 1.21
Rn. 54474	NM_022277	Casp8	3.84 ± 1.20	1.62 ± 0.89
Rn. 32199	NM_031632	Casp9	2.86 ± 0.71	1.36 ± 0.62
Rn. 67077	NM_053362	Dffb (Cad)	32.94 ± 0.78	2.72 ± 0.84
Rn. 16183	NM_152937	Fadd	2.21 ± 0.78	1.51 ± 0.73
Rn. 162521	NM_139194	Tnfrsf6 (Fas)	10.87 ± 1.77	1.79 ± 0.67
Rn. 44218	NM_053353	CD40lg	15.91 ± 0.99	3.46 ± 0.78
Rn. 160577	NM_080769	Lta (Tnfb)	28.67 ± 0.07	2.06 ± 0.68
Rn. 2275	NM_012675	TNF- α	7.17 ± 1.63	2.36 ± 1.03
Rn. 11119	NM_013091	Tnfrsf1a (TNFR1)	2.53 ± 1.48	1.22 ± 0.78
Rn. 83633	NM_130426	Tnfrsf1b (TNFR2)	5.25 ± 1.56	3.01 ± 0.99
Rn. 25180	NM_134360	Tnfrsf5 (CD40)	4.26 ± 1.84	1.99 ± 0.78
Rn. 54443	NM_030989	Tp53 (P53)	3.46 ± 1.41	-1.12 ± 0.61
Rn. 18545	XM_341671	Tradd	5.62 ± 1.13	1.46 ± 0.59
Rn. 136874	AI406530	Traf1	4.12 ± 1.34	2.06 ± 0.84

Results are expressed as mean ± SD. hUCBSC: Human umbilical cord blood stem cells; NF- κ B: Nuclear factor- κ B; SCI: Spinal cord injury. Refer Dasari *et al*^[73].

our hypothesis that the therapeutic mechanism of hUCBSC was remyelination of demyelinated axons and inhibition of the neuronal apoptosis during the repair phase of the injured spinal cord. Veeravalli *et al*^[76] reported the involvement of tissue plasminogen activator (tPA) after SCI in rats and the role of hUCBSC. The tPA expression and activity were studied *in vivo* in rats after SCI and *in vitro* in rat embryonic spinal neurons in response to injury with staurosporine, hydrogen peroxide and glutamate. Infusion of hUCBSC downregulated tPA activity *in vivo* in rats as well as *in vitro* in the spinal neurons. Furthermore, MMP-2 is upregulated after hUCBSC treatment in spinal cord injured rats and in spinal neurons injured either with staurosporine or hydrogen peroxide. Also, hUCBSC-induced upregulation of MMP-2 diminished the formation of the glial scar at the site of injury along with reduced immunoreactivity to chondroitin sulfate proteoglycans. This upregulation of MMP-2 levels and reduction of glial scar formation by hUCBSC treatment after SCI created an environment more favorable for endogenous repair mechanisms^[77] (Figure 3). Kao *et al*^[78], suggested that hUCB derived-CD34⁺ cells can induce angiogenesis and endo/exogenous neurogenesis in SCI. In addition, Chen *et al*^[79] recently showed that hUCB stem cells have the ability to secrete multiple neurotrophic factors. Their study demonstrated an elevation of neuroprotective cytokine serum IL-10 levels and a decrease in TNF- α levels after hUCB stem cells infusion. Moreover, both GDNF and VEGF could be detected in the injured spinal cord

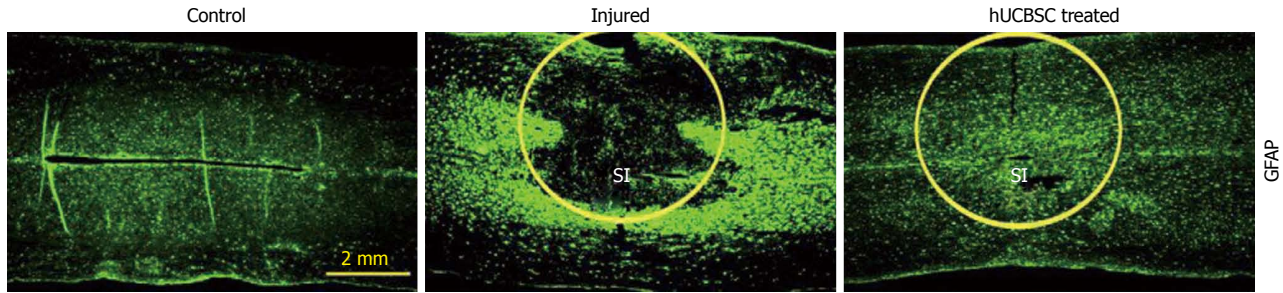


Figure 3 Reduction of inflammation in human umbilical cord blood-derived mesenchymal stem cell-treated spinal cords of rats. Immunohistochemical comparison of control, injured (21 d after spinal cord injury) and human umbilical cord blood-derived mesenchymal stem cells-treated spinal cord sections was performed to analyze the expression of reactive astrocytes at the site of injury. GFAP immunoreactivity is more evident and is localized at the lesion epicenter in the injured spinal cords. Astrogliosis is reduced in human umbilical cord blood-derived mesenchymal stem cells-treated sections. SI: Site of injury. *Neurobiol Dis* 2009; **36**: 200-212.

Table 5 Overview of effects of umbilical cord blood-derived mesenchymal stem cells after spinal cord injury

Source of MSC	Main pathological features improved/repared	Limitations/recommendations/conclusions	Ref.
Human	Stem cells migrated to injured areas, functional recovery	hUCB may be a viable source of stem cells for treatment of neurological disorders	Saporta <i>et al</i> ^[69] , 2003
	Axonal regeneration, functional recovery	HUCBs and BDNF reduced the neurological function deficit to a moderate degree for SCI rats	Kuh <i>et al</i> ^[70] , 2005
	Stem cells secrete neurotrophic hormones and remyelinating proteins, axonal remyelination	Studies on long-term survival of hUCBSC and remyelination are recommended.	Dasari <i>et al</i> ^[71] , 2007
	Repair and maintenance of structural integrity of the injured spinal cord, downregulation of apoptosis, functional recovery	Role of hUCBSC in maintaining structural integrity and thereby promoting the long-term survival of neurons and oligodendrocytes in the injured spinal cord	Dasari <i>et al</i> ^[72] , 2008
	Downregulation of neuronal apoptosis	Modulation of the micro environment of the injured spinal cord by application of hUCBSC might be a potential therapeutic modality	Dasari <i>et al</i> ^[73] , 2009
	Downregulation of elevated tPA activity/ expression in SCI rats	tPA is involved in secondary pathogenesis following spinal cord injury	Veeravalli <i>et al</i> ^[76] 2009
	Upregulation of MMP2, reduction of glial scar	hUCBSC treatment after SCI upregulates MMP-2 levels and reduces the formation of the glial scar	Veeravalli <i>et al</i> ^[77] , 2009
	GDNF and VEGF were secreted in the injured spinal cord after transplantation of CD34 ⁺ cells	CD34 ⁺ cell therapy may be beneficial in reversing the SCI-induced spinal cord infarction and apoptosis and hindlimb dysfunction	Kao <i>et al</i> ^[78] , 2008
	Serum IL-10 levels increased, TNF- α levels decreased, functional recovery	Recovery of SCI-induced hind limb dysfunction is by increasing serum levels of IL-10, VEGF and GDNF in SCI rats.	Chen <i>et al</i> ^[79] 2008
	Infarct size and blood vessel density at the injured site were significantly different in the treated group, functional recovery	Transplantation of CD34(+) HUCBCs during acute phase could promote functional recovery better than during subacute phase after SCI by raising blood vessel density	Ning <i>et al</i> ^[80] , 2013

MSC: Mesenchymal stem cell; SCI: Spinal cord injury; IL: Interleukin; TNF- α : Tumor necrosis factor- α ; hUCB: Human umbilical cord blood; hUCBSC: Human umbilical cord blood-derived mesenchymal stem cell.

after the transplantation of hUCBSC, thereby promoting angiogenesis and neuronal regeneration. Recently, Ning *et al*^[80], showed that transplantation of CD34⁺ HUCBCs during the acute phase could promote functional recovery better than during the subacute phase after SCI by raising neovascular density. These multifaceted protective and restorative effects from hUCB grafts may be interdependent and act in concert to promote therapeutic recovery for SCI (Table 5). Nevertheless, clinical studies with hUCBSC are still limited due to concerns about safety, mode of delivery, and efficiency. Among these concerns, the major histocompatibility in allogeneic transplantation is an important issue that needs to be addressed in future clinical trials for treating SCI^[16].

HUMAN WHARTON'S JELLY/UMBILICAL CORD MATRIX CELLS

There are two main populations of cells with a mesenchymal character within the human umbilical cord: Wharton's jelly mesenchymal stem cells (WJ-MSCs) and human umbilical cord perivascular cells (HUCPVCs)^[81]. Wharton's jelly cells (WJ-MSCs) can proliferate more rapidly and extensively than adult BMSCs (for a detailed review refer to Vawda and Fehlings, 2013). Yang *et al*^[82], examined the effects of human umbilical mesenchymal stem cells (HUMSC) transplantation after complete spinal cord transection in rats. They observed that transplanted HUMSCs survived for 16 wk and produced large amounts of human neuro-

Table 6 Overview of effects of Wharton's jelly/umbilical cord matrix cells after spinal cord injury

Source of MSC	Main pathological features improved/repaired	Limitations/recommendations/conclusions	Ref.
Human	Survival of transplanted HUMSCs 16 wk, secretion of human neutrophil-activating protein-2, neurotrophin-3, basic fibroblast growth factor, glucocorticoid induced tumor necrosis factor receptor, and vascular endothelial growth factor receptor 3 in the host spinal cord	Transplantation of HUMSCs is beneficial to wound healing after SCI in rats	Yang <i>et al</i> ^[82] , 2008
	Axonal regeneration, neuroprotective action by grafted cells, functional recovery	Co-grafted HUMSCs and BDNF may be a potential therapy for SCI	Zhang <i>et al</i> ^[83] , 2009
	hUCMSCs survive, migrate, and produce GDNF and neurotrophin-3, functional recovery	Studies on dose-dependent effects of hUCMSCs transplantation on SCI are required	Hu <i>et al</i> ^[84] , 2010
	Increased intensity of 5-HT fibers, increased volume of spared myelination, decreased area of cystic cavity, functional recovery	NT-3 enhanced therapeutic effects of HUMSCs after clip injury of the spinal cord.	Shang <i>et al</i> ^[85] , 2011

MSC: Mesenchymal stem cell; SCI: Spinal cord injury; hUCMSC: Human umbilical cord blood-derived mesenchymal stem cell.

phil-activating protein-2, neurotrophin-3, basic fibroblast growth factor, glucocorticoid induced tumor necrosis factor receptor, and vascular endothelial growth factor receptor 3 in the host spinal cord. Zhang *et al*^[83], used an animal model of transected SCI to test the hypothesis that co-grafted human umbilical mesenchymal stem cells-derived neurospheres (HUMSC-NSs) and BDNF can promote morphologic and functional recoveries of the injured spinal cord. Recovery of hindlimb locomotor function in SCI rats was significantly enhanced in human umbilical cord mesenchymal stem cells-grafted animals at five weeks as compared to control sham-grafted animals^[84]. Using a rat model for clip SCI, Shang *et al*^[85], showed that Neurotrophin-3 (NT-3) genetically modified human umbilical mesenchymal stem cells (NT-3-HUMSCs) promoted the morphologic and functional recovery of injured spinal cords (Table 6). Although these studies involved thoracic SCI model, these positive findings will most likely apply to cervical SCI as well^[3].

CONCLUSION

Therapeutic application of MSCs represents a promising approach in the treatment of spinal cord injury. Nevertheless, cell-based therapy for SCI in its nascent stages is facing several challenges including translational clinical issues, regulatory and ethical concerns, as well as modalities of transplantation, timing, safety and efficacy of the transplanted cells. A better understanding is also needed of the mechanisms of action and the behavior of stem cells in the pathological environment after transplantation in order to determine the best time frame and the most efficient routes for cell delivery after the injury^[86]. Although several clinical trials utilize MSCs transplantation for the treatment of SCI, the ultimate value of a translational approach needs continued exploration of basic scientific knowledge of SCI and proven therapeutic efficacy *via* rigorous controlled, randomized, double blind, multi-center clinical trials.

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WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Brain mesenchymal stem cells: The other stem cells of the brain?

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Abstract

Multipotent mesenchymal stromal cells (MSC), have the potential to differentiate into cells of the mesenchymal lineage and have non-progenitor functions including immunomodulation. The demonstration that MSCs are perivascular cells found in almost all adult tissues raises fascinating perspectives on their role in tissue maintenance and repair. However, some controversies about the physiological role of the perivascular MSCs residing outside the bone marrow and on their therapeutic potential in regenerative medicine exist. In brain, perivascular MSCs like pericytes and adventitial cells, could constitute another stem cell population distinct to the neural stem cell pool. The demonstration of the neuronal potential of MSCs requires stringent criteria including morphological changes, the demonstration of neural biomarkers expression, electrophysiological recordings,

and the absence of cell fusion. The recent finding that brain cancer stem cells can transdifferentiate into pericytes is another facet of the plasticity of these cells. It suggests that the perversion of the stem cell potential of pericytes might play an even unsuspected role in cancer formation and tumor progression.

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Key words: Stem cell; Mesenchymal stem cell; Pericyte; Brain; Cell plasticity; Cancer stem cell; Glioma; Neurodegenerative disease

Core tip: Mesenchymal stem cells (MSCs), in addition to their potential to differentiate into cells of the mesenchymal lineage, have immunomodulatory properties and can transdifferentiate to generate neural cells at least *in vitro*. These stem cells are found in almost any adult tissue, including brain. The existence of similarities between MSC and pericytes points to brain pericytes as the other stem cell population of the adult brain in addition to neural stem cells. This raises fascinating perspectives on the potential of brain pericytes in nervous system maintenance and repair. The recent finding that brain cancer stem cells transdifferentiate into pericytes is another facet of the plasticity of these cells. It suggests that the perversion of the stem cell potential of pericyte might play an even unsuspected role in cancer formation and tumor progression.

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INTRODUCTION

The history of multipotent mesenchymal stromal cells started when colony-forming unit fibroblastic cells (CFU-F) with osteogenic potential were obtained from bone marrow cultured cells^[1-3]. Accordingly, CFU-F cells were defined as self-renewing non-hematopoietic bone marrow stromal stem cells (BMSCs). They were isolated on the basis of their plastic adherence, and characterized both by their ability to form colony when plated at low-density and to differentiate into osteoblasts^[3]. Thereafter, BMSCs were shown to differentiate *in vitro* and *in vivo* into other cells of mesenchymal lineage including chondrocytes and adipocytes^[4]. Cells similar to BMSCs are also isolated from non-marrow fetal tissue such as placenta, cord blood, fetal liver and lung, as well as from adult tissues including muscle, adipose tissue, dental pulp, lung and brain^[5-8]. These fetal and adult stem cells have the same ability as BMSCs for self-renewal and for differentiation into osteoblasts, chondrocytes and adipocytes *in vitro*. They also exhibit, at least *in vitro*, transdifferentiation capacity (see below). These cells are referred as mesenchymal stem cells or as multipotent mesenchymal stromal cells (MSCs). However, the question remains if these ubiquitous cells behave *in vivo* as genuine stem cells or if their stem cell potential is a cell culture artifact^[9]. The existence of these MSCs in virtually all postnatal organs does not necessarily mean that these cells behave as stem cells during development. For example, their physiological function could be limited to postnatal regenerative processes. Hence, the concept of mesenchymal stem cell, initially well-defined and restricted to a multipotent progenitor for skeletal tissues and residing within the bone marrow has progressively evolved towards an all-encompassing concept including multipotent perivascular cells of almost any tissue^[9]. Importantly, there is not an exclusive and universal marker for immunophenotyping MSCs. Therefore, their immuno-characterization relies on a combination of both positive and negative markers. Positive markers can include CD11b, CD13, CD19A, CD73, CD105, CD146, CD271, nestin, nerve/glia antigen 2 (NG2), platelet-derived growth factor receptor β (PDGFR- β), while negative markers usually are endothelial, and hematopoietic stem cell proteins (Table 1)^[10-12]. An additional remarkable feature is that MSCs lack or have a low expression of MHC class II and of the costimulatory molecules CD40, CD80, CD86, CD134 and CD142^[13]. In relation to this, MSCs have strong anti-inflammatory and immunomodulating potentials^[14]. MSCs exert their inhibitory effects on T-cell proliferation by mechanisms involving both cell to cell contact between MSC and T lymphocytes, and secreted factors such as prostaglandin E2 (PGE2), inoleamine 2,3-dioxygenase and nitric oxide^[14]. As in many biological processes, this immunosuppressive effect is dose dependent and depends on the ratio between MSCs and T cells. Indeed low ratios of MSCs can even enhance T cell proliferation^[14]. In addition, MSCs prevent the differentiation of monocyte into dendritic cells, and modulate natural killer cell activ-

ity by the release of inhibitory factors such as PGE2 and transforming growth factor- β ^[14]. MSCs also have anti-inflammatory action by reducing the production of tumor necrosis factor (TNF)- α and interleukin (IL)-12 and by increasing the synthesis of IL-10 by macrophages^[14]. These anti-inflammatory and immuno-modulatory capacities of MSCs are already exploited *in vivo*. MSC-based treatment is beneficial in several models of graft-vs-host disease and in auto-immune diseases such as collagen-induced arthritis, experimental autoimmune encephalomyelitis, type 1 diabetes mellitus disease and inflammatory bowel disease models^[14-17]. Clinical trials are currently underway for these different pathologies^[15,18]. The ability of MSCs to home in damaged tissues, associated with their capacity to secrete bioactive molecules such as growth factors, and their immunosuppressive and anti-inflammatory properties, suggest that these cells protect tissues from damage and facilitate tissue repair independently of their capacity to generate differentiated cells^[18].

For all these reasons, MSCs became the focus of intense researches in tissue engineering and regenerative medicine. These cells could provide an answer both to the ethical concerns raised by the therapeutic use of human embryonic stem cells and to their scarce availability. Furthermore, as MSCs are easily isolated from adult tissues, they offer the advantage to allow autologous transplantation. Importantly, experimental studies performed with MSCs revealed an additional property: MSCs have a greater differentiation plasticity potential than previously envisioned. For example, they can transdifferentiate into urothelial, myocardial, and epithelial cells^[19-21]. Numerous studies also report the *in vitro* transdifferentiation of MSCs into neural and glial cells^[22-30]. At the moment, the potential of MSCs to regenerate human tissues *in vivo* is not clearly defined. Current research is ongoing to resolve this critical issue by improving MSC culture engineering and cell transplantation technology. A better characterization of the therapeutic potential of MSCs according to their tissue of origin is also a critical issue.

WHEN MSCs TRANSDIFFERENTIATE INTO NEURAL CELLS: FACTS AND ARTIFACTS

The observation that MSCs transdifferentiate into neurons was first obtained with bone MSCs, and then extended to MSCs isolated from different adult tissues including adipose tissue, bone marrow, and brain^[5,31-34]. Brain implanted marrow stromal cells also differentiate into glial cells^[25]. Importantly, grafting MSCs in several brain lesion models reduces neuronal deficits^[35-42]. However, current evidence suggests that in the experimental models used, the repair and functional improvements reported are primarily mediated by paracrine or cell-cell interactions rather than by the successful engraftment and the *in situ* transdifferentiation of implanted MSCs into neural cells^[43-47]. Regarding MSC transdifferentia-

Table 1 Major positive and negative markers used for identifying bone marrow mesenchymal stem cells and pericytes

Markers	MSCs	Pericytes	EC	HSPCs	NSPCs
CD10	+ [12]	+ [12]			
CD13	+ [12]	+ [12]			
CD29	+ [12]	+ [12]		+ [91]	+ [92]
CD44	+ [12]	+ [12]		+ [93]	+ [92]
CD73	+ [12]	+ [12]	+ [94]		
CD90	+ [12]	+ [12]		+ [95]	+ [92,96]
CD105	+ [12]	+ [12]	+ [97]		
CD140B	+ [12]	+ [12]			
CD146	+ / low [12,90]	+ [12]	+ [98]		
CD166	+ [12]	+ [12]		+ [99]	
NG2	+ [12]	+ [12]	- [11]	- [100]	
Nestin	+ [101,102]	+ [72]			+ [103]
CD14	- [12]	- [12]			
CD31	- [12]	- [12]	+ [104]		
CD34	- [12]	- [12]	+ [105]	+ [105]	
CD45	- [12]	- [12]			
CD133	- [12]	- [12]	- [106]	+ [107]	+ [108]
CD117	- [12]	- [12]		+ [109]	+ [110]
CD144	- [12]	- [12]	+ [111]		
vWF	- [112]	- [113]	+ [114]		

In the absence of any universal and specific marker to define mesenchymal stem cells, their immunophenotyping relies on the use of combinations of both positive and negative markers. Note that MSCs profile may vary depending on the cell culture conditions^[88], or with their *in situ* localization^[89]. Expression of the cell surface antigens CD73, CD90, CD105 and non-expression of CD14, CD34, CD45 are useful criteria to define bone MSCs and pericytes. MSCs: Mesenchymal stem cells; EC: Endothelial cells; HSPCs: Hematopoietic stem and progenitor cells; NSPCs: Neural stem and progenitor cells.

tion into neural cells, a notable controversy arose when it was reported that, (1) the rapid *in vitro* morphological differentiation of MSC into neuron-like cells following administration of DMSO or cAMP elevating agents such as forskolin or IBMX can be linked to actin depolymerization resulting in cytoplasm retraction and not through neurite extension^[48-50]; and (2) the transformation of MSCs into neurons *in vivo* can result from the fusion of MSCs with brain cells rather than to MSC transdifferentiation^[51]. Therefore, additional criteria are now applied when studying MSC transdifferentiation. For example, reporting neuronal differentiation of MSCs now requires observation of morphological changes, the demonstration of neural biomarkers expression, neurotransmitter responsiveness or electrophysiological recording, and absence of cell fusion^[28,33,49,52,53]. Note however, that all MSCs are not equal and that their differentiation potential can be related to their tissue of origin^[6]. This suggests that brain-derived MSCs could have a greater potential for neural differentiation than bone MSCs. Hence, the difficulty to obtain functional mature neurons by differentiating bone MSC can be explained both by their origin and by cell culture conditions which are far to provide the cues found in the brain microenvironment. Accordingly, recent experiments using brain derived MSCs instead of bone marrow MSCs, provide additional evidence on the potential of brain MSCs to transdifferentiate into neuro-

nal cells at the clonal level and on the basis of stringent criteria^[54]. A notable point is that these observations are made *in vitro*. Therefore, it remains to establish whether the transdifferentiation of MSCs is a cell culture artifact with potential applications in cell replacement therapies for implanting pre-differentiated neurons, or is it also a physiological process contributing to brain development or repair. Part of the answer might be given by determining where MSCs reside in the organism and which cell behaves as MSC *in vivo*. Recent findings show that MSCs are perivascular cells such as pericytes^[11,55,56].

MSCS ARE PERIVASCULAR CELLS

Pericytes are perivascular cells, or more strictly speaking peri-endothelial vascular mural cells (Figure 1). Pericytes form an incomplete layer on the abluminal surface of capillary endothelial cells. They wrap capillary endothelial cells and both cell types are surrounded by the basal lamina^[57] (Figure 2). For many years, pericytes have been viewed as supportive vasculature cells involved in the regulation of capillaries blood flow and contributing to the blood-brain barrier^[58]. Nowadays, known functions of pericytes also include a role in angiogenesis, in matrix proteins and bioactive molecules synthesis (vascular endothelial growth factor, placental growth factor, leukemia inhibitor factor, CXCL12, basic fibroblast growth factor, nerve growth factor, platelet-derived growth factor B...), in vessel stabilization and in the regulation of vascular tone^[59]. Importantly, these cells are now considered as a potential reservoir of stem or progenitor cells for adult tissue repair. Regarding this stem cell potential, it has been known as early as 1995 that pericytes can differentiate into an osteogenic phenotype^[60]. Ten years after, perivascular cells were also demonstrated to differentiate into adipocytes^[61]. The definitive proof that MSCs are perivascular cells such as pericytes was done in 2008 in two landmark studies showing that a subset of perivascular cells from adult tissues, identified on CD146, NG2 and PDGF-R β expression, exhibit in culture the same osteogenic, chondrogenic, adipogenic and myogenic potentials than MSCs^[11,55]. In addition, these perivascular cells express MSC markers including CD10, CD13, CD44, CD73, CD90 and CD105^[11,12]. A consequence of the demonstration of a perivascular origin for MSCs was a burst of interest in pericyte research with the number of annual entries in PubMed for the keyword “pericyte” increasing from 83 in 1993 to 445 in 2013. With hindsight, the finding that some MSCs are pericytes is not incongruous^[11,56]. Stem cells must reside in a specialized environment (the stem cell niche), and the presence of MSCs in almost all adult tissues suggests a ubiquitous distribution for MSC niches. This is consistent with the omnipresence of capillary blood vessel mural cells. In addition, this perivascular location allows the rapid recruitment of MSCs to the site of focal lesions where they could act as microenvironmental regulators for tissue regeneration^[62]. Since tissue regeneration requires functional blood ves-

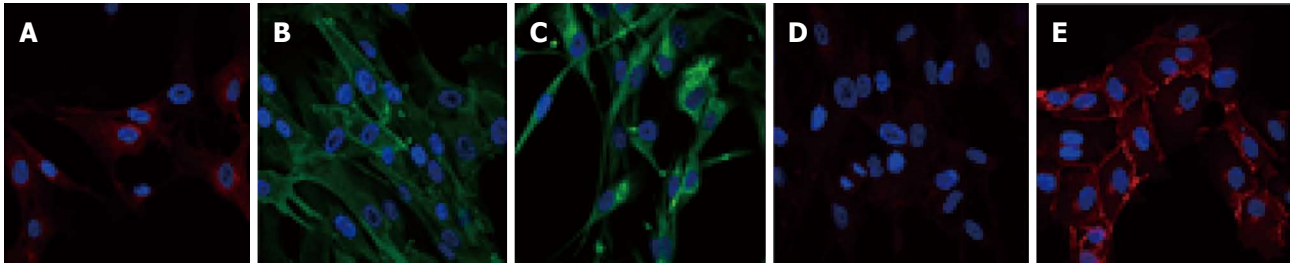


Figure 1 Pericyte immunophenotyping. Pericytes express antigens allowing their identification. However, there is currently no specific marker to identify them. Therefore, to distinguish pericytes from other cell types, both positive and negative markers are used. For example, pericytes are known to be positive for platelet-derived growth factor receptor β (PDGFR- β)/CD140b (A), Alanine aminopeptidase N/CD13 (B), and for the stem cell protein nestin (C). Pericytes are also negative for VE-Cad/CD144 (D) that is detected in human brain endothelial cells (E). Specific antigenic labeling is in green or red and nuclei are 4',6-diamidino-2-phenylindole stained (blue).

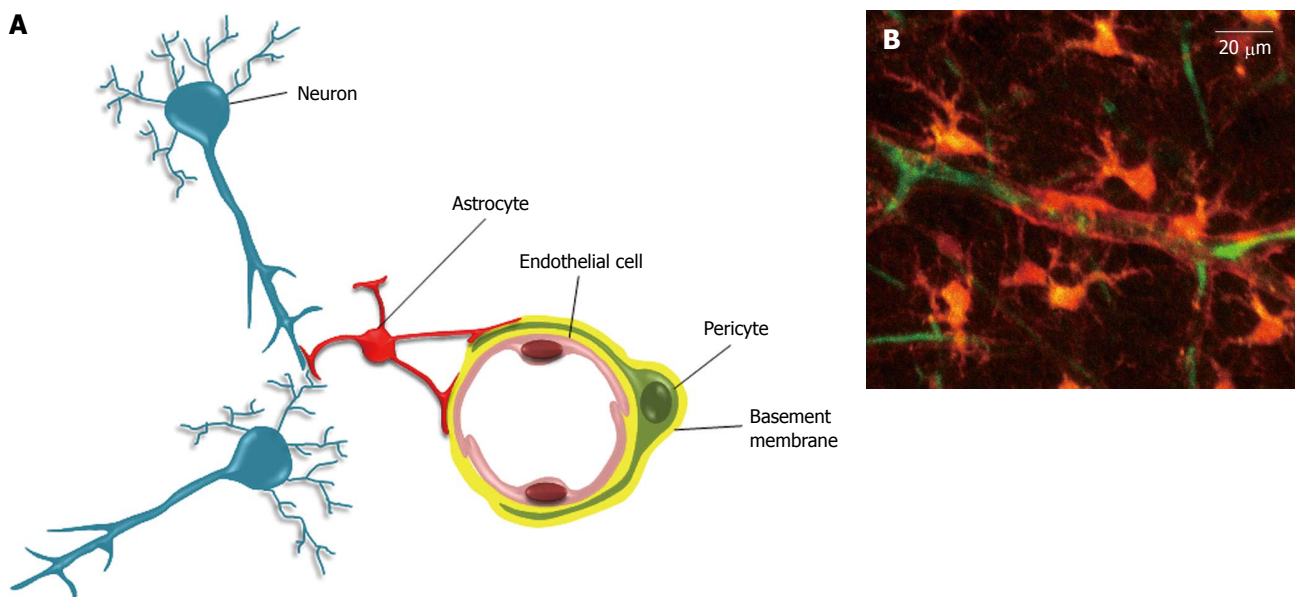


Figure 2 The neurovascular unit. A: The neurovascular unit. In the neurovascular unit, pericytes are located on the abluminal side of endothelial cells (EC). Both cells are ensheathed by the basement membrane (BM). The covering of EC by pericytes is incomplete, and interruptions in BM can allow direct contacts between pericyte and EC. These contacts occur through peg and socket structures, and adherent and gap junctions (not shown)^[59]. The abluminal side of the basement membrane is also contacted by astrocytes endfeet. In addition to these cells, the neurovascular unit also includes neurons, and microglial cells (not shown); B: Two-photon microscopy of a neurovascular unit. Following injection in the rat tail vein, the sulforhodamine-B dye crosses the blood brain barrier and stains astrocytes and pericytes in orange (reproduced from^[115]). The blood plasma is shown in green after *iv* injection of FITC-dextran (Mw 70 kDa). Neurons, endothelial and microglial cells are not shown here.

sels, associating MSCs with endothelial cells in a same “regenerative/healing unit” makes sense. Note that in addition to capillaries, MSCs are also detected in the adventitia of large vessels^[63-65].

CNS PERICYTE AND THE NEUROVASCULAR UNIT

With a human brain capillary network estimated to 400 miles length^[66], and a ratio of about one pericyte for three brain endothelial cells, the human brain pericyte population is far from negligible. Pericytes cover more than 30% of the cerebral capillary surface^[67]. These cells are well-known to be involved in the regulation of angiogenesis, vascular tone and blood brain barrier function. They constitute with endothelial cells, astrocytes and neurons a

critical brain structure named neurovascular unit (NVU). The NVU, in addition to selectively supplying nutrients and oxygen through the blood brain barrier structure, provides a permissive environment for neural stem cell homing and for their proliferation^[68-70]. Note that if most pericytes are of mesoderm origin, forebrain pericytes originate from the neural crest^[71]. The demonstration that MSCs originate at least in part from pericytes raises the question of the stem cell potential of brain pericytes. At a clonal level these cells have the potential to differentiate *in vitro* into adipocytes, chondroblasts and osteoblasts^[54]. Moreover these cells are also able to differentiate *in vitro* toward a neuronal phenotype depending on cell culture conditions^[33,54,72,73]. These observations revive the idea that CNS perivascular cells such as pericytes might contribute to brain repair either directly by generating new

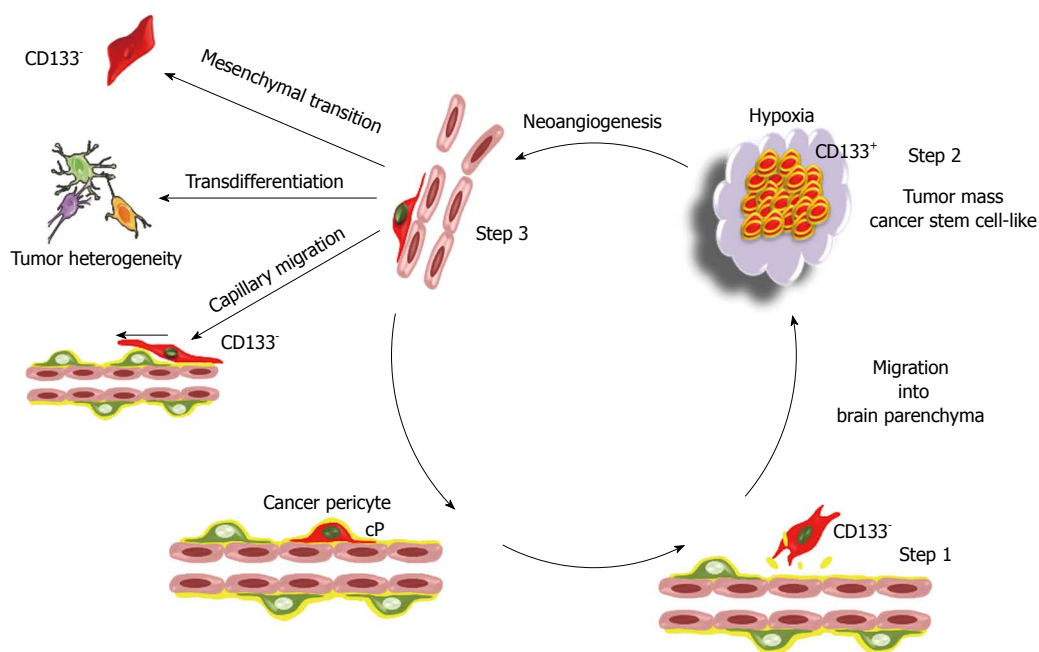


Figure 3 The cancer pericyte model: A perpetuum mobile. The proposed model of brain tumor is based on the mesenchymal stem cell potential of pericytes. In this model, the brain cancer initiating cell is a cancer pericyte (cP) harbouring oncogenic alterations and located on a brain capillary. After disruption of the basement membrane by proteases, it detaches from the vessel wall and migrates into brain parenchyma as normal pericytes do following injury^[76] (step 1). During the passage from a vascular to a neural environment the pericyte acquires a CD133⁺ neural stem cell-like phenotype, as observed *in vitro* for non-transformed pericytes^[72]. Such a transition towards a neural stem cell phenotype is already observed for non-transformed pericytes at least *in vitro*. This generates the CD133⁺ cancer stem cell pool (step 2). Amplification of the cancer stem cell pool generates hypoxia that triggers neoangiogenesis and the migration of endothelial cells towards the lesion as well as the migration of cancer stem cells towards endothelial capillaries^[83] (step 3). Cancer stem cells within this new vascular microenvironment reacquire a CD133⁺ pericyte-like phenotype. At this stage, they can either integrate into the tumor neovasculature and reinitiate a new cycle generating a perpetuum mobile, or migrate along capillaries and invade brain as previously described^[116,117]. Alternatively, due to the mesenchymal stem cell potential of pericytes, these pericyte-like cancer cells can acquire mesenchymal traits and progress towards a more aggressive mesenchymal phenotype. The transdifferentiation potential of pericyte-like cancer cells could in turn participate to the cellular heterogeneity found in glioblastoma multiforme. Since CD133 is not detected in pericytes, the existence of CD133⁺ pericyte-like cancer stem cells provides an issue to the controversy regarding the existence in glioma tumors of both CD133⁺ and CD133⁻ cancer stem cells^[118,119]. Note that this model is not exclusive. The transformation of a glial or neural stem cell might also generate cancer initiating cells.

neurons or indirectly *via* their immunomodulatory properties or the secretion of neurotrophins^[74]. Consistent with this idea is the observation that pericytes migrate away from the vascular wall and could generate neurons in response to injury^[75,76].

THE CANCER PERICYTE MODEL: A PERPETUUM MOBILE

The NVU also plays a critical role in brain cancer since a contingent of brain cancer stem cells is found near the capillaries^[77-79]. Importantly, glioblastoma stem cells are able to transdifferentiate into pericytes^[80]. According to the function of pericytes in vessel formation, these cancer pericytes contribute to the glioblastoma microvasculature^[80,81]. The recent finding that MSCs are pericytes, and that glioblastoma cells generate cancer pericytes, suggests that the stemness potential of pericytes could play a yet unsuspected role in cancer formation and progression. In the synthetic hypothetical model depicted in Figure 3, a transformed dormant pericyte harboring oncogenic mutations and lying in its vascular niche is activated and released from its vascular location as a consequence of the up-regulation of matrix proteases (Figure 3, step 1).

This activation can be triggered by inflammation or can occur following a local injury as observed *in vivo* with normal pericytes^[76,82]. In the proposed model, and in accordance with the similarities between pericytes and MSCs, this *cancer pericyte* behaves as a cancer mesenchymal stem cell. In accordance to the described potential of MSCs to generate neural stem cell-like cells^[30,72], cancer pericyte cells acquire a neural stem cell-like phenotype during their migration in brain parenchyma. This generates the cancer stem cell pool found in the tumor mass (Figure 3, step 2). Proliferation of these cancer stem cells generates hypoxia and triggers the angiogenic switch. Cancer stem cells are then recruited to develop vessels by endothelial cell-secreted cytokines such as CXCL12^[83-85] (Figure 3, step 3). In this novel vascular microenvironment made of chaotic vessels, cancer stem cells reacquire a pericyte-like phenotype as described^[80,81]. These pericyte-like cancer cells not only participate to tumor vascularization^[80,81], but also re-express their mesenchymal potential by undergoing a mesenchymal transition reminiscent to the epithelial mesenchymal transition. This generates the perpetuum mobile described in Figure 3. Indeed, MSCs have already been characterized as cancer initiating cells in gastric cancer^[86].

CONCLUSION

Since the first observation of pericyte cells by Rouget^[87], it has been a long road and winding road to get here. For many years, pericytes have been largely under-recognized and considered only as supportive cells of the vasculature. Their active role in angiogenesis and in cell-cell interactions with endothelial cells and astrocytes, as well as their *in vitro* stem cell functions, has only recently emerged. However, much remains to be done for a better understanding of the *in vivo* pericyte potential. For example, can pericytes/MSCs be considered as mobile “drugstores” migrating and delivering factors at the sites of injury^[88]? Is the pericyte/MSC transdifferentiation potential an *in vitro* artifact or is it physiologically relevant? Is it an ancient feature of more primitive organisms which has been lost during the course of evolution and which is now reactivated *in vitro*? Alternatively, could it be an emerging evolutionary trait already engaged *in vivo* in some regenerative processes? Is the neural transdifferentiation potential of brain pericyte/MSC only efficient for repairing micro-lesions, which could explain why our current experimental paradigms which generate large infarcts might not be adequate to detect this potential? Do brain pericytes/MSCs behave like “sleeping beauties” awaiting the right physiological or pharmaceutical inducers for expressing their transdifferentiating and regenerative potentials? Conversely is the perversion of this potential involved in some brain tumors? The answers to these questions promise to be fascinating.

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WCSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Adipose mesenchymal stem cells in the field of bone tissue engineering

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Abstract

Bone tissue engineering represents one of the most challenging emergent fields for scientists and clinicians. Current failures of autografts and allografts in many pathological conditions have prompted researchers to find new biomaterials able to promote bone repair or regeneration with specific characteristics of biocompatibility, biodegradability and osteoinductivity. Recent advancements for tissue regeneration in bone defects have occurred by following the diamond concept and combining the use of growth factors and mesenchymal stem cells (MSCs). In particular, a more abundant and easily accessible source of MSCs was recently discovered in adipose tissue. These adipose stem cells (ASCs) can be obtained in large quantities with little donor site morbidity or patient discomfort, in contrast to the invasive and painful isolation of bone marrow MSCs. The osteogenic potential of ASCs on scaffolds has been examined in cell cultures and animal models, with only a few cases reporting the use of ASCs for successful reconstruction or accelerated healing of defects of the skull and jaw in patients. Although these reports

extend our limited knowledge concerning the use of ASCs for osseous tissue repair and regeneration, the lack of standardization in applied techniques makes the comparison between studies difficult. Additional clinical trials are needed to assess ASC therapy and address potential ethical and safety concerns, which must be resolved to permit application in regenerative medicine.

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Key words: Adipose-derived stem cells; Bone tissue engineering; Osteogenic differentiation; Scaffold; Regenerative medicine

Core tip: The complex and dynamic process of bone tissue engineering is a challenging field in regenerative medicine. Current research is focused on the optimization and facilitation of bone regeneration by combining growth factors and mesenchymal stem cells with the many types of materials that have been studied as scaffolds. This review presents an overview of ideal scaffold properties and discusses the application of adipose-derived stem cells in bone tissue engineering and translational medicine.

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INTRODUCTION

Recent progress in the field of bone tissue engineering has led to new and exciting research concerning regenerative medicine. This interdisciplinary field is focused on the development of biological substitutes that restore,

maintain or improve tissue function by applying the principles of engineering and the life sciences^[1]. The primary target of clinical therapeutic strategies is the regeneration of bone for skeletal reconstruction of large bone defects created by trauma, infection, tumor resection and skeletal abnormalities, or cases in which the regenerative process is compromised, including avascular necrosis, atrophic non-union and osteoporosis. Strategies that stimulate bone healing to reduce or treat complications are becoming more important, due to the increase in life expectancy and ageing of the world population.

Autologous grafts represent the “ideal graft bone substitutes” and are currently the gold standard therapeutic strategy as they combine all essential components to induce bone growth and regeneration, including osteogenic cells, osteoinductive growth factors and bone-supporting matrix. Autografts are non-immunogenic and histocompatible, as they are the patient’s own tissue. Although they reduce the likelihood of immunoreaction and transmission of infection^[2], autografts are limited and commonly result in donor site morbidity as a result of the additional surgical harvesting procedures, and are accompanied by the risk of infection, hematoma and chronic pain, which can all lead to implant failure^[3-7]. An alternative approach involves the use of allogenic bone grafts obtained from human cadavers or living donors, which bypasses the complications associated with harvesting and quantity of graft materials. However, allogenic grafts are limited by tissue matching, disease transmission, batch variability and an inability to survive and integrate following implantation^[8-10].

The limited success of auto- and allografts in some clinical situations has stimulated the investigation of a wide variety of biomaterials to be used as scaffolds, and the development of promising clinical therapies^[11]. Advantages to utilizing sophisticated bone scaffolds include the elimination of the risk for disease transmission, fewer surgical procedures, and reduced risk of infection or immunogenicity. Moreover, there is an abundant availability of synthetic or natural biomaterials that can be employed, including collagen, hydroxyapatite (HA), β -tricalcium phosphate (β -TCP), calcium phosphate cements and glass ceramics. The concept of bone substitution involves the replacement of bone structure to allow the migration, proliferation and differentiation of bone cells and to promote vascularisation, thus utilizing the body’s natural biological response to tissue damage in conjunction with engineering principles. Current models of *in vitro* bone formation are based on the idea that the same factors known to play a role during embryonic development can be used to induce cellular differentiation and function in the process of regeneration^[12]. In order to engineer an environment supporting bone formation, combinations of biochemical and biophysical signals need to be presented to the cells in a three-dimensional setting in a way that allows interactions between the surrounding cells and the extracellular matrix. The complexity of signaling, with temporal and spatial gradients of

molecular and physical factors affecting bone morphogenesis, presents significant challenges to engineering fully viable, functional bone. This “diamond concept” has allowed the scientific community to consider more complex interactions between scaffolds, cells and growth factors in order to induce tissue regeneration in bone defects^[13]. This article presents a concise review regarding the main properties of scaffolds, the most recent progress in bone tissue engineering using human adipose-derived stem cells and current models used for bone regeneration.

PROPERTIES OF ENGINEERED BONE SCAFFOLDS

An ideal scaffold must address multiple physical and biological requirements in order to optimize bone regeneration. One of the most important stages of bone tissue engineering is the design and processing of a porous, biodegradable three-dimensional (3D) structure. This scaffold provides a structural and logistical template for developing tissue, which can markedly affect cell behavior. The properties of scaffolds that are important for bone formation include the size, distribution and shape of the pores, the surface roughness, the presence of cell attachment sites and the biomechanics of both the material and the scaffold structures^[14-17]. The most suitable scaffolds for bone formation are those made of osteoconductive materials, such as bone proteins and HA, with mechanical properties similar to those of load-bearing native bone that stimulate osteogenesis and have large and interconnected pores to facilitate cell infiltration and matrix deposition, and rough inner surfaces to promote cell attachment. Additionally, scaffolds should be anisotropic structures that can be fashioned into anatomically correct shapes that also have the capacity for vascularization. Scaffolds should also incorporate and control the delivery of bioactive molecules, such as growth factors or drugs that regulate cellular function, accelerating healing and preventing pathology^[18,19]. Furthermore, as scaffolds will be replaced over time by new formed bone, they should be comprised of resorbable materials, or materials that degrade in an enzymatic or hydrolytic way, such as polymers, or can be dissolved by cells such as osteoclasts^[20,21].

The majority of studies are currently focused on the development of 3D structures that mimic the anatomical and biochemical organization of cells and native matrix in order to achieve suitable mechanical properties for bone tissue^[22]. Numerous materials have been shown to support *in vitro* bone formation by human cells, including bioceramics like HA, β -TCP, bio-glasses and biodegradable polymers^[23,24], and natural or synthetic collagen, fibrin, chitosan or polyesters^[25,26]. Scaffolds containing composites of these materials provide an optimized and convenient alternative as they combine the advantages of both bioactive ceramics and biodegradable polymers^[27-31].

OSTEOINDUCTIVE BIOMOLECULES

One of the most challenging tasks for the development of bone graft substitutes is to produce scaffolds with osteoinductive properties, which can involve the application of biologically active molecules. Growth factors that naturally occur within a healthy bone matrix or are expressed during fracture healing can be used to direct the development of structures, vascularization and differentiation of bone cells^[19]. Growth factors, such as cytokines, are endogenous proteins that act on a wide variety of cells and direct their actions by binding to and activating cell-surface receptors. As developmental bone formation is an orchestrated cellular process tightly controlled by actions of growth factors, their use in engineered scaffolds is an obvious strategy when the bone integrity is compromised and bone tissue needs to be repaired^[52,33]. This strategy aims to enhance the local presence of bone-depositing osteoblasts, either by attracting the cells to the repair site or by inducing the proliferation of local undifferentiated precursor cells, followed by the transformation of precursor cells into an osteoblastic phenotype^[34].

The introduction of specific biomolecules has been shown in animal models to enhance the union of non-union type (a fracture that does not heal by itself after several months) bone fractures^[32]. Many growth factors that have been used in bone repair with some degree of success include mitogens such as platelet-derived growth factors, metabolic regulators such as insulin-like growth factors, angiogenic proteins such as basic fibroblast growth factors, and morphogens such as bone morphogenetic proteins (BMPs)^[35-39]. BMPs, which are members of the transforming growth factor beta (TGF- β) superfamily, have been the most extensively studied, as they are potent osteoinductive factors that induce the mitogenesis and differentiation of mesenchymal stem cells and other osteoprogenitors^[35,11]. They are a very promising candidate for the treatment of bone diseases and defects, as a number of experimental and clinical trials demonstrate their safety and efficacy^[40-42]. However, the clinical application of BMPs is currently limited to the use of BMP-2 for open tibial fractures and spinal fusion, and BMP-7 (OP-1) for non-unions with limited indication for spinal fusion^[43,44], which were approved by the U.S. Food and Drug Administration in 2004. The clinical and scientific utility of bone tissue engineering largely depends on the ability to create scaffolds with specific characteristics that predictably direct cells to differentiate into the right phenotypes in a spatially and temporally defined pattern guided by molecular and physical factors.

HUMAN ADIPOSE-DERIVED MSCS

The combination of engineered scaffolds with recent developments in the emerging field of stem cell science may allow the use of stem cells to repair tissue damage and, eventually, to replace organs. MSCs are non-hematopoietic cells of mesodermal derivation that are present

in a number of postnatal organs and connective tissues. The stroma of bone marrow contains bone marrow mesenchymal stem cells (BMSCs) capable of differentiating into osteogenic, chondrogenic, adipogenic and endothelial lineages^[45-48], and thus is the most well studied source of MSCs for bone regeneration. Bone marrow transplantation is also being used clinically in combination with osteoconductive materials to augment bone healing^[9].

In the last few years, MSCs have been isolated from other tissue sources including trabecular bone^[49], synovium^[50], umbilical cord^[51], periodontal ligament^[52] and other dental tissues^[53], skeletal muscle, cord blood and skin^[54-56]. Although the stem cell populations derived from these sources are valuable, common problems include limited amounts of available tissues and low numbers of harvested cells, which necessitate at least some degree of *ex vivo* expansion or further manipulation before preclinical or clinical use. In contrast, a promising population of MSCs has been identified within adipose tissue, termed adipose-derived stem/stromal cells (ASCs) by the regenerative medicine community during the Second Annual International Fat Applied Technology Society Meeting in 2004. Human adipose tissue is ubiquitous and can easily be obtained in large quantities with little donor site morbidity or patient discomfort^[45], in contrast to the invasive and painful procedure for isolating BMSCs. Moreover, stem cell yields are greater from adipose tissue than from other stem cells reservoirs, a significant factor for use in regenerative medicine. As many 1×10^7 ASCs can routinely be isolated from 300 mL of lipoaspirate, with greater than 95% purity. ASCs comprise 2% of nucleated cells in processed lipoaspirate, with a yield of 5000 fibroblast colony-forming units (CFU-F) per gram of adipose tissue, compared with estimates of about 100-1000 CFU-F per milliliter of bone marrow^[57,58]. In general, cell isolation protocols include density gradient centrifugation of the collagenase-digested tissue (lipoaspirate or minced adipose tissue)^[57-61], followed by the seeding of the pelleted stromal vascular fraction (SVF) on monolayer culture plastics. The adherent cell population can then be expanded and used in a variety of assays.

Although the study of human ASCs (hASCs) is emerging, the standardization of isolation and culture procedures could improve quality control and facilitate comparisons between different studies. There are discrepancies in the results of studies from different laboratories due to differences in the methods and quality of hASC isolation, which can affect the composition of the initial cell culture, as well as in the procedures used to culture the cells. Cell culture basal medium, generally containing 10% fetal bovine serum, is often supplemented with epidermal growth factor, fibroblast growth factor-2 and/or TGF- β ^[58,62,63]. In addition, some protocols may recommend differing initial cell seeding densities, though evidence suggests that low seeding densities and subconfluent passaging are recommended^[64,65]. Other variables that may affect the composition of the initial isolated cell culture cannot be standardized, such as donor age, gen-

der, body mass index, ethnicity and medical history^[66]. It is therefore important to standardize hASC isolation and culturing methods to maximize the reliability and reproducibility of results from different laboratories.

COMPOSITION AND CHARACTERIZATION OF CULTURED hASCs

The SVF that is obtained from processed adipose tissue contains a highly heterogeneous cell population, including non-adherent cell populations. A complete characterization of SVF cell populations was done by Yoshimura *et al.*^[64] in which they identified endothelial cells, pericytes, blood-derived cells, fibroblasts, vascular smooth muscle cells and preadipocytes, in addition to the potential hASCs. Although the adherence of hASCs allows for their selection from the SVF during subsequent tissue culture passages, other cell types, such as fibroblasts, can also adhere to the culture plastic. Thus, other cell types, or subpopulations, may compromise the proliferation and/or differentiation potential of hASCs.

To reduce the heterogeneity of cultured ASCs, a washing procedure in the beginning of the cell culture can be used, as various cell types adhere to the plastic at different time points^[66]. Additionally, flow cytometric sorting or immunomagnetic separation with specific cell surface markers can be used to isolate and purify specific subpopulations of hASCs. However, there is considerable heterogeneity in commonly analyzed hASC surface markers, which can be modified by the culturing procedure. The cell phenotype can also be influenced by differences in the cell purification procedure and by the number of passages^[66-70]. Mitchell *et al.*^[59] identified hematopoietic lineage cells from the SVF using flow cytometry based on their expression of CD1, CD14, CD45 and other markers, which were lost with progressive passages. The loss of these markers indicates that they do not represent the adherent population. Moreover, SVF cells exhibit low levels of classic stromal cell markers (CD13, CD29, CD44, CD73, CD90, CD105, CD166) in the earliest stages of isolation, and assume a more homogeneous profile with consistently high levels of stromal markers after four to five passages, a temporal expression pattern that resembles what has been reported in human BMSCs^[54]. Work from Rada *et al.*^[71] demonstrated the complexity of hASC populations by showing that they are composed of several subpopulations that express different levels of hASC markers and exhibit distinctive differentiation potentials. In their study, hASC subpopulations were isolated using immunomagnetic beads specific for CD29, CD44, CD49, CD73, CD90, CD105, p75 and STRO-1, and cultured with specific chondrogenic or osteogenic media in order to evaluate their differentiation potential into these lineages. Among all the hASC subpopulations isolated, STRO-1-containing populations had the highest osteogenic potential, with the highest chondrogenic differ-

entiation potential in populations expressing CD29 and CD105. These data clearly demonstrate that SVF from adipose tissue is comprised of several stem cell subpopulations that exhibit *in vitro* chondrogenic and osteogenic differentiation profiles. Therefore, these subpopulations should be studied in order to select those most suitable for application in bone and cartilage regenerative medicine.

APPLICATION OF hASCs AND SCAFFOLDS FOR BONE TISSUE ENGINEERING

Since the discovery of hASC osteogenic differentiation, substantial progress has been made toward the use of these cells as an optimal source for bone regeneration. Although initial applications involved the direct administration of stem cells into the target fracture site, current paradigms using scaffolds loaded with stem cells are preferred as they provide support for cell colonization, migration, growth and differentiation^[72]. Combined with the support of a scaffold, the directed osteogenesis of hASCs confirms that adipose tissue is a promising autologous source of osteoblastic cells for bone regeneration. Utilization of hASCs in scaffolds for bone tissue engineering has been heralded as the alternative strategy of the 21st century to replace or restore the function of traumatized, damaged or lost bone.

In the last ten years, several cell characterization studies have extensively described the differentiation potential and function of hASCs *in vitro*^[58,62,67,69]. Many types of materials have been used to confirm these positive hASC characteristics, which have become available for scaffold-assisted bone regeneration in a variety of tissue engineering strategies. The importance of the scaffold in hASC osteogenesis has been demonstrated in a number of studies that recommend the use of different materials, including ceramics^[73], titan alloys^[74,75], natural and synthetic polymers^[76,77], and natural or semi-synthetic grafts^[78,79], with variable porosity, roughness, and methods of fabrication for future regenerative applications. A clear trend has emerged toward the use of composite scaffolds due to their superior properties and structures^[80-82] derived from the combination of two or more materials^[83-87].

The study of hASCs for bone regeneration has largely involved the insertion of biomaterials in rat and nude mouse models^[88-92]. Furthermore, a femoral defect in nude rats is available and calvarial defect models have been described for other species, to demonstrate the application and optimization of hASCs in regenerative medicine^[93-97]. However, relatively few reports are available concerning the utilization of hASCs for human bone tissue regeneration (Table 1). The first compelling evidence supporting the clinical application of an hASC scaffold to promote fracture healing was reported by Lendeckel *et al.*^[98] in 2004. In this work, a combination of autologous hASCs obtained from the gluteal region

Table 1 Summary of current representative bone tissue engineering models combined with human adipose-derived stem/stromal cells

Scaffold origin	Type of scaffold	Active molecule	Study type	Differentiation pre-implant	Implant area	Species	Ref.
Synthetic	BCP	-	<i>In vitro</i>	Yes	-	-	[73]
Synthetic	Ti6Al4V	-	<i>In vitro</i>	Yes	-	-	[74]
Synthetic	Ti6Al4V	-	<i>In vitro</i>	Yes	-	-	[75]
Semi-synthetic	CMCA	Sr ²⁺	<i>In vitro</i>	Yes	-	-	[76]
Semi-synthetic	MPLA/CNC	-	<i>In vitro</i>	-	-	-	[77]
Semi-synthetic	Silk/fibroin	-	<i>In vitro</i>	Yes	-	-	[79]
Semi-synthetic	Apatite-coated CH/CS	rhBMP-2	<i>In vitro</i>	Yes	-	-	[80]
Synthetic	Bioactive glass	-	<i>In vitro</i>	Yes	-	-	[81]
Synthetic	PCL	-	<i>In vitro</i>	Yes	-	-	[82]
Synthetic	PLA/ β -TCP	-	<i>In vitro</i>	Yes	-	-	[83]
Synthetic	PLA/ β -TCP	-	<i>In vitro</i>	Yes	-	-	[84]
Synthetic	BCP	-	<i>In vitro/In vivo</i>	Yes	Femur	Rat	[86]
Semi-synthetic	Collagen/PCL	-	<i>In vitro</i>	Yes	-	-	[87]
Synthetic	PEG/PCL	-	<i>In vitro/In vivo</i>	-	Subcutaneous	Rat	[88]
Synthetic	HA	-	<i>In vitro/In vivo</i>	-	Subcutaneous	Rat	[89]
Synthetic	HA/ β -TCP	-	<i>In vitro/In vivo</i>	-	Subcutaneous	Mouse	[90]
Synthetic	PCL/ β -TCP	-	<i>In vivo</i>	-	Subcutaneous	Rat	[91]
Synthetic	PLA	-	<i>In vivo</i>	Yes	Palate	Rat	[92]
Synthetic	HA/ β -TCP	-	<i>In vivo</i>	-	Femur	Rat	[93]
Synthetic	Apatite-coated PLGA	rhBMP-2	<i>In vivo</i>	-	Calvaria	Mouse	[94]
Semi-synthetic	ABB/titanium	-	<i>In vivo</i>	-	Calvaria	Rabbit	[95]
Natural	Fibrin matrix	BMP-2	<i>In vivo</i>	-	Femur	Rat	[96]
Synthetic	Carbon nanotube	rhBMP-2	<i>In vitro/In vivo</i>	Yes	Subcutaneous	Mouse	[97]
Natural	Fibrin glue	-	<i>In vivo</i>	-	Calvaria	Human	[98]
Synthetic	β -TCP/titanium	rhBMP-2	<i>In vivo</i>	-	Maxilla	Human	[99]
Synthetic	β -TCP	rhBMP-2	<i>In vivo</i>	Yes	Mandibula	Human	[100]
Natural	ABB	PRP	<i>In vivo</i>	Yes	Maxilla/mandibula	Human	[101]
Synthetic	β -TCP/bioactive glass	rhBMP-2	<i>In vivo</i>	Yes	Craniofacial	Human	[103]

BCP: Biphasic calcium phosphate ceramics; Ti6Al4V: Titanium alloy; CMCA: Amidate carboxymethylcellulose; PLA: Poly(L-lactic acid); MPLA/CNC: Maleic anhydride grafted PLA/cellulose nanocrystals; CH/CS: Chitosan/chondroitin sulfate; PCL: Polycaprolactone; β -TCP: β -tricalcium phosphate; PEG: Polyethylene glycol; HA: Hydroxyapatite; PLGA: Poly(L-lactic acid-co-glycolic acid); ABB: Anorganic bovine bone; Sr²⁺: Strontium ion; rhBMP-2: Recombinant human bone morphogenetic protein; PRP: Platelet-rich plasma.

and bone grafts from the dorsal iliac crest was used for the treatment of a multi-fragment calvarial fracture in a 7-year-old girl. An autologous fibrin glue was applied using a spray adapter to keep the cells in place, and post-operative healing was uneventful after three months. In 2009, Mesimäki *et al.*^[99] described a novel method to reconstruct a major maxillary defect in an adult patient using autologous hASCs that were produced in clean room facilities free of animal-derived reagents, combined with recombinant human BMP-2 and β -TCP granules. The patient's healing was also clinically uneventful in this case, thus paving the way for extensive clinical trials using ASCs in custom-made implants for the reconstruction of bone defects. Moreover, the use of autologous cells, handled and prepared without animal-derived materials with good manufacturing practices in standard clean rooms, demonstrates that these cells can be considered safe for applications in tissue regeneration, according to the clinical cell therapy safety standards of the European Union.

Defects of the skull and jaws have been successfully reconstructed or their healing has been accelerated by the use of hASCs^[98-102], extending our limited knowledge regarding the potential use of hASCs for osseous tissue repair and regeneration. Work published in 2012 by Sándor demonstrates the synergistic effect of hASCs, resorbable

scaffolds (β -TCP and bioactive glass) and growth factors (BMP-2), in the treatment of 23 patients with craniofacial osseous defects^[103]. He has established the utility of hASCs in combination with biomaterials in 85% of the cases followed after bone reconstruction, though the long-term success of this procedure needs to be verified using a large sample.

CONCLUSION

The emerging application of hASCs on engineered scaffolds for bone tissue regeneration represents the most exciting challenge for the scientific community in future translational medicine. The ability to obtain a large quantity of MSCs from easily accessible adipose tissue, combined with the growing research on new biomaterials incorporating bioactive molecules such as drugs and growth factors, opens the way to new therapeutic applications. Although clinical trials have demonstrated the use of hASCs for the reconstruction of craniofacial defects in humans, there are many aspects that need to be examined and resolved. Further investigations are needed to standardize procedures for harvesting, isolating, cultivating and preparing hASCs for clinical applications. The differences in currently applied techniques make

comparisons across studies difficult. Moreover, the lack of guidelines for the proper utilization of different bone scaffold materials may provoke safety concerns, impeding clinical trials and the translation of scaffold technologies to the clinical environment. Prospective randomized clinical trials are needed to identify clear indications for and to demonstrate clinical outcomes of the hASC therapies. Ethical and safety concerns must be resolved to prevent human testing as the first stage in novel scaffold development.

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WCSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Purinergic receptors and nucleotide processing ectoenzymes: Their roles in regulating mesenchymal stem cell functions

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Abstract

Human mesenchymal stem cells (MSCs) are a rare population of non-hematopoietic stem cells with multi-lineage potential, originally identified in the bone marrow. Due to the lack of a single specific marker, MSCs can be recognized and isolated by a series of features such as plastic adherence, a panel of surface markers, the clonogenic and the differentiation abilities. The recognized role of MSCs in the regulation of hemopoiesis, in cell-degeneration protection and in the homeostasis of mesodermal tissues through their differentiation properties, justifies the current interest in identifying the biochemical signals produced by MSCs and their active crosstalk in tissue environments. Only recently have extracellular nucleotides (eNTPs) and their metabolites been included among the molecular signals produced by MSCs. These molecules are active on both ionotropic and metabotropic receptors present in most cell types. MSCs possess a significant display of these receptors and of nucleotide processing ectoenzymes on their plasma membrane. Thus, from their niche, MSCs give a significant contribution to the complex signaling network of eNTPs and its derivatives. Recent studies have demonstrated the multifaceted aspects of eNTP metabolism and their signal transduction in MSCs and

revealed important roles in specifying differentiation lineages and modulating MSC physiology and communication with other cells. This review discusses the roles of eNTPs, their receptors and ectoenzymes, and the relevance of the signaling network and MSC functions, and also focuses on the importance of this emerging area of interest for future MSC-based cell therapies.

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Key words: Mesenchymal stem cell; Purinergic receptors; Ectoenzymes; ATP; β -NAD; Adenosine; cADPR

Core tip: The multifaceted aspects of extracellular nucleotide metabolism (mainly ATP and β -NAD) on mesenchymal stem cell (MSC) surface has been addressed by basic researchers only recently, sometimes revealing unexpected pivotal roles for these molecules in specifying differentiation lineages and modulating MSC physiology and communication with other cells. This review discusses the roles of extracellular nucleotides, their receptors and ectoenzymes, and the relevance of their signaling network and MSC functions, and also focuses on the importance of this emerging area of interest for future MSC-based cell therapies.

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INTRODUCTION

Human mesenchymal stem cells (MSCs, also known as marrow stromal cells) are a rare population of non-he-

matopoietic stem cells with multilineage potential originally identified in the bone marrow (BM)^[1,2]. BM-derived MSCs (BM-MSCs) are still considered the gold standard for MSC applications; nevertheless, the BM has several limitations as a source of MSCs, such as low frequency in this compartment, a painful isolation procedure and the loss of differentiation potential with donor's increasing age. Thus, there is growing interest in identifying alternative sources for MSCs. To this end, MSCs obtained from the adipose tissue^[3], dental pulp^[4], placenta and Wharton's jelly^[5] have gained much attention in recent times since they can be easily isolated from tissues without any ethical concerns and which would be otherwise discarded.

Due to the lack of a single specific marker, MSCs can be recognized and isolated by a series of features such as plastic adherence, a panel of surface markers, the clonogenic and differentiation abilities^[2,6,7]. They can be expanded *in vitro* for several passages without losing their lineage properties and are commonly considered the precursors of mesodermal cell types such as osteocytes, adipocytes and chondrocytes. Whether MSCs can differentiate to non-mesodermal cell types such as hepatocytes or neurons is still under debate^[8-10].

In the BM, MSCs play a key role in providing hemopoietic progenitors (HPs) with soluble factors essential to their proliferation and differentiation^[11]. Furthermore, MSCs possess immunoregulatory functions^[12]. Actually, a number of clinical trials are currently exploring the use of MSCs in cell-based therapies of various pathological conditions, such as graft *vs* host disease, renal, neurological and cardiovascular diseases^[13,14]. The clinical benefit of MSC-based cell therapy seems mostly related to MSC-derived soluble factors possessing immunomodulating, growth-supporting and/or antiapoptotic activities, as demonstrated on animal models^[12]. Furthermore, their differentiation and tissue regeneration potential have already been used in therapeutic clinical approaches involving tissue engineering and gene therapy^[15,16].

In vitro differentiation of MSCs requires the activation of specific transcription factors, regulatory genes and signal cascades^[17,18]. Adipogenesis induction gives rise to preadipocytes with cytoplasmic accumulation of lipid droplets and release of adipokines and extracellular matrix-associated proteins^[19]. On the other hand, osteogenesis-induced osteoblasts secrete mineralized extracellular matrix, with high levels of calcium phosphate forming hydroxyapatite crystals^[20]. Since both osteoblasts and adipocytes originate from a common MSC precursor, it seems obvious that osteoblast and adipocyte differentiation pathways are regulated jointly^[21].

Although a plethora of studies^[22-24] have shown that many substances, as well as mechanical agents, are causally related to these differentiation processes, the mechanisms involved are not yet completely defined. However, a large body of evidence supports the idea that there is an inverse relationship between the differentiation of MSCs to osteoblasts or to adipocytes, *i.e.*, conditions favoring

the differentiation towards one lineage impair the differentiation to the other lineage. This seems to occur during attainment of peak bone mass^[25,26] for instance, when adipogenesis in the BM is inhibited, favoring osteogenesis, or in aging population^[27], when the BM adipocytes are predominant in respect to other cells of mesodermal origin.

MSCs regulate their fate through the complex integration of autocrine and paracrine extracellular signals (*i.e.*, hormones, cytokines, nucleotides, xenobiotics) enabling the cells to sense the external milieu and to establish a fine communication with the surrounding cell population. Hence, they calibrate their response (differentiation, immunomodulation, proliferation, migration) on the basis of the necessities of the tissue in which they reside or on the organism's physiopathological conditions.

From an evolutionary point of view, nucleotides are considered among the most ancient molecules with biological activity and they are in fact used by living organisms for many different purposes: energy metabolism, storage of genetic information, signal transduction and extracellular communication. Nucleotides can be released or leaked into the extracellular milieu by virtually every cell in the body. Extracellular nucleotides (eNTPs) comprise both extracellular purines (ATP, ADP, β -NAD, ADPR and cADPR) and extracellular pyrimidines (UTP and UDP). Once outside the cell, they either serve as signaling molecules by binding specific P2 purinergic receptors (P2X or P2Y) or are converted into other active nucleotides^[28] and finally degraded to the related nucleosides. Nucleosides, mainly adenosine, can then bind different types of P1 purinergic receptors^[29]. Nucleotide extracellular metabolism is mediated by special proteins located on the outer surface of the plasma membrane that possess an enzymatic domain in the extracellular region, called ectoenzymes^[30]. Currently, there is an accumulating body of evidence indicating that the various ectoenzymes work in concert to dismantle eNTPs. Thus, in whatsoever milieu, the balance between nucleotides and nucleosides relies on the direct outflow of such molecules from transporters and channels in the plasma membrane^[31-33], as well as on the activity of the specific ectoenzymes present on the cell surface.

It is now well established that eNTPs mediate intercellular communication in virtually all tissues. They are one of the most important indicators of cell stress in the pericellular environment^[34] and the network of extracellular nucleotides/nucleosides serves multiple functions in a balanced and finely tuned fashion^[35-37].

MSCs possess a significant display of purinergic receptors and ectoenzymes on their plasma membrane^[38-40] and these cells have been reported to actively release nucleotides such as ATP and β -NAD upon certain stimuli^[39-42] (Figure 1). Thus, from their niche, these cell types give a significant contribution to the complex network of signaling involving eNTPs and its derivatives, and accumulating literature indicates that MSC functions are also autocrinally influenced by eNTPs affecting their differ-

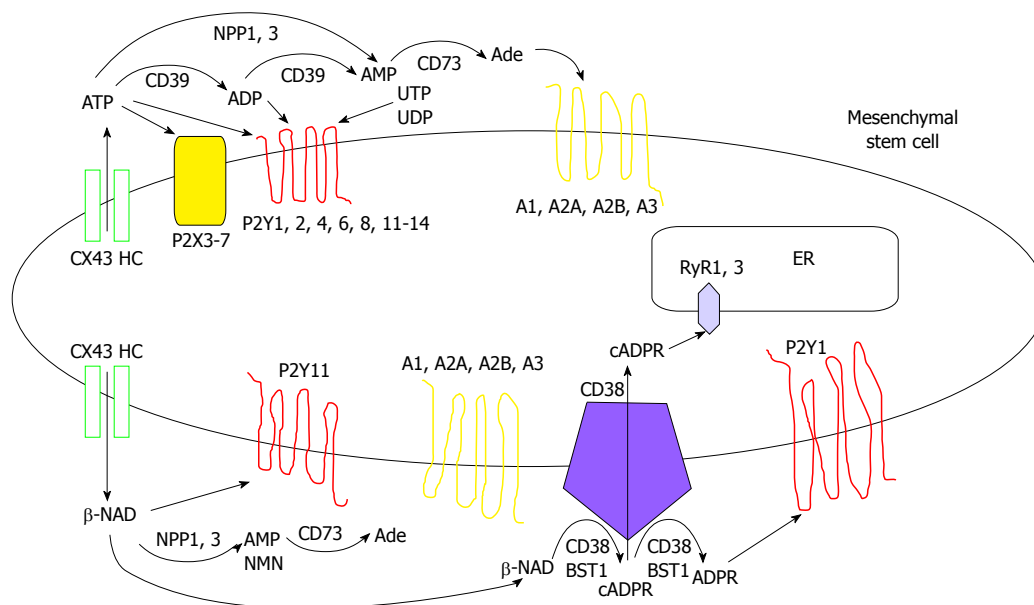


Figure 1 Surface network of purinergic receptors and nucleotide ectoenzymes on mesenchymal stem cells. On the basis of the recent findings, all the purinergic receptors and ectoenzymes whose presence has been ascertained on mesenchymal stem cells through qPCR analyses and/or demonstration of a clear physiological function (see text for references) are shown. Furthermore, both ATP and β -NAD stimulation mechanisms and metabolisms are summarized as an example of the finely tuned extracellular balance between nucleotides and nucleosides and their pleiotropic effects. CX43 HC: CX43 hemichannels; Ade: Adenosine; NMP: Nicotinamide monophosphate; ER: Endoplasmic reticulum; RyR1,3: Ryanodine receptors 1 and 3.

entiation properties as well as their immunomodulatory activity.

Here, the role of eNTPs, its receptors and converting ectoenzymes and the relevance of this signaling network in MSC functions are discussed, also focusing on the importance of this emerging area of interest for future MSC-based cell therapies.

P1 RECEPTORS IN MSC

Purinergic receptors (PRs) are plasma membrane receptors specific for adenosine, purine and pyrimidine nucleotides, which are expressed throughout the mammalian organism in all cell types. Upon their physiological agonist, Ps can be classified into P1 receptors, whose natural ligand is adenosine, and P2 receptors, whose recognized natural ligands are nucleotides (mainly ATP and UTP, see Figure 1)^[29]. The adenosine receptors are G protein-coupled seven-transmembrane proteins, further classified into the A₁R, A_{2A}R, A_{2B}R and A₃R subtypes^[29]. In particular, the P1 signaling pathway involves cyclic adenosine monophosphate (cAMP) synthesis upon A_{2A}R and A_{2B}R activation, or cAMP inhibition upon A₁R and A₃R activation^[29].

Adenosine can be directly released by cells^[31,32] or generated by the dephosphorylation of adenine nucleotides, which in many tissues are dephosphorylated to AMP by the ectonucleoside triphosphate phosphohydrolase (CD39). AMP is then further dephosphorylated to adenosine by ecto-5'-nucleotidase (CD73)^[30]. The resulting adenosine has an essential role in the attenuation of inflammation and in damaged tissue healing. Furthermore, it mediates diverse cardioprotective, neuroprotective, vasodilatory and angiogenic responses^[43-46], in many

cases counteracting the ATP inflammatory/stress signal triggered by P2 purinergic receptor activation.

Several studies in the last decade have established the presence of both P1 and P2 receptor family members on MSC surface (Figure 1), trying to elucidate their role in the homeostasis and differentiation properties of this cell type both *in vitro* and *in vivo*.

Adenosine receptor presence and function on MSC surface was first evidenced by Evans and coworkers^[47], demonstrating the formation of extracellular adenosine by an osteoprogenitor cell line and by MSCs for the first time. On that occasion, the presence of all four adenosine receptor subtypes, especially A_{2B}R, was ascertained, demonstrating a causal role of their activation in active secretion of the inflammatory cytokine IL-6 and of the osteoclastogenesis inhibitory factor osteoprotegerin. These data indicate that adenosine production, as well as its activity through adenosine receptors, could be a potential target for pharmacological interventions in the bone for many diseases, including osteoporosis^[48].

A further study^[49] demonstrated that adenosine signaling affects proliferation and development of BM-MSCs. Perhaps the most significant finding of this work is the demonstration that adenosine A_{2A}R deletion or blockade diminishes the number of colony-forming unit-fibroblasts (CFU-F) in cultured BM-MSCs. Thus, the authors speculated that adenosine, targeting the A_{2A}R, could increase the proliferation of MSCs, as also reported for other cell types^[50,51]. Alternatively, they suggest that since A_{2A}R stimulation has been shown to diminish apoptosis in other cell types^[52,53], an increased survival of MSCs could enhance CFU-F yield from freshly isolated adult stem cells. Interestingly, they confirmed that A_{2A}R and

CD73 are coordinately regulated in MSCs as in other cell types^[54], strengthening the idea of an active crosstalk in adenosine signaling between the adenosine receptor and the ectoenzymes able to generate the nucleoside in the pericellular space.

More recently, both *in vitro*^[55] and *in vivo*^[56,57] studies have evaluated the contribution of adenosine signaling in MSC differentiation. Gharibi *et al*^[55] in particular investigated the *in vitro* expression of adenosine receptor subtypes and the adenosine metabolism as they differentiated MSCs into osteoblasts or adipocytes. They found differential expression of the adenosine receptor subtypes during differentiation as well as in mature cells. Differential expression was related both to the progression of lineage specificity (A_{2B}R dominant in osteoblast differentiation; A₁R and A_{2A}R in adipogenic differentiation) and to the maintenance of specialized features in the two lineages (A_{2A}R essential to ALP expression in osteoblasts; A₁R involved in lipogenic activity in adipocytes).

These data suggest that useful strategies could include the targeting of the adenosine signaling pathway in cases of diseases associated with an imbalance in the differentiation and function of these two lineages. This research will be useful in preventing or treating conditions with insufficient bone or excessive adipocyte formation^[25-27].

Finally, an essential role of adenosine signaling through A_{2B}R in *in vivo* osteoblast differentiation and bone formation seems to be definitely confirmed in recent reports^[56,57]. Both studies suggest that the pharmacological stimulation of this signaling pathway may enhance bone density and bone fracture healing in variously compromised situations, such as non-healing fractures in osteoporosis^[56] and osteolytic bone lesions in multiple myeloma^[57]. In general, all the above-mentioned studies confirm an essential, functional role of extracellular adenosine and its signaling pathway in MSC physiology, homeostasis and intervention in bone and adipose tissue reconstitution, allowing the identification of new pharmacological targets.

P2 RECEPTORS IN MSC

Extracellular nucleotides have been definitely recognized as autocrine/paracrine signaling molecules^[58] released from cells in response to physiological and pathological stimulation, such as mechanical stress, hypoxia, inflammation and other agonists. The mechanisms of nucleotide release comprise exocytosis, ATP-binding cassette transporters, connexin hemichannels and voltage-dependent anion channels^[33]. Many signaling roles for nucleotides have been demonstrated in several tissues, including: neurotransmission^[33]; rhythm regulation in the myocardium^[59]; gastrointestinal and liver function^[60]; regulation of epithelial cell responses^[61]; blood flow distribution, oxygen delivery and endothelial barrier integrity^[62,63]; immune responses^[43,64]; and activation of platelets at sites of vascular injury^[65]. Besides acute signaling events, there is increasing evidence that purines and pyrimidines also

have potent long-term roles in cell proliferation and growth^[34], induction of apoptosis and anticancer activity^[43] and atherosclerotic plaque formation^[66]. These effects are mediated by extracellular stimulation of P2 purinergic receptors, of which two major subfamilies, P2X and P2Y, have been described. The ionotropic P2X receptors are ligand-gated channels that gate extracellular cations in response to ATP and comprise seven receptor subtypes (P2X₁-P2X₇)^[29]. Conversely, the metabotropic P2Y receptors are G-protein-coupled proteins that alternatively couple to G_q (P2Y₁₋₂, P2Y₄, P2Y₆ and P2Y₁₁) and therefore activate phospholipase C-β, or to G_i (P2Y₁₂₋₁₄), that inhibit adenylyl cyclase and regulate ion channels^[29]. Notably, P2Y₁₁ receptor is dually coupled to phospholipase C and adenylyl cyclase stimulation.

P2Y receptors can be divided into: (1) adenine nucleotide-preferring receptors, mainly responding to ATP and ADP (P2Y₁, P2Y₁₁₋₁₃); (2) uracil nucleotide-preferring receptors (P2Y₄ and P2Y₆) responding to both UTP and UDP; (3) receptors of mixed selectivity (P2Y₂); and (4) nucleotide sugar-preferring P2Y₁₄ receptor responding to UDP-glucose and UDP-galactose^[29]. Finally, the P2Y₁ and P2Y₁₁ receptors have also been described as β-NAD receptors with diverse functional activities^[64,67,68]. In particular, P2Y₁ is also a receptor for ADPR, a β-NAD metabolite generated by the cycling/hydrolyzing activity of CD38 and BST1/CD157 ectoenzymes^[36,68].

P2 receptors and the related activating nucleotides have been the object of investigation in relation to MSC functions (Figure 1) only recently. In earlier reports^[41,42], the spontaneous release of ATP from MSCs *via* gap junction hemichannels was assessed, on one occasion demonstrating a direct stimulation of P2Y₁ receptor triggering intracellular Ca²⁺ oscillations^[41], while showing the concurrent activation of P2X and P2Y receptors by ATP in another, resulting in a modulation of the proliferation rate at early passages of MSC cultivation^[42].

The presence of the G-protein coupled P2Y₂ receptor has also recently been demonstrated on rat MSCs, as well as its activation by the preferred agonist UTP inducing intracellular Ca²⁺ oscillations or elevating Ca²⁺ levels depending on cell density, and suggesting that these different Ca²⁺ responses in MSCs may be correlated with cell cycle progression^[69].

More recently, different investigations have been directed to the pleiotropic effects of P2 receptor activation by ATP, focusing on MSC functionality in the hematopoietic niche and on the differentiation properties of these cells^[70-73]. In a recent paper analyzing the effects of ATP on MSC functions, Ferrari and collaborators^[70] observed a downregulation of genes related to cell proliferation and anti-inflammatory cytokines and concurrently an upregulation of pro-inflammatory cytokines and cell migration related genes. These data confirm the *in vitro* inhibitory activity of ATP on MSC proliferation, as already observed in a previous work^[42], and demonstrate an *in vivo* potentiated homing capacity to the BM of ATP-pretreated MSCs that could be useful in supporting thera-

pies for BM engraftment.

The role of ATP during MSC differentiation has also been addressed in the last years^[38,71-73]. The related studies indicate that: (1) a variety of metabolically active P2X (P2X3-7) and P2Y (all subtypes) receptors are detectable in MSCs (Figure 1) and are up- or downregulated during adipogenic and osteogenic differentiation. In particular, P2Y₄ and P2Y₁₄ seem to be important for the onset of MSC commitment (regulated both in adipogenic and in osteogenic differentiation), P2Y₁ and P2Y₂ are downregulated in osteogenic differentiation, while P2Y₁₁ is significantly upregulated in adipogenic commitment^[38]; (2) significant ATP release by MSCs, especially observed during shockwave treatment, is able to promote osteogenic differentiation through P2X₇ receptor activation with a significant positive impact in bone healing^[71]; and (3) ATP treatment modulates the expression of several genes governing adipogenic and osteogenic differentiation of MSCs which can be tuned from one lineage to the other by specific culture conditions in the presence of this nucleotide^[72]. In addition, evidence from Ciciarello and coworkers^[72] seems to indicate that ATP is able to promote adipogenesis through its triphosphate form, while osteogenic differentiation seems to be induced by its nucleoside adenosine, as also proposed by others^[55-57], resulting from ATP degradation by the CD39/CD73 system or directly released by cells. Thus, based on these findings, it is proposed that adipogenic differentiation is mainly mediated by activation of P2Y₁ and P2Y₄ receptors, while stimulation of the adenosine receptor subtype A_{2B}R is involved in osteogenic differentiation. In another recent investigation, P2Y₁₃ receptor has been implicated in *in vivo* osteogenic differentiation through the study of impaired bone turnover in a P2Y₁₃-KO mouse model^[73]. In this study, P2Y₁₃ activation and consequent osteogenic induction, at the expenses of adipocyte differentiation, seems to be orchestrated by ADP stimulation and not ATP, thus complicating the picture of nucleotide involvement in the MSC differentiation process.

Together, all these data provide new insights into the molecular regulation of MSC differentiation and demonstrate the necessity to further deepen this topic of investigation in order to better understand the pleiotropic effects of ATP and its derivatives on MSC differentiating abilities and to finally merge current, sometimes contrasting, observations.

Besides ATP and its derivatives, the dinucleotide β -NAD has also been shown to activate P2 receptors (P2Y₁ and P2Y₁₁), its effects mainly investigated in cell types of the immune system and in neuromuscular transmission^[64,67,68]. Interestingly, it has been recently demonstrated that this nucleotide also has a significant impact on MSC functions^[39]. In particular, β -NAD can be released in the extracellular milieu upon stimuli able to open CX₄₃ hemichannels in MSCs (*i.e.*, low extracellular calcium, shear stress, inflammatory stimuli) and this release is functional to increase MSC proliferation, migration and production of immunomodulatory cyto-

kines without compromising the differentiation abilities of these cells. Such effects are observable in MSCs in the presence of β -NAD, both extracellularly added or autocrinally released, and are dependent on P2Y₁₁ activation (Figure 1). Thus, as for adenosine and its preferential receptors, β -NAD through its specific P2Y₁₁ target can also exert a beneficial role in modulating cell protective functions relevant to MSC-based cell therapies.

NUCLEOTIDE-DEGRADING

ECTOENZYMES IN MSC

Ectoenzymes are a family of cell surface molecules whose catalytic domain lies in the extracellular region. A subset of this family, the nucleotide-metabolizing ectoenzymes, are key components in the regulation of the extracellular balance between nucleotides and nucleosides, together with equilibrative transporters and channels enabling direct outflow of these molecules^[31-33].

Following the signal transduction, eNTPs need to be rapidly inactivated, mainly to adenosine which in turn has other pharmacological/counteracting properties. Nucleotide hydrolyzing enzymes include the nucleoside triphosphate diphosphohydrolase (NTPDase) family^[74], the nucleotide pyrophosphatase/phosphodiesterase (NPP) family^[75,76] and ecto-5'-nucleotidase^[77].

NTPDases are capable of hydrolyzing a broad range of nucleoside tri and diphosphates, but not monophosphates. Namely, half of the eight different NTPDase genes (NTPDase1, 2, 3 and 8) are expressed as cell surface-located enzymes. The prototypic member of the NTPDase family is the cell activation antigen CD39 (NTPDase1)^[78] whose expression has been demonstrated on a variety of cells, vascular endothelial and smooth muscle cells^[79], exocrine pancreas^[80], dendritic cells^[81], lymphocytes^[82] and recently MSCs^[40] (Figure 1). On the other hand, The NPP family consists of seven related ectoenzymes possessing surprisingly broad substrate specificity capable of hydrolyzing pyrophosphate and phosphodiester bonds generating, for instance, AMP from ATP, or AMP and NMN (nicotinamide monophosphate) from β -NAD^[83]. The first three members of this family, NPP1-3, hydrolyze various nucleotides and are therefore relevant in the purinergic signaling cascade^[75]. In particular, human NPP1 is highly expressed in bone and cartilage and less in other organs and tissues^[75]. In bone tissue, NPP1 acts as a PPi-generating ectoenzyme ensuring normal bone matrix mineralization and soft tissue calcification^[84]. The presence and enzymatic activity of NPP1 and NPP3 has been recently demonstrated in MSCs^[39] (Figure 1), attesting to the existence of an active and complex extracellular nucleotide metabolism in these cells once more.

Extracellular AMP, generated either from ATP or from β -NAD degradation, can be further metabolized by the ecto-5'-nucleotidase CD73 releasing adenosine^[77]. CD73 is expressed to a variable extent in different tissues, with abundant expression in the colon, kidney, brain,

liver, heart, lung and large vessel endothelium^[77,85,86]. Notably, CD73 is coexpressed with CD39 on the surface of CD4⁺ T_{reg} cells, being an important constituent of the suppressive machinery that converts ATP to the anti-inflammatory mediator adenosine with subsequent inhibition of T cell proliferation and cytokine secretion^[82]. Interestingly, this situation closely resembles that of MSCs whose immunomodulatory activity has also been recently related to the CD39/CD73 enzymatic axis actively producing extracellular adenosine, also with paracrine/immunosuppressive effects in these cells^[40] (Figure 1). These data may indicate a key role of adenosine in switching the stem cell properties of MSCs towards an immunomodulatory/pro-healing phenotype which in so many occasions has demonstrated its utility^[14], suggesting a possible pharmacological use of adenosine in potentiating these features in cell-based therapies.

Although CD73 is one major cell surface marker defining MSCs according to the International Society for Cellular Therapy (ISCT), it is surprising how little is known about the enzymatic function of CD73 in these cells^[87]. Notably, CD73 expression is regulated by Wnt- β -catenin signaling, one of the major pathways in stem cell and bone homeostasis^[88]. Recently, CD73 has been reported to be involved in osteogenic differentiation where loss of this ectoenzyme causes a lower bone mineral content in mouse trabecular bone with decreased osteocalcin serum levels and reduced expression of osteogenic mRNA markers^[89]. Little is known about the role of CD73 in chondrogenesis, except that CD73 is downregulated during differentiation^[90,91]. In a recent investigation, further insights into CD73 in relation to osteogenic/chondrogenic differentiation have been added to the literature^[92] using an *in vitro* model of MSCs differentiated after cyclic-compressive loading. In these conditions, Ode *et al.*^[92] observed increased chondrogenic differentiation accompanied by a decreased CD73 expression; in addition to that, they found that inhibition of CD73 enzymatic activity further increased chondrogenic matrix deposition. In contrast, in the same experimental setting but in conditions of osteogenic induction and in the presence of a CD73 inhibitor, MSCs showed a reduction of osteogenic marker expression and of mineral matrix deposition, suggesting that CD73 and its metabolite adenosine, as well as P1 receptors, belong to alternative differentiation pathways in MSCs whose expression enhance (osteogenic) or inhibit (chondrogenic) specific cell lineages. So far, and to our knowledge, no investigations have been undertaken to test the role of CD73 as an ectoenzyme during adipocyte differentiation in MSCs. Since it is known that this protein is expressed on mature adipocytes and that CD73-derived adenosine is functionally involved in body fat homeostasis, mainly inhibiting lipolysis^[93], it is highly probable that this topic will be eventually addressed in the near future, hopefully adding new bricks to the comprehension of adipose tissue formation mechanism and complex homeostasis.

Another well-known class of ectoenzymes are β -NAD-

consuming surface proteins, primarily represented by the CD38-BST1 system^[36]. The CD38 gene codes for a type II transmembrane protein distributed in a broad range of cell types^[36]. The other member of the family is BST1/CD157, which differs in structure and tissue distribution^[36]. The dual cycling/hydrolyzing metabolism of β -NAD by CD38 leads to the generation of potent intracellular Ca²⁺ mobilizing compounds, including cADPR (from cycling activity) and ADPR (from both cycling and hydrolyzing activities)^[94].

It has been recently demonstrated that MSCs show both a significant β -NAD release from CX43 hemichannels and an active extracellular metabolism of this dinucleotide due not only to NPP1/3 and CD73 degradation to adenosine, but also to CD38-BST1 secondary metabolite production^[39] (Figure 1). The release of β -NAD in the BM milieu from MSCs is essential not only for autocrine physiological and immunomodulatory functions^[39], but also for HP proliferation and stem cell niche maintenance^[95-97]. Thus, the bilateral nucleotide network generated upon β -NAD release from MSCs in the BM comprises the following enzymatic steps and functional effects: (1) β -NAD released in the BM milieu directly stimulates MSC and HP functions through the purinergic receptor P2Y₁₁^[39,98]; (2) extracellular β -NAD can be a substrate of various ectoenzymes present either on MSCs, possessing both NPP-CD73 and CD38-BST1 ectoenzymes, or on HP displaying the CD38 activity^[39,99-101]; and (3) these enzymatic activities are able to release secondary metabolites in the BM milieu, namely adenosine, ADPR and cADPR, which again can exert autocrine and paracrine regulatory effects on MSCs and HPs^[28,39,99-102]. Indeed, nanomolar/low micromolar concentrations of cADPR, such as those produced by variously stimulated CD38-BST1 positive BM cells^[99,100], significantly increase the *in vitro*^[99-102] and *in vivo*^[96,103] proliferation and engraftment of human HPs and MSCs, indicating a relevant role for this network of nucleotide-responding and nucleotide-metabolizing proteins in the BM.

CONCLUSION

The increasingly recognized role of MSCs in the homeostasis of mesodermal tissues through their proliferation/differentiation properties and in the regulation of hemopoiesis and cell-degeneration protection through the production of paracrine signals justifies the current interest in identifying the biochemical signals produced by MSCs and their active crosstalk in tissue environments. Only recently, such signals have been shown to also belong to the network of eNTPs and their metabolites produced by specialized ectoenzymes^[39,40,87,89-92,99] and active on both ionotropic^[41,71] and metabotropic receptors^[38,39,42,69,70,72,73] in MSCs (Figure 1). Researchers have just begun to uncover the multifaceted aspects of the eNTP network on MSCs, sometimes revealing unexpected pivotal roles for these molecules and their derivatives in specifying differentiation lineages and in modulating MSC physiology and

signaling towards other cells.

Thus, while extracellular β -NAD and cADPR signaling seem to be more related to MSC homeostasis/proliferation and to the maintenance of an optimal stem cell niche for the harmonious growth of HPs and MSCs in the BM^[39,95-97,99-103], ATP and adenosine demonstrate more pleiotropic roles affecting both the immunomodulatory properties of these cells and their lineage commitment. In particular, the nucleotide has been more frequently associated with inhibition of proliferation^[42,69], pro-inflammatory and cell migration properties^[70], as well as to an enhancement of both adipogenic and osteogenic differentiation^[38,71-73] in MSCs. Conversely, adenosine has been associated with an autocrine protective^[49] as well as a paracrine immunosuppressive^[40] activity counteracting ATP stimulation. Furthermore, in MSCs, adenosine seems to have a significant role in alternative lineage specification by concomitant promotion of bone formation^[55-57,72,90-92] and inhibition of cartilage production^[92]. In agreement with this, it has been suggested that the positive effect of ATP on osteocyte differentiation could be just a consequence of adenosine production on MSCs through surface activity of degrading ectoenzymes^[72].

The prosecution of these studies, on the basis of what has been discovered until now and is summarized in this review, seems to be essential for a thorough comprehension of MSC physiology and in the future will enable researchers to precisely define the involvement of these cells in tissue repair and to finally address the current clinical issues related to their use in cell-based therapies.

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WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Mesenchymal stem cells help pancreatic islet transplantation to control type 1 diabetes

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Abstract

Islet cell transplantation has therapeutic potential to treat type 1 diabetes, which is characterized by autoimmune destruction of insulin-producing pancreatic islet β cells. It represents a minimal invasive approach for β cell replacement, but long-term blood control is still largely unachievable. This phenomenon can be attributed to the lack of islet vasculature and hypoxic environment in the immediate post-transplantation period that contributes to the acute loss of islets by ischemia. Moreover, graft failures continue to occur because of immunological rejection, despite the use of potent immunosuppressive agents. Mesenchymal stem cells (MSCs) have the potential to enhance islet transplantation by suppressing inflammatory damage and immune mediated rejection. In this review we discuss the impact of MSCs on islet transplantation and focus on the potential role of MSCs in protecting islet grafts from early graft failure and from autoimmune attack.

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Key words: Mesenchymal stem cell; Islet transplantation; Type 1 diabetes; Vascularization; Immune modulation

Core tip: Type 1 diabetes is caused by a cell-mediated autoimmune destruction of pancreatic β cells. The transplantation of pancreatic islets provides a cure for this disorder. In this review, we first summarize the current knowledge on the pathogenesis of type 1 diabetes and on the therapeutic options for this disorder. Next we discuss the impact of mesenchymal stem cells on vascularization and immunomodulation of islet transplantation.

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INTRODUCTION

Type 1 diabetes results from the autoimmune destruction of insulin-producing pancreatic islet β cells and is usually diagnosed in children and young adults. β cell replacement therapies using either pancreas or islet transplantation represent a therapeutic alternative to the administration of exogenous insulin.

Islet transplantation is advantageous compared with whole pancreas transplantation because it is relatively non-invasive. However, a decline in islet cell survival, after transplantation, remains a significant obstacle in successful islet transplantation. It has been suggested that the complete lack of islet vasculature and hypoxic environment in the immediate post-transplantation period contribute to the acute loss of islet by ischemia^[1].

Moreover, graft failure continues to occur because of immunological rejection, despite the use of potent immunosuppressive agents.

Mesenchymal stem cells (MSCs) are non-hematopoietic multipotent stromal cells that can differentiate in a variety of tissues^[2]. The ability of MSCs to secrete trophic and angiogenic factors may help to prevent early islet damage and assist islet engraftment. MSCs may also attenuate autoimmunity through their immunomodulatory properties while secreting cytokines to control autoreactive T cells. All these properties could be used for *in vivo* co-transplantation to improve islet engraftment^[3]. Here we discuss the impact of MSCs on islet transplantation from both early graft failure and from autoimmune attack, to prevent immune rejection and promote long-term islet allograft survival.

PATHOGENESIS OF TYPE 1 DIABETES

Type 1 diabetes is a fast-growing global problem with enormous social, health, and economic consequences. This metabolic disorder is characterized by the irreversible destruction of insulin-secreting β cells. Death of the pancreatic β cells is associated with hyperglycaemia, which is the main determinant of long-term complications in diabetic patients. Exogenous insulin administration is required to control glucose levels in the blood. The pancreatic islets are the targets of an autoimmune assault, where islets are invaded by mononuclear cells that cause an inflammatory reaction called “insulinitis”, leading to loss of most of β cells. β cell death in the course of insulinitis is probably caused by direct contact with activated macrophages and T-cells, and/or exposure to soluble mediators secreted by these cells, as cytokines, nitric oxide (NO), and oxygen free radicals^[4].

Type 1 diabetes is a multifactorial disease where a genetic predisposition combines with environmental factors to induce the activation of the specific autoimmune destruction of β cells. Different known genetic risk factors can predict type 1 diabetes but autoantibodies are the most important preclinical markers. Autoantibodies include: islet cell autoantibodies, autoantibodies to insulin, autoantibodies to GAD (GAD 65), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2 β . In 85%-90% of patients affected by juvenile diabetes, these autoantibodies are detectable^[5]. Several genetic loci have been associated with type 1 diabetes but the HLA (human leukocyte antigen) region, located on chromosome 6p, with its multiple genes is the strongest link to immune-mediated diabetes susceptibility. More than 200 identified genes are located in the HLA region, over half of which are predicted to be expressed^[6]. Non-genetic factors also contribute to the risk of type 1 diabetes. This is supported by the fact that the overall concordance rate for type 1 diabetes among monozygotic twins is only about 10%-40%^[7]. Environmental factors play a substantial role in the development of type 1 diabetes. They include specific infectious agents, dietary factors,

perinatal factors, socioeconomic factors, and psychosocial factors^[8].

THERAPEUTIC OPTIONS FOR TYPE 1 DIABETES

The treatment of type 1 diabetes mellitus includes different therapeutic approaches. The aim of clinical intervention is to arrest or prevent the β cell destruction due to autoimmunity, reverse this process and restore normal blood glucose level and immune homeostasis. Insulin therapy was the first therapy and represented the primary breakthrough treatment for type 1 diabetes, however, frequent hyper- and hypo-glycaemia episodes seriously affect the quality of life of these patients. Recent technological innovations such as insulin analogue formulation, devices for insulin delivery and glucose monitoring systems have allowed diabetic patients to improve their glycaemic control^[9]. Intensive insulin therapies *via* insulin pens, subcutaneous or intraperitoneal insulin infusions using pumps reduce the onset and progression of diabetic complications, risks of hypo- or hyper-glycaemia, and increase the patient's quality of life.

β cell replacement is the only way to restore euglycaemia and ameliorate the progression of diabetic complications. Pancreas or pancreatic islet transplantation represents therapeutic alternatives to the administration of exogenous insulin to treat patients with type 1 diabetes. At the current time pancreas transplantation can produce long-term exogenous insulin independence, however, it remains a major surgical undertaking, associated with sizeable early morbidity and mortality, and with mandatory life-long immunosuppression^[10]. Islet transplantation also offers glycaemic control and prevention of hyperglycaemia without the need for exogenous insulin administration^[11]. As islets make up only 1%-2% of the pancreas, islet transplantation provides a much smaller transplant mass than whole pancreas transplant and is therefore a much less invasive procedure, and presents a smaller load of immunogenic tissue.

New therapeutic strategies for type 1 diabetes focus on three important points: residual β cell prevention, β cell restoration and β cell immune protection^[12]. An achievable goal could be to develop a new cellular source for β cell. Different studies focus on immortalization and expansion of β cells from deceased donor pancreas^[13,14], reprogramming or transdifferentiation of other pancreatic cells to β cells^[15], differentiation from pancreatic progenitor cells in the adult pancreas^[16] and differentiation and maturation from embryonic stem cells and induced pluripotent stem cells^[17]. All these cellular sources appear promising in developing potential new candidates for beta-cell substitution and therapy for patients.

Immunoprotection strategies include immunomodulatory therapies and immunoisolation techniques. Immunotherapies aim to down-regulate the autoimmune

response to pancreatic self-antigens and arrest beta-cell destruction. Ideally, this type of technique would induce prolonged remission from type 1 diabetes and achieve a cure^[18]. As regards the separation of implanted cells from the host immune system, this has been recognized as an experimental strategy to prevent immunorecognition, rejection and avoid lifelong immune suppression. A bioartificial pancreas tries to create a barrier to immune cells while allowing sufficient oxygen and nutrients transfer. Immunoisolation strategies facilitate islet transplantation without the need of immunosuppression^[19].

Islet transplantation as a cure for type 1 diabetes

Transplantation of pancreatic islets is a less invasive procedure than pancreas transplantation, with a shorter hospital stay and lower morbidity. This therapeutic option is reserved for patients with severe glycaemic variability, progressive diabetic complications and life threatening hypoglycemia^[19]. Successful islet transplantation was established in the early 70s in diabetic rats^[20] and rhesus monkeys^[21]. Najaran *et al.*^[22] reported the first significant case of human islet transplantation in patients with chronic pancreatitis. These patients underwent total or near total pancreatectomy, followed by autologous islet transplantation which prevented the development of diabetes. Thereafter, allograft was attempted in selected patients with type 1 diabetes. Unfortunately, out of the 237 allografts transplanted from 1990 to 1999, only 16% have resulted in insulin-independence for more than 1 week, and only 11% for more than 1 year^[23]. Important progress was made thanks to improvements in techniques for isolating human islets^[24,25] and to the availability of new and more effective immunosuppressive agents.

A positive turn in islet transplantation occurred in 2000, when James Shapiro and his colleagues treated 7 diabetic patients with severe hypoglycemia with allogeneic islets and a novel immunosuppressive regimen, obtaining insulin-independence in all the transplanted patients at a median follow-up period of 11.9 mo^[11]. This success was due to several changes in the transplantation procedure, such as the large number of infused islets (from 2-4 donors for each recipient), an immunosuppressive regimen with inclusion of sirolimus and without glucocorticoids and the limited cold ischemia time of the recovered pancreases. A follow-up report monitored 65 transplant recipients for a period of 5 years. This study showed that 80% of the transplanted patients remained insulin-independent at 1 year, but only 10% retained an insulin-free status at 5 years. However partial graft function allowed improvement of glycaemic control with a decreased occurrence of hypoglycemic episodes. Recent results for islet transplantation demonstrate major improvement in outcomes. Analysis of transplantations performed by Collaborative Islet Transplant Registry (CITR) from 1999 to 2010 showed that the insulin independence rate at 3 years after transplantation increased from 27% in 1999-2003 to 44% in 2007-2010 and at 4

years approximately 90% of the grafts showed some degree of function^[26].

All these studies indicated that islet transplantation is a promising strategy for treatment of type 1 diabetes. However, there are several challenges limiting widespread application. The disadvantages of the current approach is the limited supply and suboptimal yields of procurement and isolation of islets, graft failure and relatively high requirements to achieve prolonged insulin independence and glucose stability^[27]. Poor vascularization and hypoxia of the transplanted islets^[28], destruction by autoimmunity and allojection^[29] and exposure to the toxic effects of immunosuppressive agents^[30] are thought to contribute to early graft failure. Better protection of the transplanted islets and improved immunosuppression are current strategies under investigation that could substantially advance islet transplantation as an acceptable alternative treatment. Mesenchymal stromal cells have been proposed to be one possible means to enhance islet transplantation protocols^[31].

ROLE OF MSCS IN VASCULARIZATION AND IMMUNOMODULATION OF ISLET TRANSPLANTATION

MSCs are multipotent, self-renewing cells that reside mainly in the bone marrow, representing only 0.001%-0.01% of nucleated marrow cells. They can be also isolated from other tissues, including skeletal muscle^[32], adipose tissue^[33], amniotic fluid^[34] and umbilical cord blood^[35] and expanded for several passages without losing their self-renewing capacity^[36,37]. The International Society for Cellular Therapy has defined criteria to define the MSC population, including adherence to plastic in culture, expression of cell surface markers, such as CD105, CD73 and CD90, and lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules^[38]. MSCs have been well characterized for their ability to differentiate into several cell types of mesenchymal origin, such as osteoblasts, adipocytes and chondrocytes^[38], but it has been also demonstrated that they have the capacity to differentiate into cell types of endodermal and ectodermal lineages, including lung epithelial cells^[39], retinal pigment^[40], skin^[41], sebaceous duct cells^[42], renal tubular cells^[43], neural cells^[44], hepatocytes^[45] and insulin producing cells^[46]. However, an intense debate about the contribution of MSCs to form functional tissue through transdifferentiation processes is still open^[47]. Aside to their ability to differentiate into many types of cells, MSCs can also have a reparative effect through the migration to the site of injury^[48] and the release of paracrine factors that affect cell migration, proliferation and survival of the surrounding cells^[49]. In addition, MSCs have been shown to contribute to repair processes through the secretion of pro-angiogenic molecules, thus promoting the formation of new blood vessels *in vivo*^[50]. Moreover, MSCs have

emerged as a useful cell population for immunomodulation therapy thanks to their ability to secrete a large amount of bioactive molecules that affect immune and inflammatory responses^[51]. The combination of tissue regenerative potential and immunomodulatory or immunosuppressive activity has prompted therapeutic interest.

MSCs promote islet graft revascularization

Normally pancreatic islets have a rich vascular supply within the pancreas to support their metabolic activity and to facilitate rapid dispersal of secreted hormones. Large islets are supplied by 1-3 arterioles that penetrate the B cell-rich islet core and distribute into a dense network of sinusoidal capillaries connected to venules in the islet periphery^[52]. Islets receive considerably more blood flow than surrounding pancreatic exocrine tissue^[53] and islet capillaries are much more permeable than exocrine capillaries due to the presence of 10 times as many small pores within their endothelial cells^[54]. Relatively strong expression of VEGF by islets is probably responsible for the high degree of vascularization and fenestration. Depletion of VEGF in β cells in mice reduces vascular density and permeability to the level of exocrine tissue and partly impairs insulin secretion^[55]. The islet vasculature degenerates during the process of isolation and transplantation and the islets must initially rely on diffusion of oxygen and nutrients from the culture medium and from vessels in the transplant environment for their survival^[56,57]. This condition leads to prolonged hypoxia that, at the early post-transplant stages, is considered a major reason for early islet graft loss. The vessel density and oxygen tension in transplanted islets are less than half compared with islets in the native pancreas^[58]. Further compromising islet graft survival in this context is their vulnerability to oxidative stress, a consequence of relatively low expression of antioxidant enzymes^[59]. Thus, transgenic islet expression of antioxidant enzymes, such as glutathione peroxidase, could be a possible solution. However, a potential drawback of this approach is that glutathione peroxidase removes H₂O₂, an inducer of VEGF synthesis^[60], and thus may further impair islet graft revascularization. The net result of oxidative and other challenges is that more than 70% of islets transplanted intraportally fail to become stably engrafted^[61].

VEGF is a multi-functional angiogenic regulator that stimulates blood vessel formation, endothelial cell survival and epithelial cell proliferation^[62]. The receptors of VEGF are predominantly expressed on vascular endothelial cells^[62] and are also expressed in pancreatic islets^[63]. Several lines of evidence indicate that insufficient expression of VEGF limits the rate and extent of islet graft revascularization. Transplanted islets show a significant reduction of VEGF expression at day 3-4 after transplantation^[64] while an over expression of VEGF markedly improves the degree of revascularization and function of islet grafts. Mouse islets transfected with an adenovirus carrying the cDNA for the human VEGF₁₆₅ isoform were transplanted under the kidney capsule of diabetic nude mice. Vascular

endothelial growth factor (VEGF) expression resulted in an increase in both islet graft mass and revascularization and, unlike vector-transfected grafts, rapidly returned recipient to stable normoglycaemia^[65].

Several bone marrow subpopulations, such as endothelial progenitor cells and MSCs may be able to differentiate into one or more of the cellular compartments of the vascular bed^[66,67]. MSCs are known to secrete VEGF and other growth factors and to enhance proliferation of endothelial cells and smooth muscle cells^[68]. MSCs release a wide array of cytokines that support hematopoietic stem and progenitor cell development, as well as the secretion of other cytokines that are relevant to increasing blood flow to ischemic tissue^[69]. Moreover, MSCs secrete several important arteriogenic cytokines, including VEGF and monocyte chemoattractant protein-1 (MCP-1). In mice undergoing distal femoral artery ligation, a model of hind limb ischemia, local injection of MSCs increased adductor muscle levels of VEGF and fibroblast growth factor (FGF) proteins compared with controls, and co-localization of VEGF and transplanted MSCs within adductor tissue was demonstrated^[68].

Recently it has been reported that in animal models, MSCs are able to enhance survival and function of islet graft by increasing islet revascularization^[70]. Consistent with these studies, our group showed that cultured MSCs express high level of VEGF and that transplantation of those MSCs elicited a robust host angiogenic response leading to neovascularization of syngeneic islet grafts in diabetic rats. This effect may serve to increase local perfusion of the islets and ameliorate their metabolic activity^[71]. Similar results were obtained in a preclinical model by Berman *et al.*^[72] that demonstrated enhanced islet engraftment and function at 1 mo post-transplant in a cynomolgus monkey model of allogeneic islet-MSCs transplantation. The authors hypothesized that MSCs enhance islet engraftment by staying in proximity to the islets at the time of cotransplantation, providing revascularization and regenerative signals. MSCs provided an important approach for enhancement of islet engraftment, thereby decreasing the numbers of islets needed to achieve insulin independence^[72].

In summary, MSCs cotransplanted with islets in type 1 diabetic recipients can facilitate islet revascularization, engraftment and improved islet function. Consequently, the presence of MSCs permit to reduce the islet number required for reversal of diabetes. Therefore, cotransplantation of MSCs with islets could facilitate islet engraftment and improve islet graft function in clinical islet transplantation.

Immunomodulation of islet transplantation by MSCs

One of the most promising aspects of MSCs regards their dynamic role in modulating the immune system. MSCs are not only immunoprivileged cells, due to the low expression of class II Major Histocompatibility Complex (MHC-II) and co-stimulatory molecules in their cell surface, but they also interfere with different

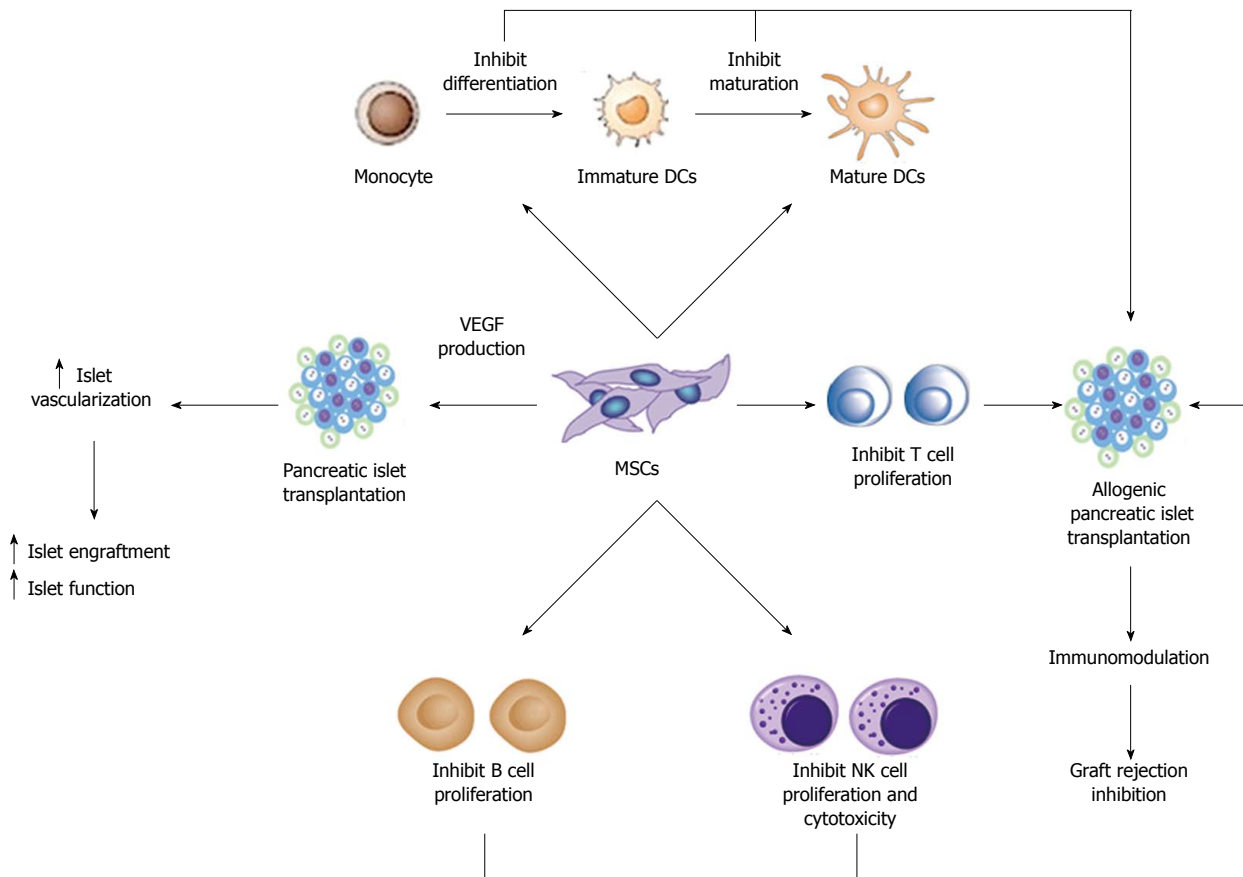


Figure 1 Schematic representation of the role of mesenchymal stem cells in islet transplantation. MSCs: Mesenchymal stem cells; VEGF: Vascular endothelial growth factor; DCs: dendritic cells.

pathways of the immune response by means of direct cell-to-cell interactions and soluble factor secretion. As schematically represented in Figure 1, it is well established that MSCs can exert immunosuppressive activity on T cells^[73] and interfere with dendritic cell (DC) maturation^[74]. Furthermore, MSCs may modulate natural killer (NK) cell cytotoxic activity, B cell proliferation and immunoglobulin production.

MSCs have been shown to suppress autoreactive T-cell responses in models of autoimmunity such as experimental autoimmune encephalomyelitis^[75], collagen-induced arthritis^[76] and autoimmune enteropathy^[77]. Type 1 diabetes is one of the most prevalent autoimmune diseases in childhood. The effector mechanisms of immune-mediated destruction of islet β cells are complex, but an essential early event is the activation of islet cell antigen reactive T cells. Recently, the therapeutic benefit of MSCs has been evidenced in the treatment of type 1 diabetes. Lee *et al.*^[78] used immunodeficient recipient mice chemically induced by streptozotocin to study the effect of human MSCs in the development of diabetes. Infusion of hMSCs reduced glycaemic levels and increased peripheral insulin levels. In the pancreas of these mice the islets appeared larger compared with islets from untreated diabetic mice^[78]. In experimental mouse models, intravenously infused MSCs are capable of migrating to pancreatic islets^[48]. However, the role of MSCs in β cell

replacement is controversial. Some evidence suggests the possibility that MSCs differentiate into islet β cells^[48]. In addition, similar results were reported by Ezquer *et al.*^[79] in a model of streptozotocin-induced diabetes. Reversion of hyperglycemia and glycosuria was observed after injection of MSCs, with increased morphologically normal β pancreatic islets. Other reports have contradicted these findings suggesting that MSCs could be feeder cells for islet differentiation, proliferation and vascularization, but do not differentiate into β cells^[80].

MSCs may also offer therapeutic opportunities in transplantation by directly targeting alloreactive T cells. MSCs are immunosuppressive *in vitro* and, in mixed-lymphocyte reactions, suppress T-cell proliferation^[73] through soluble factors, including 2,3-dioxygenase (IDO), prostaglandin-E2 (PGE2), nitric oxide, transforming growth factor β (TGF β) and hepatocyte growth factor (HGF)^[81,82]. Neutralizing antibodies against TGF β and HGF can restore the MSC-induced suppression of T cell proliferation^[73]. In a model of allogeneic pancreatic islet transplantation, the administration of MSCs resulted in the prolonged survival of islets and led to long-term stable normoglycemia^[83]. In this study MSCs were colocalized at the graft site where they locally produced immunosuppressive matrix metalloproteinase-2 and -9 that block the activation and expansion of alloreactive T cells^[83]. In a most recent paper, using

a rat model of streptozotocin induced diabetes, the authors found that MSCs significantly improved glycemic control and reduced graft infiltration by immune cells in either allogeneic or syngeneic pancreatic islet transplantation^[84]. They found that MSCs were effective when administrated either locally or systematically. The modulation of acute rejection that the authors observed after islet transplantation may indicate that soluble factors are released by MSCs to several organs after their systemic administration.

Additional studies revealed that MSCs might produce this anti-proliferative effect *via* induction of anergy in the T cell population^[85], T cell tolerance^[75], or by inducing proliferation of regulatory T cell populations^[86]. Berman *et al.*^[72] first reported increased numbers of Treg in a MSC allogeneic islet transplant preclinical model. MSCs treatment significantly enhanced islet engraftment and function at 1 mo post-transplant, as compared with animals that received islets without MSCs. Additional infusions of donor or third-party MSCs resulted in reversal of rejection episodes and prolongation of islet function. Stable islet allograft function was associated with increased numbers of regulatory T-cells in peripheral blood^[72].

The immune response is related not only to T cells, but to the interaction between DC cells and T cells^[87]. DCs are antigen-presenting cells (APCs) capable of stimulating both naïve and memory T cells. MSCs affect the differentiation, maturation and function of DCs at different levels^[74,88]. MSCs have also been shown to alter the cytokine secretion profile of DCs toward up-regulation of regulatory cytokines, such as IL-10, and down regulation of inflammatory cytokines such as IFN γ , IL-12 and TNF α , inducing a more anti-inflammatory or tolerant dendritic cell phenotype^[74,89]. Studies in animal models suggest that DC based immunotherapeutic strategies might also be utilized to facilitate islet transplant tolerance^[90,91]. Li *et al.*^[92] demonstrated that in mice with combined transplantation of pancreatic islets and MSCs, the expression of CD11c (DCs phenotype derived from monocytes) and CD83 (mature DCs phenotype) was down regulated markedly. This finding showed that MSCs inhibit the maturation of DCs and the stimulation of T cell was weakened, resulting in survival of transplanted pancreatic islets.

Autoimmunity also involves B cells by antibody production. The interaction between MSCs and B cells is not yet completely understood. However, co-culture experiments with these two cells using both mouse and human cells showed that MSCs inhibit B cell proliferation^[93]. They also observed that MSCs affect chemotactic properties of B cells while B-cell co-stimulatory molecule expression and cytokine production were unaffected by MSCs.

Finally, NK cells are key effector cells of innate immunity. MSCs alter the function of NK cells by suppressing their proliferation, and cytotoxicity. Spaggiari *et al.*^[88] demonstrated that cytokine induced proliferation of

freshly isolated NK cells was inhibited in the presence of MSCs.

Thanks to their interactions with many different types of immune cells, MSCs administrated in conjunction with islet cell transplantations could prevent immune rejection and promote long term islet allograft survival.

CONCLUSION

In summary, current data suggest that MSCs have the potential to aid in the treatment of type 1 diabetes and overcome some of the current limitations to islet transplantation. These cells may exert beneficial pro-angiogenic and immunomodulatory effects when co-transplanted with pancreatic islets. The pro-angiogenic effects result from the release of angiogenic factors from MSCs that have been shown to improve islet vascularization and graft function in islet transplantation. The immunomodulatory properties of MSCs may help in reducing inflammatory damage to the islets in the early peritransplant period. MSCs may also reduce autoimmunity through their capacity to inhibit T cell proliferation and suppress differentiation and maturation of dendritic cells.

These data encourage further preclinical co-transplantation of MSCs and pancreatic islets to improve the outcome of allogeneic islet transplantation in the treatment of type 1 diabetes. However, some key issues need to be addressed before MSC based therapies become a safe option for clinical studies. Most importantly, it is unclear if co-transplanted MSCs engraft and differentiate at the implantation site. Thus, the long-term stability of MSC activity and function after transplantation should be assessed *in vivo*. In addition, the selection of a suitable donor MSC source may differ if the treatment aims at modulating the autoimmune disease or enhancing pancreatic islet engraftment and vascularization. Therefore, whether autologous or allogeneic MSCs are suitable as a donor source should be selected according to the specific aim of the study.

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WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Mesenchymal stem cells in treating autism: Novel insights

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ing candidates in regenerative medicine. MSC therapy may be applicable to several diseases associated with inflammation and tissue damage, where subsequent regeneration and repair is necessary. MSCs could exert a positive effect in ASDs through the following mechanisms: stimulation of repair in the damaged tissue, *e.g.*, inflammatory bowel disease; synthesizing and releasing anti-inflammatory cytokines and survival-promoting growth factors; integrating into existing neural and synaptic network, and restoring plasticity. The paracrine mechanisms of MSCs show interesting potential in ASD treatment. Promising and impressive results have been reported from the few clinical studies published to date, although the exact mechanisms of action of MSCs in ASDs to restore functions are still largely unknown. The potential role of MSCs in mediating ASD recovery is discussed in light of the newest findings from recent clinical studies.

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Key words: Autism spectrum disorders; Autism treatment; Cell therapy; Mesenchymal stem cells

Abstract

Autism spectrum disorders (ASDs) are complex neurodevelopmental disorders characterized by dysfunctions in social interactions, abnormal to absent verbal communication, restricted interests, and repetitive stereotypic verbal and non-verbal behaviors, influencing the ability to relate to and communicate. The core symptoms of ASDs concern the cognitive, emotional, and neurobehavioural domains. The prevalence of autism appears to be increasing at an alarming rate, yet there is a lack of effective and definitive pharmacological options. This has created an increased sense of urgency, and the need to identify novel therapies. Given the growing awareness of immune dysregulation in a significant portion of the autistic population, cell therapies have been proposed and applied to ASDs. In particular, mesenchymal stem cells (MSCs) possess the immunological properties which make them promis-

Core tip: Autism spectrum disorders are still untreatable pathologies. Mesenchymal stem cells possess the immunological properties which make them promising candidates as a novel therapeutic option.

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AUTISM SPECTRUM DISORDERS

Autism spectrum disorders (ASDs) are complex neurodevelopmental disorders. Indeed, this term refers to

a heterogeneous group of varied conditions characterized by dysfunctions in social interactions, skills, and communication, restricted interests, and repetitive stereotypic verbal and non-verbal behaviors, influencing the ability to relate to others. Cognitive, emotional and neurobehavioral abnormalities characterize the core symptoms^[1,2]. The prevalence of these disorders has dramatically increased in the last years, with present rates of 11.3 per 1000 (one in 88) children aged 8 years in the United States, according to Centers for Disease Control^[3]. ASDs are presumed to be a lifelong disability with multiple impacts on child and adult health. Indeed, adult autistic individuals show limited independence because of their learning disability. In adulthood, communication is still impaired, as reading and spelling abilities are poor. Stereotyped behaviors and restricted interests still persist. The children affected require special and intensive parental, school, and social support^[4]. ASD results in a substantial impact on a person's quality of life and that of their family^[5]. Given the total lifetime societal cost of caring for one individual with autism, estimated in \$3.2 million^[6], autism should be considered as an urgent public health priority^[2].

Together with the cognitive, emotional and neurobehavioral abnormalities, ASDs are disorders characterized by a broad range of biochemical, toxicological and immune involvement, including: oxidative stress, endoplasmic reticulum stress, decreased methylation capacity, limited production of glutathione, mitochondrial dysfunction, intestinal dysbiosis, increased toxic metal burden, and immune dysregulations including autoimmunity^[7].

Currently, only a handful of medications are licensed for treating a limited number of autism-related symptoms^[8]. Moreover, prescribed pharmaceuticals (*i.e.*, antipsychotics) fail to address the ASD core symptoms, have the potential of markedly adverse effects, and are at best palliative^[9-12]. The alternative treatments for ASDs are diverse and include: behavioral, nutritional, and biomedical approaches. Thus the need for a definitive and effective therapy is an unfulfilled priority for autism research.

MESENCHYMAL STEM CELLS

Presently, cell therapies and cell-based biopharmacies offer a valid intervention for several otherwise untreatable human diseases. Stem cells appear to represent the greatest potential for the future of molecular and regenerative medicine^[13,14]. Among the various stem cell subtypes, mesenchymal stem cells (MSCs) provide a useful tool for the treatment of several diseases associated with inflammation, tissue damage, and subsequent regeneration and repair^[15].

MSCs are multipotent stem cells that possess the capacity to differentiate *in vivo* or *in vitro*, under specific conditions, into cells of connective tissue lineages, including bone, fat, cartilage and muscle^[16]. They are distinct from the hematopoietic lineage, and were initially described by

Alexander Friedenstein in the 1960s after he extracted MSCs from bone marrow^[17]. It is common practice for clinical and research applications, to acquire MSCs from bone marrow aspirates of the superior iliac crest under local anesthesia. The cells are then isolated by their adherence to plastic and amplified through passage in culture, where they exhibit a great replicative capacity^[18].

In order to achieve a detailed classification of this type of stem cell, the International Society for Cellular Therapy has proposed the following minimal criteria to identify human MSCs: they must grow in standard, plastic-adherent culture conditions; must express the cytospecific markers CD73, CD90 and CD105, without expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules; and must be capable of *in vitro* differentiation into osteoblasts, adipocytes and chondroblasts^[19].

Interestingly, MSCs seem to be the most promising clinical candidate for immune-modulatory cell-based therapy^[20]. MSCs show immunomodulatory capacities, as they are able to induce tolerance in immunocompetent allotransplants or even xenotransplant recipients^[21]. Interacting with a wide range of immune cells, probably through a cell-to-cell contact mechanism^[22], MSCs are able to modulate T-cell phenotype and immune-suppress the local environment^[23].

Their unique properties of immunomodulation, multipotency, and rapid self-renewal proliferation rate, distinguish them as useful tools for application in immunomodulatory therapy and neurological disorders. In addition, other desirable characteristics of MSCs, *e.g.*, genetic stability, stable phenotype, and easy procedures for collection, storage and shipping from the laboratory to the bedside^[24], direct us to MSC-based therapies as a potent intervention.

In clinical settings, MSCs can be transplanted directly without genetic modification or pretreatments (*i.e.*, immunosuppressants). No host *vs* graft rejection has been observed^[25]. Importantly, there is an absence of uncontrollable growth or tumorigenesis with MSCs, in contrast to the potential problems intrinsic to embryonic stem cells^[26]. Crucially, MSCs create no moral objection or ethical-religious controversies, unlike embryonic or fetal stem cells^[27].

MESENCHYMAL STEM CELLS IN TREATING AUTISM: THE RATIONALE

The potential application of cell therapy, in particular MSCs, for ASDs has already been discussed by our group^[28,29]. After a brief description of MSC-mediated ameliorative effects in ASDs, we will review recent and ongoing clinical trials using MSC transplantation in ASD patients.

We hypothesize that MSCs exert a positive effect in ASDs through the following mechanisms: stimulation of the plastic response in the host damaged tissue (*e.g.*, inflammatory bowel disorders); synthesizing and releas-

ing anti-inflammatory cytokines and survival-promoting growth factors (paracrine and biopharmacy); integrating into existing neural and synaptic network (engrafting), and restoring plasticity^[28,29]. Following transplantation, MSCs target and migrate to the site of injury. In some cases these cells respond to the local environment with appropriate secretion of soluble factors to ameliorate inflammation and promote repair^[30]. This paracrine mechanism offers potential in ASD treatment.

ASDs are characterized by a coexistent, if not etiological, immune system dysregulation^[31]. Changes in innate and adaptive immune responses have been reported in ASD patients^[32]. Characteristically, ASD cases show alterations in both T cell- and B cell-mediated immunity, as well as an imbalance in CD3⁺, CD4⁺, and CD8⁺ T cells and natural killer (NK) cells^[33]. On these bases, the regulatory effects mediated by MSCs present an optimal way to restore immune balance, which cannot otherwise be obtained through pharmaceutical interventions. Through inhibition of the proliferation of CD8⁺/CD4⁺ T lymphocytes and NK cells, suppression of the immunoglobulin production by plasma cells, and inhibition of the maturation of dendritic cells (DCs), MSC transplantation appears ideally suited to provide a unique therapeutic application for ASDs^[34,35].

In addition, MSCs are able to inhibit T lymphocyte pro-inflammatory cytokine production^[36]. MSCs function as an implanted biopharmacy: after homing in to the targeted tissue site, they synthesize and release a broad range of bioactive molecules^[35,37], *i.e.*, anti-inflammatory cytokines, trophic and growth factors, interleukin (IL)-6, IL-7, IL-8, IL-11, IL-12, IL-14, IL-15, macrophage colony-stimulating factor, Flt-3 ligand, and stem-cell factor^[38], which in turn could be responsible for activating endogenous restorative mechanisms within injured tissues. This strong paracrine activity of MSCs seems to be the most plausible and reasonable mechanism for the functional benefit derived from MSC transplantation. Furthermore, transplanted MSCs can induce the host tissue to upregulate the production of anti-inflammatory molecules, such as IL-10, in this way restoring the pro-inflammatory processes noted in ASDs^[39,40].

MESENCHYMAL STEM CELLS IN TREATING AUTISM: CLINICAL EVIDENCE

Despite insufficient pre-clinical models of MSC therapy for ASDs^[41], several clinical studies on humans have been conducted. Recently, a non-randomized, open-label, controlled, single-center phase I / II clinical trial to examine the treatment safety and efficacy of transplantation of human cord blood mononuclear cell (CBMNCs) and/or human umbilical cord-derived mesenchymal stem cells (UCMSCs) in children with autism has been performed^[42]. Stem cell administration was carried out via intravenous and intrathecal infusions. Autistic children transplanted with cells were followed for 24 wk. According to the authors, the cell treatment was safe, well toler-

ated without immediate or long term side effects, and no allergic, immunological reactions or other serious adverse events were observed at the time of injection or during the whole follow-up period. The cell transplantation showed efficacy; improvements were observed in visual, emotional and intellectual responses, body use, adaption to change, fear or nervousness, non-verbal communication and activity level, as measured by Childhood Autism Rating Scale, as well as in lethargy/social withdrawal, stereotypic behavior, hyperactivity and inappropriate speech evaluated by the Aberrant Behavior Checklist^[42]. They noted that the group receiving CBMNCs and UCMNCs demonstrated a more robust therapeutic effect than the group receiving mono-cell line therapy, which may be attributed to the action of CBMNCs and UCMSCs in synergy. It has been proposed the synergistic mechanism is related to increased cell-mediated perfusion in brain areas and/or the regulation of immune dysfunction.

Intrathecally transplanted autologous bone marrow-derived mononuclear cells were efficacious in improving the quality of life in a 14-year-old boy with severe autism^[43]. A detailed cell-sorting analysis was not done, but the cell extract contained a percentage of MSCs. We know bone marrow is comprised of a heterogeneous population of stem cells, encompassing hematopoietic stem cells, MSCs, endothelial progenitor cells, and very small embryonic-like stem cells. The bone marrow cell transplantation was safe, the patient had no noted side-effects and showed some immediate improvements within a week (eye contact and attention, fine motor activities). Significant improvements were observed over a period of 6 mo to 1 year (social interaction and emotions, impulse control, reading skills, tracing, recognition of all shapes and following commands, and hyperactivity). Interestingly, comparisons of pre/post cell therapy brain positron emission tomography scans revealed a markedly increased uptake in bilateral temporal lobes and bilateral calcarine cortices with mild increased uptake in the left medial pre-frontal cortex^[43].

Transplanted stem cells therefore seemed to ameliorate neural hypoperfusion in the previous case report. Hypoperfusion may be a consequence of focal inflammation and would likely result in low-grade ischemic consequences: hypoxia, abnormal metabolites, neurotransmitters dysregulation, and potential neural tissue damage.

In the light of these encouraging, but limited observations, the authors launched an open-label proof-of-concept study using autologous bone marrow-derived mononuclear cell transplantation in 32 patients with autism^[44]. The average number of intrathecally injected cells was 8×10^7 cells. Cell treatment was determined to be safe and adverse events were transient (hyperactivity). They hypothesize that the intrathecal administration route is able to enhance homing of the transplanted cells into the central nervous system. Clinical improvements after cellular therapy were observed in social relationships and reciprocity, emotional responsiveness, communication and behavior. As a putative mechanism of action,

the authors further hypothesized that cellular transplantation was able to restore function to ASD patients by neuroprotection, neural circuit reconstruction, neural plasticity, neurogenesis, and immunomodulation.

The hypothesis that intrathecal administration increases the efficacy of stem cell therapies is not actually evaluated by these various studies. Clearly, it is a testable hypothesis and future studies should include arms with and without intrathecal administration to compare the therapeutic efficacy of the more invasive intrathecal implantations.

PROBLEMS

Despite these early clinical trials with MSCs, there are no apparent pre-clinical studies on the use of MSCs in ASD models^[41]. Thus, more research into the mechanisms of action post transplantation is required to adequately understand the route, dosing and safety. However, the parental perspective is unlikely to wait on more detailed scientific studies. Stem cells are readily available from many centers in numerous countries, with various cell types and methodologies being utilized. Families recognize the devastating nature of autism on their children and are already seeking stem cell therapies. Based on a simple scan of the internet sites advertising cell therapies, it appears hundreds of ASD children have already been treated.

Another complexity in the research arises from stem cell sourcing. Some protocols use allogeneic (derived from a different person or collection of donors), while others use autologous donor (self-derived) cell types^[15]. Some protocols for ASD also use expanded autologous MSCs (United States Patent Application: 20060182724). This adds another dimension to the discussion and a potential source of laboratory contamination. Expansion requires medium for growth from which the cells must then be isolated, and any medium washed sufficiently to prevent a reaction in the recipient. Typically bovine serum is used. This creates the further risk of prion infection of the medium. To avoid this, one group has proposed using pooled human serum^[45]. This xeno-free methodology has many desirable features, but retains the concerns about human pathogen transmission. The group, however, screened extensively for contamination and it appeared they were able to ascertain that the samples were free of any disease vectors. This process should be considered for any use of expanded MSCs for ASD therapies.

Another challenge in the standardization of dosing derives from the varying efficacy amongst allogeneic donors in terms of: vitality, potency, and expansion potential. Every donor is different and this could affect efficacy and also paracrine effects. Indeed, it seems that the secretion of bioactive molecules could differ by a factor of 10 between different donors of matched age and gender^[15]. Recently, in order to increase the adequate supply of stem cells from donor tissues, it has been demonstrated that a 3D co-culture system with murine-derived hematopoietic stem cells co-cultured with MSCs produces 3D-microag-

gregates of stem cells. These 3D-microaggregate systems support the expansion of approximately twice as many hematopoietic stem cell candidates as the 2D controls. In addition, the MSCs maintained in 3D aggregates are able to express significantly higher levels of hematopoietic niche factors compared with 2D cultures^[46].

Finally, there are complex hurdles to overcome from the legal and regulatory restrictions placed by governments seeking to control cell therapies^[27]. Several countries (*i.e.*, United States and EU area) have attempted to create uniformity within the regulations governing cell trials, while creating very stringent regulations on cell culture conditions, diseases to be treated, and patient safety. However, in some other countries (*e.g.*, Ukraine, China, Dominican Republic, Panama, and Mexico) the access to cell therapy is more readily available.

CONCLUSION

The rapidly increasing prevalence of ASDs worldwide is creating an urgent need for effective restorative therapies. The lack of safe and effective psychopharmaceuticals and other definitive medical therapies, together with the limited understanding of the pathophysiology, has created an urgency to identify novel and more effective therapies^[47]. MSCs appear to offer a greater potential in regenerative medicine for complex disorder like autism than existing pharmaceutical protocols. Promising and impressive early results have been achieved from a few clinical studies, although the exact restorative mechanisms of action of MSCs in ASDs are still largely unknown.

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WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Neurotrauma and mesenchymal stem cells treatment: From experimental studies to clinical trials

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Abstract

Mesenchymal stem cell (MSC) therapy has attracted the attention of scientists and clinicians around the world. Basic and pre-clinical experimental studies have highlighted the positive effects of MSC treatment after spinal cord and peripheral nerve injury. These effects are believed to be due to their ability to differentiate into other cell lineages, modulate inflammatory and immunomodulatory responses, reduce cell apoptosis, secrete several neurotrophic factors and respond to tissue injury, among others. There are many pre-clinical studies on MSC treatment for spinal cord injury (SCI) and peripheral nerve injuries. However, the same is not true for clinical trials, particularly those concerned

with nerve trauma, indicating the necessity of more well-constructed studies showing the benefits that cell therapy can provide for individuals suffering the consequences of nerve lesions. As for clinical trials for SCI treatment the results obtained so far are not as beneficial as those described in experimental studies. For these reasons basic and pre-clinical studies dealing with MSC therapy should emphasize the standardization of protocols that could be translated to the clinical set with consistent and positive outcomes. This review is based on pre-clinical studies and clinical trials available in the literature from 2010 until now. At the time of writing this article there were 43 and 36 pre-clinical and 19 and 1 clinical trials on injured spinal cord and peripheral nerves, respectively.

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Key words: Neurotrauma; Stem cell therapy; Mesenchymal stem cells; Pre-clinical studies; Clinical trials

Core tip: Basic and pre-clinical studies have highlighted the positive effects of mesenchymal stem cell (MSC) treatment after spinal cord injury (SCI) and nerve trauma. There are many pre-clinical studies on MSC treatment for SCI and nerve injuries. However, the same is not true for clinical trials, particularly those concerned with nerve trauma. As for clinical trials for SCI, the results obtained so far are not as beneficial as those described in experimental studies. For these reasons basic and pre-clinical studies dealing with MSC therapy should emphasize the standardization of protocols that could be translated to the clinical set with consistent and positive outcomes.

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SPINAL CORD LESION: MECHANISMS OF DEGENERATION AND REGENERATION

Spinal cord injury (SCI) causes motor and sensory deficits that impair functional performance, and significantly impacts expectancy and quality of life of affected individuals. The estimated annual global incidence of SCI is 15-40 cases per million inhabitants^[1]. In addition to the sensory and functional deficits, spinal cord injury also causes great economic impact on the whole society and it is estimated that this impact is greater than 4 billion dollars per year^[2].

SCI results from primary and secondary injury mechanisms. Primary injury refers to the immediate physical injury to the spinal cord as a consequence of laceration, contusion, compression, and contraction of the neural tissue^[3]. Pathological changes resulting from primary injury mechanisms include severed axons, direct mechanical damage to cells, and ruptured blood vessels. Secondary injury is responsible for the expansion of the injury site which, in turn, limits the restorative process^[4,5]. Specific secondary sequel include alterations in local ionic concentrations, loss of regulation of local and systemic blood pressure, reduced spinal cord blood flow, breakdown of the blood-brain barrier, penetration of serum proteins into the spinal cord, inflammatory responses (alterations in chemokines and cytokines), apoptosis, excitotoxicity, calpain proteases activation, neurotransmitter accumulation, production of free radicals/lipid peroxidation, and imbalance of activated metalloproteinases. These changes lead to demyelination, ischemia, necrosis, and apoptosis of spinal cord parenchyma^[5]. These intrinsic responses to tissue injury contribute to an environment that is inhibitory to axonal regrowth^[6]. As a consequence of these negative influences when axons in the central nervous system (CNS) are damaged they mount a poor regenerative response.

An injury in the central nervous system generally leads to transection of some nerve fibers as well as damage to the surrounding tissues. The distal ends of the damaged axons form dystrophic growth cones that are exposed to a glial hostile microenvironment. During the initial phase of lesion, inhibitors associated with intact myelin oligodendrocyte and myelin debris, such as Nogo (no go), MAG (myelin associated glycoprotein) and OMgp (oligodendrocyte myelin glycoprotein) proteins can restrict axonal growth^[7]. In addition, the recruitment of inflammatory cells and astrocytes, in an attempt to restore the blood-brain barrier, leads to the formation of glial scar, which is usually accompanied by cavities filled with astrocytes secreted substances, such as chondroitin sulfate proteoglycans, which also acts as axon growth inhibitory molecules^[8,9]. Furthermore, there is also a lack of trophic factors in the lesion milieu due

to intrinsic changes in neurons such as atrophy and death after axonal injury. Together, all these inhibitory molecules form a glial microenvironment which is hostile to axonal repair^[2,4,10,11].

Although effective treatments for SCI remain limited, there have been many studies in recent years that have promised for the future from a clinical translational perspective. In general, basic science, preclinical, and clinical studies are aimed at overcoming the factors that are involved in impeding recovery from SCI. Current research is aimed at preventing secondary injury, promoting regeneration, and replacing destroyed spinal cord tissue. In particular, a variety of therapies have been addressed to alter neuro-inflammation^[12-14], reduce free radical damage^[15-17], reduce excitotoxic damage to neurons^[18,19], improve blood flow^[20,21], and counteract the effects of local ionic changes^[20,22-25]. Current experimental studies and the knowledge of clinical situations provide us with a better understanding of the complex interaction of the pathophysiological events after SCI. Future approaches should involve strategies aimed at blocking the multiple mechanisms of progressive pathogenesis in SCI and therefore promoting neuroregeneration.

Methylprednisolone (MP), a glucocorticoid, is the only current pharmacotherapy approved for SCI in the human clinic. Although therapy with methylprednisolone has been shown to be protective, its efficacy is limited and it only marginally improves outcomes^[14]. Recent advances in SCI research have led to a variety of novel experimental therapeutic strategies. The approach based on cell therapy using various lineages of stem cells has been considered as the most potential for the treatment of spinal cord injuries^[26]. Cell transplantation after spinal cord injury has several goals, among them, filling the cavity of the lesion to make a bridge that joins the edges of conserved areas, restore dead cells (neurons or myelinating cells) and make a favorable environment for axonal regeneration. Our laboratory employed *in vivo* experiments using predifferentiated embryonic stem cells^[27], human dental pulp stem cells^[28], and mesenchymal stem cell (MSC) (data not published) as a therapy for compressive spinal cord injury in mice, and our results show that these treatments lead to positive and similar functional and morphological responses. Among these lineages, mesenchymal stem cells have strengths such as easy extraction and cultivation, and do not involve ethical and moral issues, making them one of the favorite lineages for spinal cord injury treatment.

MSC THERAPY FOR SPINAL CORD LESION: FROM EXPERIMENTAL STUDIES TO CLINICAL TRIALS

MSC transplantation has been extensively investigated by several groups and these cells can be considered a feasible candidate for treatment of central nervous system diseases because they have characteristics that address the multifactorial events that occur after SCI. These cells have anti-inflammatory, immunomodulatory^[29] and neu-

roprotective^[30] effects. It has also been shown that MSC can secrete trophic factors thus exerting a paracrine effect that can stimulate axon regeneration contributing to functional recovery enhancement.

Concerning the paracrine effect, some groups have identified the ability of these cells in secreting pro-survival factor such as insulin-like growth factor (IGF) brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), granulocyte-macrophage colony stimulating factor (GM-CSF), fibroblast growth factor-2 (FGF2) and transforming growth factor beta (TGF- β)^[31-33]. In addition, MSC can be combined with gene therapy, by introducing genes to generate molecules with great therapeutic potential in promoting neuron survival and regeneration^[34]. Table 1 is a summary of preclinical studies using MSC for spinal cord injury, from 2010 until now.

Sources of MSC

MSC reside in a range of adult tissues that are easily accessible such as bone marrow, adipose tissue, skin, and even peripheral blood^[34]. Most of the studies in SCI use MSC derived from bone marrow and adipose tissue, but it is also possible to get MSC from a perinatal source like umbilical cord blood, umbilical cord matrix^[74], amniotic fluid and placenta^[75-77]. MSC can be extracted from these tissues and plated to be used in autologous transplantation, minimizing the rejection risk.

Studies using MSC extracted from bone marrow in rodents have demonstrated a beneficial effect of cell transplantation after SCI. The beneficial effect of MSC is usually attributed to secretion of neurotrophic factors^[78,79] and anti-inflammatory cytokines^[71,80,81]. Studies performed with pigs^[82] and monkeys^[83] showed that MSC can promote axonal growth and sprouting, corroborating the previous results in rodents, thus supporting the clinical use of MSC.

MSC extracted from adipose tissue is considered an attractive source of cells due to easiness of isolation, obtention of a large amount of cells per donor, and also due to the fact that this tissue is usually discarded after liposuctions. In SCI models, treatment with these cells have resulted in cell survival, neuroprotection, attenuation of secondary damage, axonal regeneration, decrease of gliosis, angiogenesis and enhanced functional recovery^[61,84-90]. A comparative study using MSC extracted from both bone marrow and adipose tissue after SCI found that both sources of MSC expressed similar surface protein markers, but animals that received adipose tissue cells presented higher levels of tissue BDNF, increased angiogenesis, higher number of preserved axons and a decrease in the number of macrophages, suggesting that the use of MSC extracted from adipose tissue is a better candidate for SCI treatment^[41]. However, this is not a consensus and should be further investigated because in another comparative study published in 2012, the authors did not find any difference between animals that received MSC derived from bone marrow or adipose tissue, in

terms of axonal regeneration, neuroprotection and functional recovery after a compression lesion in dogs^[51].

Despite being less investigated in terms of SCI treatment, MSC extracted from perinatal tissues also present a therapeutic potential. Human umbilical cord blood cells (hUCBC) transplantation in rats submitted to an injury, resulted in differentiation of these cells into neural cells and downregulation of the fas/caspase-3 pathway in neurons and oligodendrocytes, and also increased levels of anti-apoptotic proteins^[91,92].

The umbilical cord matrix, also known as Wharton's jelly, possesses a stem cell population that present some advantages in comparison to other sources because they can proliferate more rapidly and extensively than adult MSC^[76,93] and also because they are easily obtained after normal and cesarean births, with low risk of viral contamination^[94,95]. Other advantage is the possibility of using them for allogenic transplantation because they act by suppressing immune response and are, therefore, considered non-immunogenic cells^[96]. Some studies using umbilical cord matrix-derived MSC indicated that these cells can survive in the injury site and promote repair and recovery after SCI. This improvement is attributed to immunomodulatory and trophic effects through secretion of glial-derived neurotrophic factor (GDNF), BDNF and nerve growth factor (NGF) which are known as supporters of cell survival and regeneration^[54,97].

The amniotic fluid cells constitute another source of MSC, which are obtained from discarded post-partum tissue, without any ethical objections about their use. They present similar proliferation and differentiation patterns in comparison to adult MSC^[98,99]. According to few studies, these cells are able to enhance cell survival and axon myelination and improve hind limb function, after transplantation in SCI models^[100]. Some studies have also demonstrated the immunomodulatory effect and trophic support provided by these cells after SCI^[101,102].

Issues regarding the quantity and best via of administration of MSC for SCI

Two important questions that should be addressed when we discuss MSC and its efficacy in treating central nervous system disorders are: the ideal quantity of cells and the best administration *via*. Concerning the cell quantity, the literature presents several studies using different amount of cells. In terms of cell administration, most transplantation is delivered directly into the injury site or adjacent to it, by injecting few microliters of cell suspension^[103]. Attempts have been made to inject cells intravenously or intraperitoneally in order to decrease tissue damage and, thus, avoiding subjecting the individual to another surgical intervention.

There are several studies that injected different quantity of cells with similar results. Apart from the difference on the quantity of cells, there are other points that make the comparison among these studies difficult, such as the diversity of lesion models, animal types and route of cell administration. For example, Cizkova and colleagues^[104]

Table 1 Summary of pre-clinical studies using mesenchymal stem cell for spinal cord injury

Animal	Lesion type	Cells source	Route of administration	Effects on CNS regeneration	Ref.
Rat	Contusion	Human mesenchymal precursor cells	Lesion site	Improvement in functional recovery and tissue sparing and reduction of cyst volume	[35]
Rat	Contusion	Human bone marrow-MSC	Lesion site , intracisternal, intravenous	Improvement in functional recovery	[36]
Rat	Hemisection	Bone marrow-MSC induced into Schwann Cells	Lesion site	Improvement in locomotor and sensory scores, axonal regeneration and remyelination	[37]
Rat	Contusion	Bone marrow-MSC	Lesion site , intravenous	Improvement in locomotor scores and NGF expression	[38-40]
Rat	Transection to the dorsal columns and tracts	Bone marrow-MSC, adipose derived-MSC	Lesion site	Improvement in locomotor scores, increased angiogenesis, preserved axons, decreased numbers of ED1-positive macrophages and reduced lesion cavity formation	[41]
Rat	Hemisection	Human umbilical cord-derived MSC	Lesion site	Suppress mechanical allodynia, and this effect seems to be closely associated with the modulation of spinal cord microglia activity and NR1 phosphorylation	[42,43]
Rat	Hemisection	Human bone marrow-MSC	Lesion site	Improvement in locomotor scores, shorter latency of somatosensory-evoked potentials and differentiation into various cells types	[44]
Rat	Hemisection	Bone marrow-MSC	Lesion site	Improvement in locomotor scores and reduced lesion cavity formation	[45]
Mouse	Compression	Bone marrow-MSC	Lesion site	Improvement in locomotor and sensory scores and reduced lesion volume	[46]
Rat	Contusion	Human bone marrow-MSC	Lesion site	Improvement in functional recovery, tissue sparing and reduction in the volume of lesion cavity and in the white matter loss	[35,47-49]
Rat	Contusion	Human umbilical cord-MSC	Lesion site	Improvement in functional recovery, reduction of the extent of astrocytic activation and increased axonal preservation	[50]
Dog	Compression	Bone marrow, adipose, Wharton's jelly, umbilical cord derived-MSC	Lesion site	Improvement in functional recovery, increased numbers of surviving neurons, smaller lesion sizes and fewer microglia and reactive astrocytes in the epicenter of lesion	[51]
Rat	Compression	Bone marrow-MSC	Intravenous	Improvement in functional recovery, increase of NGF expression, higher tissue sparing and density of blood vessels	[52]
Rat	Contusion	Human umbilical cord-MSC	Lesion site	Improvement in functional recovery, endogenous cell proliferation and oligogenesis, and smaller cavity volume	[53,54]
Rat	Transection	Human-MSC	Lesion site	Improvement in functional recovery, increased amplitude of motor-evoked potentials, differentiation into neural cells	[55,56]
Rat	Contusion	Bone marrow-MSC	Lesion site	Improvement in functional recovery, preservation of axons, less scar tissue formation and increase in myelin sparing; higher levels of IL-4 and IL-3 and higher numbers of M2 macrophages, and reduction in TNF- α and IL-6 levels, and in numbers of M1 macrophages	[57-60]
Dog	Compression	Neural-induced adipose derived-MSC	Lesion site	Improvement in functional recovery and neuronal regeneration, and reduction of fibrosis	[61]
Mouse	Transection	Bone marrow-MSC	Lesion site	Improvement in functional recovery and neuronal survival, reduction of cavity volume and attenuation of inflammation, promotion of angiogenesis and reduction of cavity formation	[62-64]
Rat	Compression	Bone marrow-MSC	Lesion site	Improvement in functional recovery, up-regulation of VEGF mRNA expression, increase in angiogenesis and prevention of tissue atrophy	[65-67]
Rat	Compression	Human umbilical cord-MSC	Lesion site	Improvement in functional recovery, increase in the intensity of 5-HT fibers and in the volume of spared myelination; decrease in the area of the cystic cavity	[68]
Dog	Compression	Umbilical cord-MSC	Lesion site	Improvement in functional recovery, promotion of neuronal regeneration and reduction of fibrosis	[69]
Dog	Compression	Human umbilical cord-MSC	Lesion site	Improvement in functional recovery and remyelination	[70]
Rat	Contusion	Bone marrow-MSC	Intrathecal	Improvement in functional recovery	[71]
Rat	Contusion	Human bone marrow-MSC	Lesion site , lumbar puncture	Improvement in functional and sensory recovery	[72]
Rat	Contusion	Neural differentiated and undifferentiated MSC	Lesion site	Improvement in functional recovery and reduction of cavitation	[73]

CNS: Central nervous system; MSC: Mesenchymal stem cell; TNF: Tumor necrosis factor; IL: Interleukin; NGF: Nerve growth factor; VEGF: Vascular endothelial growth factor.

demonstrated cell survival and enhancement in locomotor performance after MSC transplantation delivered by intravenous injection (one million cells in a volume of 0.5 mL of DMEM) in a model of balloon compressive injury in rats, while Sheth *et al.*^[105] performed cell transplantation (600000 cells in a volume of 6 μ L) directly into the injury site after contusive injury in rats, and also observed an enhancement in locomotor function and a decrease in the lesion volume, indicating a neuroprotective effect of these cells. Thus, it is still difficult to determine the ideal quantity of cells and the best *via* for stem cell transplantation after SCI. The questions that arise from these studies are: Is there a minimum number of transplanted cells that can be used and yet giving the best results in terms of functional recovery? Can we get similar results with cells injected systemically in comparison to local injection? Studies using the same type of lesion and different amount of cells and administration *via* should be further undertaken in order to better clarify this issue.

Time point for cell transplantation

Other crucial issue that should be further addressed here is the time point for cell transplantation after lesion. This is important because the environment created after SCI is hostile for regeneration and can negatively influence cell survival and differentiation. Thus, depending on the time that the treatment is performed the results can be completely different. Most studies have been performed in acute or sub-acute phases, which means immediately or 1-2 wk after injury, respectively^[35,103]. There are fewer studies in the SCI chronic phase, when cells are delivered in later stages, when the glial scar is already present^[38,41].

Clinical trials

The clinical trials conducted for SCI comprise three different phases with human participation in all phases. The phase 1 trial begins with the administration of the cell transplants to a human subject with the aim to investigate the presence of adverse or toxic effects and treatment safety. People who participate in these trials may experience some risks and have limited benefits. In phase 2, the objective is to determine the potential and variability of a therapy in comparison with a control group. The participants are usually recruited and randomly assigned to the groups (experimental or control) and both, participants and investigators, do not know to which study they have been assigned to. The phase 3 clinical trials are usually the definitive clinical trial. The aim is to confirm the preliminary results obtained at the phase 2, with a statistically significant clinical benefit of the therapeutic intervention. The number of subjects is also larger and multiple study centers are involved^[106,107]. The majority of the studies using MSC transplantation after spinal cord injury are in phase 1 or 2.

At the time of writing this article there were twenty clinical trials being either completed, ongoing or in the recruitment stage, using either adult or perinatal sources of mesenchymal stem cells in different phases of the dis-

ease, and most of them use autologous transplantation to minimize the risk of rejection. Table 2 list the clinical trials listed on the clinical trials.gov.

The number of clinical trials using MSCs for treatment of SCI is increasing, indicating that despite several questions that still need to be addressed at basic and pre-clinical levels, the MSC are considered potentially beneficial for translational studies.

According to PubMed database, in the last three years only three studies were published in “clinical trials” category, using MSC transplantation after SCI. One of them transplanted autologous bone marrow-derived MSC into the cerebrospinal fluid of patients with complete SCI. The authors described that 45% of the patients showed a recovery, but there was no difference between these patients and those from control groups; they emphasized that despite the fact that results were not positive, the transplantation was a feasible and safe technique, since patients did not present any adverse reaction^[108]. On the other hand, Park *et al.*^[109] using the same cell source, and repeated cells injections directly into the spinal cord, demonstrated that three of ten patients presented a motor improvement, and significant magnetic resonance changes and electrophysiological results. These results are similar to those obtained by Dai *et al.*^[110] who also demonstrated a clinical improvement in patients that received autologous MSC transplantation. The results of these studies are not conclusive, and, unfortunately, not as good as those obtained in pre-clinical experiments. In spite of that, all of them emphasize mesenchymal stem cell clinical potential.

WALLERIAN DEGENERATION AND NERVE REGENERATION IN THE PERIPHERAL NERVOUS SYSTEM

Traumatic injury to nerves in the peripheral nervous system (PNS) is a large-scale problem annually affecting more than one million people worldwide. These injuries often result in pain and disabilities, owing to reduction in motor function and sensory perception. Moreover, the trauma can cause emotional, social and work-related disorders, and the affected individuals undergo a reduction in their quality of life^[111,112].

While it is widely accepted that the PNS has an inherent potential for regeneration, functional recovery after a lengthy peripheral nerve injury (PNI) remains unsatisfactory^[113]. After an extensive traumatic nerve injury with a large gap between the proximal and distal nerve stumps, a long period of time is required for regenerating axons to cross that gap. During that time, the ability of axotomized neurons to regenerate declines and Schwann Cells (SC) can no longer support regenerating neurons and their axons. As a result, regenerating axons fail to reach their target organs and the injury cannot be successfully repaired. In order to accelerate the rate of axonal growth many therapeutic strategies are being developed and in-

Table 2 Summary of clinical trials studies using mesenchymal stem cell for spinal cord injury

Title	Lesion type	Cells source	Phase of the study	Status	Effects on CNS regeneration
Clinical study of treatment for acute SCI using cultured bone marrow stromal cells	Cervical SCI	Autologous Bone marrow-MSc	Terminated	1/2	Rapid and remarkable recovery of ASIA B and C patients, but gradual or limited in ASIA A patients.
Autologous mesenchymal stem cell in SCI patients	Complete cervical or thoracic SCI	Autologous bone marrow-MSc	Enrolling by invitation	2	Not informed
Different efficacy between rehabilitation therapy and umbilical cord derived MSCs transplantation in patients with chronic SCI in China	Traumatic SCI	Umbilical cord derived-MSc	Recruiting	3	Not informed
A phase III/IV clinical trial to evaluate the safety and efficacy of bone marrow-derived MSC transplantation in patients with chronic SCI	Cervical SCI	Autologous bone marrow-MSc	Recruiting	1/2	Not informed
Phase I / II trial of autologous bone marrow derived MSCs to patients with SCI	Traumatic thoracic or lumbar SCI	Autologous bone marrow-MSc	Recruiting Completed	1/2 1	Not informed
Safety of autologous adipose derived MSCs in patients with SCI	Clinical diagnosis of SCI (ASIA A to C)	Autologous Adipose derived-MSc			
The effect of intrathecal transplantation of autologous adipose tissue derived MSCs in the patients with SCI, phase I clinical study	Clinical diagnosis of SCI	Autologous Adipose derived-MSc	Recruiting	1	Not informed
Phase I, single center, trial to assess safety and tolerability of the intrathecal infusion of ex-vivo expanded bone-marrow derived MSCs for the treatment of SCI	Clinical diagnosis of SCI (ASIA A)	Autologous bone marrow-MSc	Active, not recruiting	1	Not informed
Study the safety and efficacy of bone marrow derived autologous cells for the treatment of SCI	Clinical diagnosis of SCI	Autologous bone marrow-MSc	Recruiting	1/2	Not informed
Surgical transplantation of autologous bone marrow stem cells with glial scar resection for patients of chronic SCI and intra-theal injection for acute and subacute injury-a preliminary study	Complete spinal cord trans-section	Autologous bone marrow-MSc	Completed	1/2	Not informed
To study the safety and efficacy of autologous bone marrow stem cells in patients with SCI	SCI below C5 (ASIA A to C)	Autologous bone marrow-MSc	Recruiting	1/2	Not informed
Safety of autologous stem cell treatment for SCI in children	Clinical diagnosis of SCI (ASIA A to D)	Bone marrow-MSc	Recruiting	1	Not informed
Autologous bone marrow derived cell transplant in SCI patients	Traumatic SCI	Autologous bone marrow-MSc	Completed	1/2	Not informed
Phase 1 study of autologous bone marrow stem cell transplantation in patients with SCI	Traumatic thoracic or lumbar SCI	Autologous bone marrow-MSc	Not informed	1	Not informed
Phase I pilot study to evaluate the security of local Administration of autologous stem cells obtained from the bone marrow stroma, in traumatic injuries of the spinal cord	Traumatic SCI between C3 and L1	Autologous bone marrow-MSc	Recruiting	1	Not informed
Feasibility and safety of umbilical cord blood cell transplant into injured spinal cord: an open-labeled, dose-escalating clinical trial	Chronic SCI between C5 and T11 (ASIA A)	Umbilical cord blood mononuclear derived-MSc	Active, not recruiting	1/2	Not informed
Efficacy difference between rehabilitation therapy and umbilical Cord derived transplantation in patients with acute or chronic SCI in China	Clinical diagnosis of SCI	Umbilical cord derived-MSc	Not informed	2	Not informed
Safety and feasibility of umbilical cord blood cell Transplant Into Injured Spinal cord: an open-labeled, dose-escalating clinical trial	Chronic SCI between C5 and T11 (ASIA A)	Umbilical cord derived-MSc	Active, not recruiting	1/2	Not informed
Safety and effect of lithium, umbilical cord blood cells and the combination in the treatment of acute and sub-acute spinal cord injury : a randomized, double-blinded placebo-controlled clinical trial	Acute or Subacute traumatic SCI between C5 and T11 (ASIA A)	Umbilical cord derived-MSc	Active, not recruiting	1/2	Not informed

MSC: Mesenchymal stem cell; CNS: Central nervous system; SCI: Spinal cord injury.

vestigated. The identification of crucial elements responsible for successful regeneration in injured peripheral nerves will be quintessential in improving regenerative outcomes after peripheral and central nerve injuries.

Nerve trauma elicits a cascade of molecular, cel-

lular, and ultrastructural responses which are necessary for degeneration and posterior regeneration, including: disruption of axonal conduction; increase in cell body metabolism and protein synthesis; degeneration of the distal stump of injured axons; dedifferentiation and pro-

liferation of SC; degradation of the myelin sheath; recruitment of macrophages to the site of injury^[114], as well as the release of cytokines, neurotrophins and growth factors^[115-117]. These events will allow rapid and efficient removal of the growth inhibitory cellular debris present in the injured peripheral nerve milieu, producing a favorable microenvironment for axonal growth^[118].

After an injury the axon is divided into two segments: a proximal stump that remains in contact with the cell body, and a distal stump which is separated from the rest of the neuron. The distal nerve stump undergoes a cascade of events called "Wallerian degeneration"^[119,120], which is initiated within 24 to 48 h by the entry of calcium in the axoplasm. Calcium influx activates proteases, such as calpains that promote cytoskeletal degradation and disintegration of axoplasm, myelin and axolemma^[121,122]. The rupture of the blood-nerve barrier allows the entry of macrophages into the site of injury and, together with SC, these cells initiate intense phagocytosis and removal of degenerating axon and myelin debris. The barrier permeability decreases two weeks after the injury and then, in the fourth week, increases again in order to regain homeostasis after Wallerian degeneration^[118].

Immediately after injury, the SC in the distal stump of the nerve begin the process of dedifferentiation. Even before axonal degeneration occurs, SC can modify its gene expression^[123] and 48 h after injury, they decrease myelin protein expression, acquire a non-myelinating phenotype and begin to express genes related to regeneration, like growth associated protein 43 (GAP-43), neurotrophic factors and their receptors, neuroregulins and their receptors, and assume an intense proliferative activity^[124,125]. About four days after injury SC reach their proliferation peak. These proliferative cells are confined within the tube formed by its own basal lamina and align forming the so called bands of Büngner. These bands columns will form a supportive substrate, providing clues that will guide axon growth toward the target organ, through the release of trophic factors. When SC contact the regenerating axons, the process of re-myelination is started^[126].

The injury also causes a rapid arrival of signals from the damaged axons to the neuronal body resulting in an extraordinary change from a transmitting to a growth promoting phenotype. Cell body suffers a process called chromatolysis, which is characterized by swelling of the neuronal body and by dispersion of Nissl corpuscles^[127,128]. These changes reflect variations in the metabolic activity of neurons which, as a result, fail to synthesize proteins required for neurotransmission, and start producing substances that are important for axonal sprouting and growth^[129]. The regeneration that follows occurs *via* different mechanisms: the elongation of the distal end of injured axons and the growth of collateral axons from nodes of Ranvier in the proximal stump. However, the success of regeneration and target organ reinnervation depends mostly on the enhancement of the number of regenerating axons, the velocity of axon

growth and on the ability of affected neurons to survive and acquire a regenerative phenotype.

In the clinical settings, reconstruction of transected peripheral nerve requires accurate microsurgical repair that connects the proximal and distal stumps of the nerve in a tension-free manner. In cases of injury with tissue loss, autologous peripheral nerve grafts, *i.e.*, autografts, is considered by neurosurgeons the gold standard technique, but unfortunately, even in these cases, the clinical results remain disappointing and, therefore, the search for better strategies is an urgent necessity. In cases of digital nerve lesions, biodegradable artificial nerve conduits are being used in the clinical settings, but their use is still limited to these thin nerves. An advantage of the use of these conduits is that they can be combined with other pro-regenerative strategies, such as the local injection of neurotrophic factors and cells.

New therapeutic approaches should have as a goal an increase of the intrinsic regenerative capacity of transected nerve fibers and a decrease of the extrinsic factors that limit regeneration of severed nerve fibers, thus creating an appropriate environment in which, axon elongation, remyelination and proper reinnervation of target organ may occur. A stem cell-based therapy represents an important new strategy to manage peripheral nerve injury. In the next part of this review we will discuss the potential use of mesenchymal stem cells, in promoting nerve regeneration.

MSC THERAPY IN PNS: FROM EXPERIMENTAL STUDIES TO CLINICAL TRIALS

A number of experimental studies have shown the potential of MSC to improve peripheral nerve regeneration following traumatic injuries^[130-135]. These cells may act on nerve regeneration mainly by paracrine, neuro/axonoprotective, or immunomodulatory effects; by transdifferentiation into SCs; by cell-to-cell contact; or even by a combination of the above mechanisms^[134]. However, most of the beneficial effects exerted by the MSC are strongly correlated with the production of neurotrophic substances, such as FGF, NGF, ciliary neurotrophic factor, BDNF, GDNF among others^[132,133,136,137].

Our group showed the presence of high levels of NGF-b in the in MSC *in vitro* suggesting that they are also able to express this potent neurotrophic factor *in vivo*; this result could represent one mean by which these cells acted on the enhancement of axon regeneration and remyelination, consequently contributing to the observed return of motor function^[133]. In agreement with these findings, bone marrow-MSC locally injected in the mouse ischiatic nerve resulted in improvement of regeneration of sensory and motor axons^[134]. Because these authors also observed that these cells were capable of increasing neurite outgrowth *in vitro* through NGF releasing, and that they presented low potential to differentiate into SC

in vivo, they suggested that the beneficial effects exerted by the implanted cells were mainly dependent on their trophic activity rather than their stemness potential^[134]. In another work, our group also observed the benefits of bone marrow-MSC locally injected in the mouse median nerve following transection and conduit repair. This cell system was capable of increasing the number of both myelinated and unmyelinated fibers, preventing the muscle atrophy and, most importantly, improving functional performance^[130].

It is also possible that MSC can act indirectly on nerve regeneration by modulating cellular behaviors such as inducing SC to survive, proliferate, produce neurotrophic factors and promote remyelination. A coculture system with rat bone marrow-MSC conditioned media and SC demonstrated cell-cell interactions despite no direct contact between the two population of cells. MSC not only favored survival and proliferation of SC but also induced them to express NGF, BDNF and NGF receptors^[138]. This is an important MSC feature as it might indicate that MSC can relay and magnify neurotrophic function from stem cells to glia cells, thus improving peripheral nerve regeneration.

Besides rodents, larger animal models have also been used to investigate the effects of MSC-based therapy on more challenging nerve gaps. Few authors have shown the successful bridging of a 30 mm-long ischiatic nerve defect by means of a biodegradable conduit in dogs^[139]. After six months of MSC implantation, they observed the reconstruction of ischiatic nerve trunk with restoration of nerve continuity, functional recovery for conducting electrical impulses and transporting materials, and muscle re-innervation, which lead to improvement of locomotion activities. Even more challenging, using a two-fold nerve gap in a similar experimental model but with addition of autologous MSC, the same group^[140] demonstrated that the cellular treatment improved nerve regeneration and functional recovery in a manner comparable to the autograft-treated animals, which is considered by neurosurgeons the current gold standard for peripheral nerve repair.

As aforementioned, the great majority of the experimental studies of mesenchymal stem cell-based therapy on the peripheral nerve regeneration use rodents (mainly mice and rats) as animal models^[130,133,134,138], perhaps because they are small size mammals and, consequently, easy to handle; also, they have been extensively used in the field of genetic engineering for a diversity of experimental trials of gain and loss of function as well as reporter assays. However, there are few studies using non-human primates such as cynomolgus and rhesus monkeys, which share high level of sequence homology with human genome, that have confirmed the feasibility of this cell system for improving nerve regeneration after severe nerve lesions. MSC transplantation into either allogeneic nerve grafts^[141] or artificial conduits^[142] for bridging severe upper extremity nerve defects in higher primates yielded structurally and functionally regenerated

nerves; these studies proved to be safe and effective, thus giving great insight into the use of MSC in human clinics.

MSC obtained from human subjects have also been used in pre-clinical studies for promoting nerve regeneration, yielding promising results^[143-145]. These studies are of great relevance because they address human MSC properties, clarifying their mechanisms of action, and also provide insight into their effects on peripheral nervous tissue recovery. Interestingly, the authors of these studies demonstrated that human MSC-based therapy improved peripheral nerve regeneration as well as functional recovery. However, McGrath *et al.*^[145] showed that MSC survived in the conduit and enhanced axonal regeneration only when transplantation was combined with the immunosuppressive treatment, cyclosporine A. As these results provide evidence of the nerve regeneration potential of human MSC, and taking into account that one of the great advantages of MSC is the possibility of auto transplantation without donor-site morbidity, they might encourage the use of this cell system for treating human peripheral nerve trauma.

Thus, the results of pre-clinical studies highlighting the improved outcomes yielded by using MSC with the aim to repair a large nerve gap may increase the feasibility of translation of MSC-based therapy to clinical trials for peripheral nerve applications.

Table 3 summarizes the studies using MSC for nerve injuries, either in pre-clinical or clinical trials, since 2010 until now. To date, only one clinical trial has used autologous bone marrow mononuclear cells within silicone tubes to repair human median or ulnar nerves^[146]. In this study scores for motor function, sensation and the effect of pain on function were better than those obtained from individuals that had the tubular nerve repair only; However, a possible limitation in this study is the fact that there was a difference between groups regarding the age of individuals and the length of follow-up after treatment, which could represent biases in this study. So, the interval between injury and treatment was always longer than 75 d, which could possibly limit the positive effects exerted by the cells on the nerve regeneration process. Another possible disadvantage of this work is that nerve conduits were made of silicone, a non-biodegradable material, thus requiring a second surgery to remove the conduit. In spite of these limitations cells-treated patients presented a better recovery compared to the untreated. The results of this study will, hopefully, encourage subsequent clinical studies to be conducted safely, with fewer biases, and with the association of the cellular treatment with suitable biodegradable conduits, thus preventing discomfort and complications generated from the use of silicone material.

Although important advances have been achieved in the use of stem cells for improving nerve regeneration, they are still limited to basic and pre-clinical trials. In addition, there are several variables among these studies, such as tissue source; methods of cell isolation, expansion and characterization; route of cell delivery; number

Table 3 Summary of pre-clinical and clinical studies using mesenchymal stem cell for peripheral nerve injury

Animal	Nerve	Lesion type	Cells source	Route of administration	Ref.
Mouse	Median	Transection	Bone marrow-MSC	Local	[147]
Mouse	Ischiatic	Transection	Bone marrow-MSC	Local	[148]
Mouse	Ischiatic	Transection	Embryonic stem cell derived-MSC	Local	[144]
Mouse	Ischiatic	Crush	Adipose derived-MSC	Intravenous	[149]
Mouse	Ischiatic	Transection	Bone marrow-MSC	Local	[131]
Rat	Ischiatic	Crush	Amniotic fluid derived-MSC	Local	[150]
Rat	Ischiatic	Crush	Amniotic fluid derived-MSC	Intravenous	[151-153]
Rat	Facial	Transection	Bone marrow-MSC	Local	[154,155]
Rat	Ischiatic	Crush	Bone marrow-MSC	Local	[156]
Rat	Ischiatic	Transection	Adipose derived-MSC	Local	[157-159]
Rat	Ischiatic	Transection	Umbilical cord derived-MSC	Local	[160,161]
Rat	Ischiatic	Transection	Bone marrow-MSC	Local	[145,158,162-169]
Rat	Cavernous	Traction	Bone marrow-MSC	Intracavernousal injection	[170]
Rabbit	Facial	Transection	Bone marrow-MSC	Local	[171]
Rabbit	Ischiatic	Traction	Bone marrow-MSC	Local	[172,173]
Pig	Ischiatic	Transection	Bone marrow-MSC	Local	[174]
Dog	Ischiatic	Transection	Bone marrow-MSC	Local	[140,175]
Dog	Ischiatic	Transection	Adipose derived-MSC	Local	[176]
Monkey	Median	Transection	Bone marrow-MSC	Local	[142]
Human	Median or ulnar	Transection	Bone marrow mononuclear cell fraction-MSC	Local	[146]

MSC: Mesenchymal stem cell.

of transplanted cells; therapeutic time window; animal and nerve models; type of injury; number of transplanted cells; and immunogenicity. These variables represent an important obstacle for comparing and contrasting study outcomes from different groups, thus hindering progress in the field.

In 2006, The International Society for Cellular Therapy proposed the development of a set of minimal criteria (adherence to plastic in standard culture conditions, expression of a number of markers and multipotent differentiation potential into osteoblasts, adipocytes and chondroblasts) for defining the MSC for research purposes^[177]. Although this action represented a great attempt to allow for comparison of scientific studies among different groups, the criteria for mesenchymal cells from different species should be further considered and well-defined, in particular the non-human and human primate MSC.

CONCLUSION

Pre-clinical studies have shown the beneficial effects of MSC therapy in the neurotrauma field. Unfortunately, these effects are not usually seen in the clinical trials, and the results are far from being as good as those described in experimental studies. Therefore, there is an urgent need to seek for standardization of protocols in terms of source of cells, culture conditions, time of treatment after injury, number and *via* of administration of cells, plasticity and capability of human MSC after extraction and expansion in culture, among other concerns. Basic and pre-clinical studies focusing on these important points will, hopefully, be of great help in terms of their successful implementation in clinical trials.

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WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Umbilical cord-derived mesenchymal stem cells: Their advantages and potential clinical utility

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Abstract

Human umbilical cord (UC) is a promising source of mesenchymal stem cells (MSCs). Apart from their prominent advantages, such as a painless collection procedure and faster self-renewal, UC-MSCs have shown the ability to differentiate into three germ layers, to accumulate in damaged tissue or inflamed regions, to promote tissue repair, and to modulate immune response. There are diverse protocols and culture methods for the isolation of MSCs from the various compartments of UC, such as Wharton's jelly, vein, arteries, UC lining and subamnion and perivascular regions. In this review, we give a brief introduction to various compartments of UC as a source of MSCs and emphasize the potential clinical utility of UC-MSCs for regenerative medicine and immunotherapy.

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Key words: Umbilical cord; Mesenchymal stem cells; Wharton's Jelly; Multipotency; Immunotherapy

Core tip: Human umbilical cord (UC) is a promising source of mesenchymal stem cells (MSCs). UC-MSCs have shown the ability of faster self-renewal and to differentiate into three germ layers, to accumulate in damaged tissue or inflamed regions, to promote tissue repair, and to modulate immune response. There are diverse protocols and culture methods for the isolation of MSCs from the various compartments of UC, such as Wharton's jelly, vein, arteries, UC lining membrane and subamnion and perivascular regions. In this review, we introduce various compartments of UC and discuss the potential clinical utility of UC-MSCs for regenerative medicine and immunotherapy.

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INTRODUCTION

Mesenchymal stem cells (MSCs) originate in the human embryo and are considered multipotent stem cells. MSCs are a heterogeneous subset of stromal stem cells, which can be isolated from the bone marrow^[1], mobilized peripheral blood^[2], cord blood^[3], umbilical cord (UC)^[4,5], placenta^[6], adipose tissue^[7], dental pulp^[8], and even the fetal liver^[9] and lungs^[10]. UC contains two umbilical arteries (UCAs) and one umbilical vein (UCV), both embedded within a specific mucous connective tissue, known as Wharton's jelly (WJ), which is covered by amniotic epithelium (Figure 1). UC is considered medical waste and the collection of UC-MSCs is noninvasive; furthermore, the access to UC-MSCs has not been encumbered with ethical problems. UC-MSCs, similarly to MSCs derived

from other sources, have distinct capacity for self-renewal while maintaining their multipotency, *i.e.*, the ability to differentiate into adipocytes, osteocytes, chondrocytes, neurons and hepatocytes, although some differentiation abilities are known to be partial^[11-13]. Moreover, UC-MSCs have also attracted great interest because of their immunomodulatory properties. Nowadays, UC-MSCs are proposed as a possible versatile tool for regenerative medicine and immunotherapy.

HISTORY OF UC-MSCs

During pregnancy, the fetus and mother are connected by UC. UC prevents umbilical vessels from compression, torsion and bending, while providing good blood circulation. McElreavey *et al.*^[4] for the first time reported isolation of fibroblast-like cells from WJ of human UC in 1991. The UC-derived cells have the similar surface phenotype, plastic adherence and multipotency as those of MSCs derived from other sources. It was 3 years earlier that the first cord blood (CB) transplantation was performed in France in 1988^[14]. After that, together with the development of CB banking, CB transplantation has become the alternative source of hematopoietic stem cells. Although CB-derived MSCs cannot be consistently isolated^[15-18], MSCs were considered to circulate in the blood of preterm fetuses and able to be successfully isolated and expanded^[3]. Where these cells home at the end of gestation is not clear^[13]. Thus, UC has inevitably become a focus of attention as a source of MSCs because it contains CB^[18]. One key study appeared concerning CB-derived MSCs appeared around 2003^[19]. Mitchell *et al.*^[20] successfully isolated matrix cells from UC WJ using explant culture and Romanov *et al.*^[19] isolated MSCs-like cells from the subendothelial layer of UCV.

ADVANTAGES OF UC-MSCs

Stem cell populations can be isolated from embryonic, fetal and adult tissues. Embryonic stem cells (ESCs) are a leading candidate for tissue engineering because of their high self-renewal capacity and pluripotency (ability to differentiate into all germ layers) *in vitro* and *in vivo*. Nonetheless, in addition to ethical restrictions, the clinical applications of ESCs are severely limited by technical difficulties with the depletion of immature cells that may result in the formation of a teratoma.

In contrast, adult stem cells, such as those in the skin, bone marrow (BM) and adipose tissue, may have wider clinical applications. BM-MSCs have been used for autologous and allogeneic purposes. Recently, successful clinical application of autologous BM-MSCs was reported for conditions such as cardiac infarction^[21], graft-versus-host disease (GVHD)^[22,23], Crohn's disease^[24] and bone tissue engineering^[25]. On the other hand, the autologous use was sometimes limited by cell numbers and age-related changes such as decreased growth and differentiation capacity^[26,27].

Compared with BM-MSCs and ES cells, UC-MSCs show a gene expression profile more similar to that of ESCs and faster self-renewal rather than BM-MSCs^[11,12].

It is easy to obtain a substantial number of UC-MSCs after several passages and extensive *ex vivo* expansion^[28]. The most appreciable advantage is that the collection procedure is noninvasive and ethically acceptable.

Similar to BM-MSCs, UC-MSCs can be considered for autologous and allogeneic use. Autologous UC-MSCs might be used as gene therapy for genetic diseases and as regenerative or anti-inflammatory therapy for neonatal injury, such as cerebral palsy or hypoxic brain damage. On the other hand, allogeneic UC-MSCs can be expanded and cryopreserved in a cell bank for patients in need. The only disadvantage is that physicians need to confirm the baby's health as a donor because it cannot be ascertained in advance whether the donor will grow normally without health problems; thus, genomic or chromosomal tests need to be performed. In contrast, in the case of a BM donor, the physician can directly see and examine the donor and then decide to collect BM. In the case of CB banking, many CB banks monitor the baby's health after birth. Thus, it is important to know the advantages and disadvantages of UC-MSCs for each clinical application.

DIFFERENT METHODS FOR ISOLATION OF MSCs FROM DIFFERENT COMPARTMENTS OF UC

There are two methods to obtain MSCs from various UC compartments or from the whole UC: the explant method and the enzymatic digestion method.

The explant method

UC, or its compartments, is manually minced into small fragments 1-2 mm³. These fragments are aligned and seeded regularly on the tissue culture-treated dishes. After the tissue fragments are attached to the bottom of the dish, the culture medium is poured slowly and gently, so as not to detach the fragments, and the culture is started^[29-31]. The culture medium is replaced every 3-7 d for 2-4 wk until fibroblast-like adherent cells reach 80%-90% confluence. The adherent cells and tissue fragments are rinsed once with PBS and detached using a trypsin solution, followed by washing with the medium. The cells and tissue fragments are filtered to remove the tissue fragments.

The disadvantage of this method is that the fragments often float in the medium, resulting in poor cell recovery. No MSCs can be obtained from the floating fragments. To collect a consistent number of MSCs each time, it is important to prevent the exfoliation of the tissue fragments from the bottom of plastic dishes.

The enzymatic digestion method

WJ is either directly exposed to enzymatic solutions to

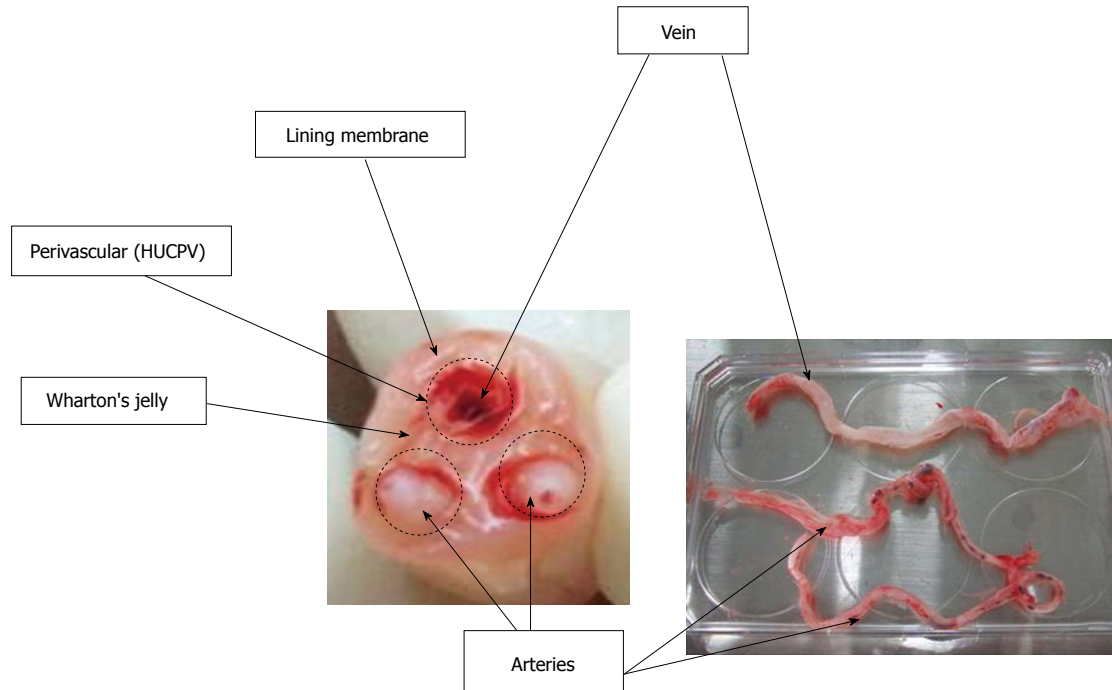


Figure 1 Various compartments of umbilical cord from which mesenchymal stem cells can be isolated. HUCPV: Human umbilical cord perivascular.

release the cells or it is cut into small pieces followed by enzymatic digestion. The enzymes used for digestion vary from simple collagenase^[31,32] to a combination of either collagenase and hyaluronidase with or without trypsin^[33,34] or collagenase, dispase II and hyaluronidase^[33]. The digestion time and concentrations varied by researchers.

There are four compartments of UC as a source of MSCs: (1) Whole UC-MSCs: the whole UC is cut into smaller pieces followed by either an explant procedure or enzyme digestion^[30,35,36]; (2) UCWJ-, UCA- and UCV-MSCs: UCWJ-MSCs are obtained after removing umbilical vessels. Umbilical vessels [two arteries (UCAs) and one vein (UCV)] can also be minced into 1-2 mm³ fragments. The fragments are aligned regularly on the plate and cultured until MSCs start growing; (3) UC lining and subamniotic-derived MSCs: the subamniotic region of UC lining membrane is removed with a razor blade and then cut into small pieces. These fragments are plated in plastic culture dishes until MSCs start growing (explant method). With this method, however, it might be difficult to remove the adjacent region underneath the amniotic epithelium completely^[37,38]; and (4) Human UC perivascular stem cells (HUCPVC): the vessels are extracted from UC and tied at both ends into loops. The loops are then placed into an enzymatic solution for a defined period of time to allow the cells to separate from the perivascular region. The detached cells are cultured and collected as HUCPVCs^[26,28,39].

It is still controversial whether the isolation of the cells from the whole or some compartment is superior to others with respect to their proliferation ability, differentiation ability and immunosuppressive capacity.

Proliferation assays

The frequency of colony-forming unit fibroblasts (CFU-F)

is significantly higher in whole UC-derived MSCs than in BM-MSCs with limiting dilution^[26,30,40]. The authors first compared UCWJ-MSCs, UCA-MSCs and UCV-MSCs. UCV-MSCs exhibited a significantly higher frequency of CFU-F than UCWJ-MSCs and UCA-MSCs, but the doubling time was not different among these cell types^[5]. The Mennan group also reported that there are no significant differences among the various compartments of UC, although the cells derived from any UC compartment proliferate significantly faster than BM-MSCs, with mean doubling times of 2-3 d at P0 through P3^[41]. Depending on the purpose, researchers need to select either a compartment or the whole UC.

Biomarkers of UC-MSCs

The immunoprofile of UC-MSCs is analyzed using flow cytometry, according to the standard definitions for MSCs described by the position paper of the International Society for Cellular Therapy^[42]. There are no single specific markers that can be used to identify multipotent MSCs. MSCs are positive for adhesion markers such as CD29 and CD44; mesenchymal markers such as CD90, CD73 and CD105; and human leukocyte antigen class I (HLA-ABC). MSCs are negative for endothelial cell marker CD31; hematopoietic cell markers such as CD34, CD45 and CD117; and human leukocyte differentiation antigen class II (HLA-DR)^[43]. Among the different UC compartments, UCWJ-, UCV- and UCA-derived MSCs show a similar fibroblast-like spindle shape and the MSCs from these three types of tissues demonstrate no significant differences in the immunoprofile. These cells are positive for CD13, CD29 (integrin β 1), CD73 (SH3), CD90 (Thy-1), CD105 (SH2; endogrin) and HLA-ABC at the cellular frequency greater than 90% and are negative for CD34,

CD45, CD133 and HLA-DR, with the cellular frequency less than 1%^[5]. Mennan *et al*^[41] also confirmed that MSC immunophenotypes showed no significant differences among different sources: BM, umbilical cord arteries, vein, WJ and UC lining membrane. Even although the authors could not find any major differences in their immunophenotypes, the cell populations derived from the different compartments may consist of different proportions of multipotent stem cells. Karahuseyinoglu *et al*^[44] demonstrated that CD73 is expressed throughout the vessels and endothelium and is absent in the perivascular region, but the strongest expression is observed in the epithelial and subepithelial regions of WJ. CD90 is positive in most compartments but negative in the endothelial lumen lining. A high expression of vimentin, CKs (1, 4, 5, 6, 8, 10, 13, 18 and 19), desmin and SMA has been detected in the subamniotic layer and the perivascular region. Schugar *et al*^[45] reported that CD146 (endothelial progenitor marker) is expressed in the vessel walls (100%) and the perivascular region of UC (62%) but is no longer expressed in UCWJ-MSCs^[26,46]. These markers might aid in determining the multipotency of the isolated cell population. Phenotypic characterization of UC-MSCs might be influenced by the culture passage number, culture medium and culture method.

Furthermore, ESC markers such as Oct4, Nanog, Sox-2 and KLF4 are expressed only at low levels in UC-MSCs^[47]. This suggests that MSCs are primitive stem cells, somewhere between ESCs and mature adult stem cells. Nonetheless, a precise isolation of pluripotent MSCs using specific markers remains a challenge.

The role of SSEA3 and SSEA4 in MSCs remains controversial. Gang *et al*^[48] reported that SSEA4⁺ cells proliferate predominantly when the culture is initiated from primary BM cells in the medium supplemented with special cocktails of cytokines. In contrast, the authors failed to reproduce the same phenomena in UCWJ-MSCs in the medium consisting of α -MEM and 10% FBS. Furthermore, SSEA4 expression in UCWJ-MSCs significantly correlates with the FBS concentration in the culture medium, whereas SSEA3 expression was inversely correlated. We concluded that SSEA4 in UCWJ-MSCs is not a marker of either proliferation capacity or multipotency^[31]. Schrobback *et al*^[49] assessed SSEA4 expression in human articular chondrocytes, osteoblasts and BM-derived MSCs and characterized their differentiation potential. Their results showed that SSEA4 levels in these cells are not related to the capacity for chondrogenic and osteogenic differentiation and the proliferation potential *in vitro*^[49].

THE ABILITY OF UC-MSCs TO DIFFERENTIATE INTO ADIPOGENIC, CHONDROGENIC AND OSTEOGENIC LINEAGES

UC-MSCs originating from the extraembryonic meso-

derm and their capacity for differentiation into adipogenic, chondrogenic and osteogenic lineages have been extensively studied^[50]. Regarding the osteogenic differentiation ability, Hsieh *et al*^[11] demonstrated that the gene profiles of UC-MSCs are close to ESCs; UC-MSCs show delayed and insufficient differentiation into osteocytes. On the other hand, BM-MSCs have already expressed an osteogenic gene profile and can easily differentiate into osteocytes. Among the three compartments, UCWJ, UCV and UCAs, UCWJ-MSCs demonstrate an obviously defective ability to differentiate into osteocytes, even although the expression of osteocyte-related genes is detected by reverse-transcriptase PCR, at levels similar to those in the other two tissues/compartments^[5]. Mennan *et al*^[41] compared the osteogenic differentiation among cord regions in six samples and found that the best differentiation is seen with UCWJ-MSCs and whole UC-derived MSCs, rather than with UCA-, UCV- and UC lining MSCs.

As for adipocytic differentiation, Mennan *et al*^[41] reported that UC-MSCs produce small lipid vacuoles, whereas BM-MSCs produce more mature adipocytes (unilocular lipid vacuoles). UC-MSCs might maintain their multipotency for longer periods than BM-MSCs can^[51], although there were no obvious differences among MSCs derived from UC compartments in our research^[5].

With respect to chondrogenic differentiation, UC-MSCs show no apparent differences among the different cord regions (sources)^[41]. Moreover, the comparison of the chondrogenic potential between BM-MSCs and UC-MSCs revealed that UC-MSCs produce thrice as much collagen as BM-MSCs; this finding indicates that the former may be a better option for fibrocartilage tissue engineering^[52].

In relation to other differentiation abilities, UCWJ-MSCs are the most studied cell type among various UC compartments and many papers have been published^[53,54]. In addition to differentiating into osteocytes, chondrocytes and adipocytes, UCWJ-MSCs can differentiate into cardiomyocytes (with the gene expression of N-cadherin and cardiac troponin I^[55]), neurons and glia^[20], oligodendrocytes^[56] and hepatocytes^[57]. Recently, clinical trials have been conducted using UC-MSCs for neurogenic disorders (spinocerebellar ataxia and multiple system atrophy of the cerebellar type)^[58] and liver disorders^[59,60].

IMMUNOSUPPRESSIVE PROPERTIES OF UC-MSCs

Immunosuppressive effects have now become the most popular property of MSCs for potential clinical use. First, MSCs themselves are weakly immunogenic owing to the lack of HLA-DR and low expression of MHC class I molecules. MSCs have been shown to have immunomodulatory properties *in vitro*^[61]. Furthermore, MSCs lack both CD80 and CD86 proteins^[36,62], which

are costimulatory molecules inducing T cell activation and survival. The lack of HLA-DR, CD80 and CD86 suggests that MSCs do not elicit acute rejection and are suitable for allogeneic cell-based therapy.

Second, UC-MSCs have immunosuppressive properties *in vitro* and *in vivo*. Many studies have been published about the immunosuppressive effect of UCWJ-MSCs^[63], UC lining-MSCs^[37,64], HUCPV^[65] and whole UC-derived MSCs^[66]. The immunosuppressive effect of UC-MSCs is mediated by soluble factors and cell-to-cell contacts. PGE2, galectin-1 and HLA-G5 are released from MSCs and serve as effective factors of immunosuppression^[67]. Among these factors, indoleamine 2,3-dioxygenase (IDO) is one of the most relevant because it is inducible by IFN- γ and catalyzes conversion from tryptophan to kynurenine^[62,68]. This depletion of tryptophan from the environment can suppress T cell proliferation. UCWJ-MSC-mediated immunosuppression may require preliminary activation by proinflammatory cytokines, such as IFN- γ , with or without TNF- α , IL-1 α or IL-1 β .

It was recently suggested that the inflammatory environment produced by the upregulation of cytokines such as IFN- γ and TNF- α might alter the biological activity of MSCs from immunosuppression to immunostimulation^[68]. In this case, UC-MSCs may not prevent GVHD *in vivo*. It is known that upon stimulation by activated immune cells or cytokines (priming), MSCs are primed and become functional immunosuppressors. The extent of immunosuppression is greater with UCWJ-MSCs than with BM-MSCs^[62]. Polchert *et al.*^[68] demonstrated that MSCs primed with IFN- γ are effective in a mouse GVHD model despite upregulated MHC class II molecules. In order to ensure the effective and safe therapeutic use of UC-MSCs, more *in vivo* experiments need to be conducted because of the many discrepancies with *in vitro* data.

CONCLUSIONS

Compared with the counterparts of other origins, UC-MSCs have attractive advantages as MSCs and as UC-derived cells: (1) a noninvasive collection procedure for autologous or allogeneic use; (2) a lower risk of infection; (3) a low risk of teratoma; (4) multipotency; and (5) low immunogenicity with a good immunosuppressive ability. It is still unclear which compartment in UC is the best for clinical use; nonetheless, the era of the clinical use of UC-MSCs is approaching quickly.

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WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Differentiation of mesenchymal stem cells into gonad and adrenal steroidogenic cells

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Abstract

Hormone replacement therapy is necessary for patients with adrenal and gonadal failure. Steroid hormone treatment is also employed in aging people for sex hormone deficiency. These patients undergo such therapies, which have associated risks, for their entire life. Stem cells represent an innovative tool for tissue regeneration and the possibility of solving these problems. Among various stem cell types, mesenchymal stem cells have the potential to differentiate into steroidogenic cells both *in vivo* and *in vitro*. In particular, they can effectively be differentiated into steroidogenic cells by expressing nuclear receptor 5A subfamily proteins (steroidogenic factor-1 and liver receptor homolog-1) with the aid of cAMP. This approach will provide a source of cells for future regenerative medicine for the treatment of diseases caused by steroidogenesis

deficiencies. It can also represent a useful tool for studying the molecular mechanisms of steroidogenesis and its related diseases.

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Key words: Steroid hormone; Adrenal; Gonad; Steroidogenic factor-1; Liver receptor homolog-1; Mesenchymal stem cells; Differentiation

Core tip: Stem cells can be a potential source of cells for regenerative medicine for diseases caused by steroidogenesis deficiency. Among various stem cell types, mesenchymal stem cells have the potential to differentiate into steroidogenic cells both *in vivo* and *in vitro*. This system can also provide a powerful tool for studying the molecular mechanisms of steroidogenesis and its related diseases.

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INTRODUCTION

In mammals, steroid hormones are produced from cholesterol mainly in adrenal glands and gonads. Steroid hormones are essential for glucose metabolism, the stress response, fluid and electrolyte balance, sex differentiation and reproduction via binding to cognate receptors in target tissues. Therefore, a steroidogenesis abnormality can often be life threatening. Congenital adrenal hyperplasia (CAH) is one of the most common disorders caused by deficiency of any enzyme involved in steroidogenesis in adrenal glands^[1,2]. Impaired cortisol and aldosterone pro-

duction increases adrenocorticotrophic hormone (ACTH) secretion from the pituitary gland, leading to adrenal hyperplasia and accumulation of adrenal androgens. Female patients are prenatally virilized because of excess androgen and neonates of both genders may suffer from a life-threatening Addisonian crisis. Steroid hormone deficiency also occurs in aging people by hypogonadism. In males, testosterone concentrations decline with age, causing various clinical symptoms such as obesity and hypertension^[3-6]. Postmenopausal women often suffer from osteoporosis caused by estrogen deficiency^[7,8]. Hormone replacement therapy has been well established for the treatment of such patients, although they require hormone replacement for their entire lifetime. In addition, these patients suffer from various side effects (liver and kidney damage, immune system dysfunction) and risks associated with long-term replacement therapy (cancer). Therefore, another therapy is needed to resolve these problems. Stem cells represent an innovative tool for tissue regeneration and gene therapy, which could possibly solve these problems. In this review, we provide an overview of differentiation and regeneration of steroidogenic cells using mesenchymal stem cells (MSCs), preceded by a description of the development of steroidogenic organs. We also describe molecular events, such as coactivator function and epigenetic modifications, which occur during differentiation.

DEVELOPMENT OF STEROIDOGENIC ORGANS AND NUCLEAR RECEPTOR 5A SUBFAMILY

Steroidogenesis begins with conversion of cholesterol into pregnenolone in mitochondria by the P450 side chain cleavage enzyme (P450_{sc}/CYP11A1/Cyp11a1), a rate-limiting enzyme in the synthesis of all steroid hormones. Thereafter, various hormones are synthesized by tissue-specific P450 hydroxylases and hydroxysteroid dehydrogenases^[9,10]. Although adrenal glands and gonads produce various steroid hormones in adult life, they have a common developmental origin, a so-called adrenogonadal primordium (AGP) that mainly originates from the intermediate mesoderm and is localized on the coelomic epithelia of the developing urogenital ridge^[11-13]. As development proceeds, AGP separates into two distinct populations, adrenocortical and gonadal primordia, characterized by the existence of chromaffin cell precursors and primordial germ cells, respectively, which originate and migrate from other germ layers. During differentiation, adrenal glands and gonads synthesize tissue-specific steroid hormones by specific expression patterns of steroidogenic enzymes.

Steroidogenic factor-1 (SF-1, also known as Ad4BP) is one of the earliest markers of the appearance of AGP^[11,14]. Because SF-1 knockout mice fail to develop adrenal glands and gonads, SF-1 represents a master regulator of the development of these organs^[15-17]. SF-1/

Ad4BP is also important for steroidogenesis by regulating the transcription of steroidogenic genes. SF-1/Ad4BP was originally discovered by Keith Parker and Ken Morohashi as a transcription factor that binds to the Ad4 sequence in promoter regions of all cytochrome P450 steroid hydroxylase genes for transactivation^[18,19]. They concluded from the expression of SF-1 in steroidogenic cells and its regulation of all steroid hydroxylase genes that SF-1 is a determinant factor in cell-specific expression of steroidogenic enzymes. In addition to steroidogenic enzymes, diverse groups of SF-1 target genes, such as other steroidogenic genes, pituitary hormones and cognate receptors, and sex differentiation-related genes have been identified thus far^[17,20,21]. SF-1 belongs to the nuclear receptor (NR) superfamily. NRs are lipophilic ligand-dependent and independent transcription factors and essential for various physiological phenomena^[22,23]. A large number of family members have been identified from invertebrate to mammals. There are a total of 48 family members on the human genome. They share a common structural organization: zinc finger DNA-binding domain and a carboxyl-terminal ligand-binding domain. The NR superfamily can be broadly divided into four classes based on their characteristics (steroid hormone receptors, RXR heterodimers, dimeric orphan receptors and monomeric orphan receptors). SF-1 is categorized into monomeric orphan receptors, although Ingraham and colleague argued the possibility that phosphatidylinositols are ligands for SF-1^[24]. SF-1 is very similar to liver receptor homolog-1 (LRH-1). LRH-1 was originally identified in the liver^[25] and is known to function in metabolism, cholesterol and bile acid homeostasis by regulating the transcription of a number of genes^[26-29]. In addition to the liver, LRH-1 is highly expressed in tissues of endodermal origin. It is also expressed in gonads and involved in steroidogenesis; in particular, its ovarian expression levels are the most abundant among tissues^[30]. These factors constitute one of the NR subfamilies and are designated as NR5A proteins (Table 1, SF-1 is NR5A1 and LRH-1 is NR5A2). SF-1 and LRH-1 have various common characteristics, such as binding sequences, target genes and cofactors^[24,31-38].

Consistent with its role in steroidogenesis, SF-1 expression is detected in adults in three layers of the adrenal cortex (zona reticularis, zona fasciculata and zona glomerulosa), testicular Leydig and Sertoli cells, ovarian theca, granulosa cells and, to a lesser extent, in the corpus lutea^[39,40]. In the corpus lutea, LRH-1 rather than SF-1 is highly expressed and is important for progesterone production^[36,41,42]. LRH-1 is also expressed in testicular Leydig cells^[12,43,44].

SF-1 knockout mice die shortly after birth because of adrenal insufficiency and exhibit male-to-female sex reversal in external genitalia^[15]. These phenotypes are caused by the complete loss of adrenal glands and gonads. Although the initial stages of adrenal and gonadal development occur in the absence of SF-1, they regress and disappear during the following developmental stage.

Table 1 Summary of the characteristics of steroidogenic factor-1 and liver receptor homolog-1

Nuclear receptor	Expressing tissues	Function	Phenotypes of knockout mice
SF-1/ Ad4BP/ NR5A1	Testis, ovary, adrenal,	Steroidogenesis Sex differentiation Energy homeostasis	Adrenal and gonadal agenesis Sex reversal in external genitalia Impaired expression of pituitary gonadotropins Abnormality of ventromedial hypothalamic nucleus
LRH-1/ NR5A2	Ovary, testis, liver, pancreas, intestine, early embryo	Steroidogenesis Ovulation Bile acid synthesis Glucose metabolism	Embryonic lethal around E6.5-7.5 d

Because gonads disappear prior to male sexual differentiation, the internal and external urogenital tracts of SF-1 knockout mice are of the female type, irrespective of genetic sex. Heterozygous SF-1 knockout mice show decreased adrenal volume associated with impaired corticosterone production in response to stress^[45-47], whereas transgenic overexpression of SF-1/Ad4BP increases adrenal size and ectopic adrenal tissue in the thorax^[48,49]. Total SF-1 disruption in mice demonstrated that SF-1 is crucial for the determination of steroidogenic cell fate *in vivo*. It has also been shown in Leydig cell and granulosa cell-specific knockout (LCKO and GCKO, respectively) models that SF-1 plays important roles in steroidogenesis following the development of steroidogenic organs. In LCKO mice, testicular steroidogenic acute regulatory protein (StAR) and Cyp11a1 expression is impaired, indicating a defect in androgen production^[50]. Consistent with this hypothesis, the testes fail to descend (an androgen-dependent developmental process) and are hypoplastic. In GCKO mice, the ovaries are hypoplastic, adults are sterile and ovaries show reduced numbers of oocytes and lack corpora lutea^[51]. Gonadotropin-induced steroid hormone production are also markedly reduced in this model.

LRH-1 knockout mouse embryos die around E6.5-7.5 d^[52,53]. Moreover, heterozygous and GCKO models revealed the importance of LRH-1 in steroidogenesis^[41,54,55]. In heterozygous Lrh-1-deficient male mice, testicular testosterone production is decreased along with the expression of steroidogenic enzymes and the development of sexual characteristics^[54]. In addition, GCKO mice are infertile because of anovulation with impaired progesterone production^[41]. It has also been demonstrated that LRH-1 has a broader role beyond steroidogenesis in these cells as they fail to luteinize.

Although SF-1 and LRH-1-deficient models revealed a common function in gonadal steroidogenesis, both factors cannot compensate for the deficiency of the other factor, even in cells expressing both factors. These facts indicate that even although SF-1 and LRH-1 control transcription by binding to the same response sequences,

each has selective actions on the pattern of gene expression in the development of steroidogenic cells and steroidogenesis.

DIFFERENTIATION OF MSCS INTO STEROIDOGENIC CELLS

In an early study, forced expression of SF-1 has been shown to direct differentiation of murine embryonic stem cells (ESCs) toward the steroidogenic lineage and then Cyp11a1 mRNA was expressed after the addition of cAMP and retinoic acid^[56]. However, the steroidogenic capacity of these cells is very limited and they do not undergo *de novo* synthesis because progesterone is the only steroid hormone produced in the presence of the exogenous substrate, 20 α -hydroxycholesterol. In addition, major differences between these differentiated cells and natural steroidogenic cells have been shown in cholesterol delivery and the steroidogenic pathway, including deficiencies of StAR (cholesterol delivery protein from the outer to inner mitochondrial membrane in steroidogenic cells) and steroidogenic enzymes, except for Cyp11a1 and Hsd3b1^[56-58]. It is also very difficult to isolate clones expressing SF-1 from ESCs and induced pluripotent stem cells^[37,57,59] because SF-1 (and LRH-1) overexpression is cytotoxic to these cells. These studies clearly indicate that SF-1 initiates the fate-determination program of the steroidogenic lineage in stem cells, although it is not completed in pluripotent stem cells.

Based on these results, we focused on MSCs^[57], multipotent adult stem cells that have been shown to differentiate into mesodermal lineages, such as adipocytes, chondrocytes, osteoblasts and hematopoietic-supporting stroma, both *in vivo* and *ex vivo*^[60-63]. Furthermore, MSCs are able to generate cells of all three germ layers, at least *in vitro*. Although MSCs were originally discovered in bone marrow (BM-MSCs)^[60,64-66], they have also been isolated from various origins, such as fat, placenta, umbilical cord blood and other tissues^[62,63,67-69]. In addition to their multipotency, MSCs have attracted considerable interest for use in cell and gene therapies because they can be obtained from adult tissues and suppress immune responses^[70,71]. Indeed, their therapeutic applicability has been assessed in some cases and particularly in bone tissue engineering^[72,73].

Induction of MSC differentiation into steroidogenic cells *in vivo* and *in vitro*

To investigate the potential of MSCs to differentiate into steroidogenic cells, BM-MSCs from GFP-transgenic rats were transplanted into prepubertal testes (Figure 1A)^[57]. In testes, there are two different steroidogenic populations, fetal and adult Leydig cells^[74-76]. Even although the cells in these two populations share a common characteristic of producing androgen, they are different in their origin, ultrastructure, lifespan, steroidogenic pathway and its regulation. Fetal Leydig cells have multiple origins and

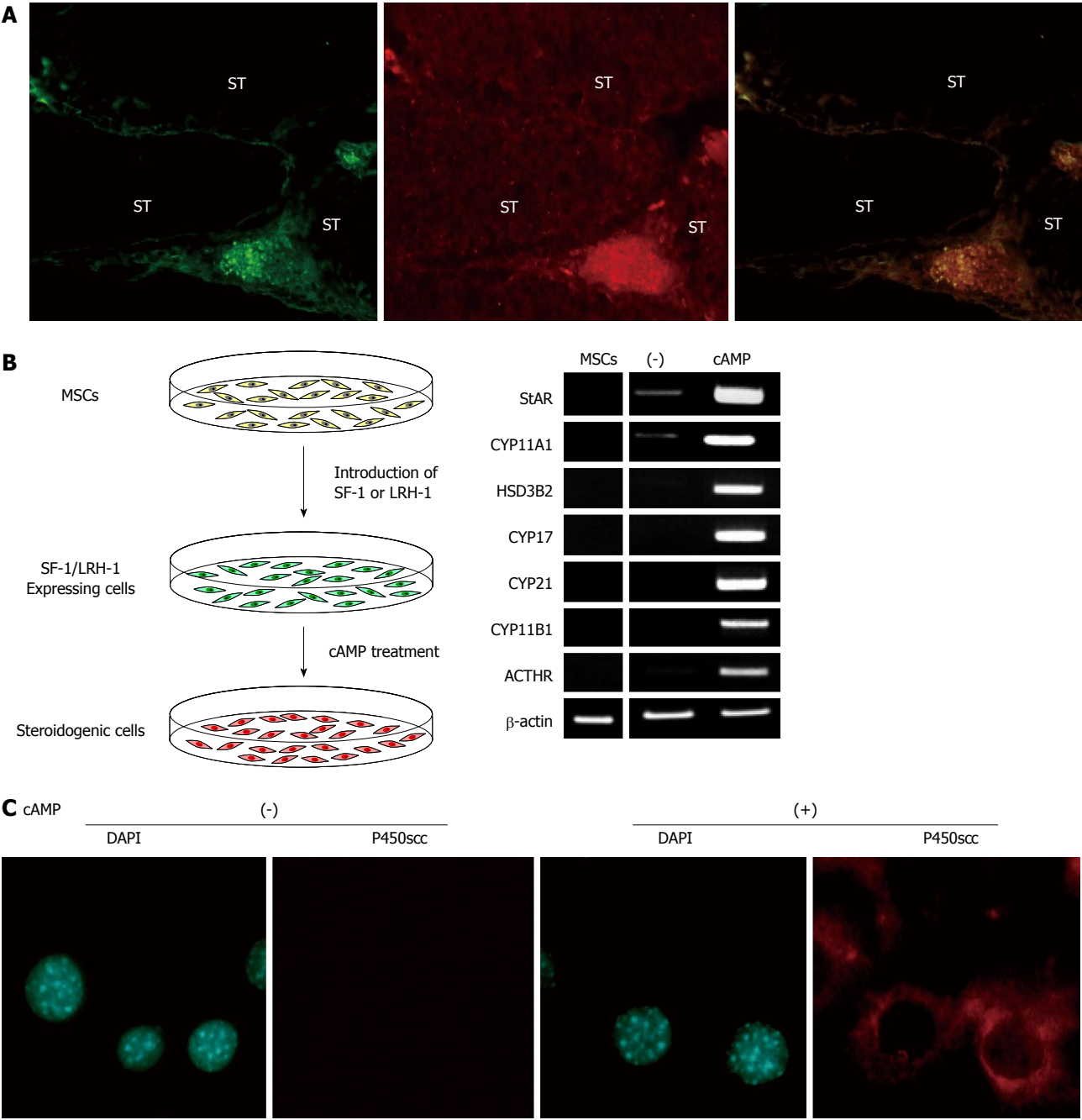


Figure 1 Differentiation of mesenchymal stem cells into steroidogenic cells. A: Transplantation of GFP-positive MSCs into prepubertal testis. Double staining of frozen sections from the testis 5 wk after MSC transplantation with anti-GFP and anti-P450scc antibodies; B: Protocol for generating steroidogenic cells from MSCs, and gene expression pattern of steroidogenic cells derived from hBM-MSCs; C: Fluorescence images of DAPI staining and P450scc immunostaining of SF-1 introduced BM-MSCs cultured with or without cAMP. ST: Seminiferous tubule. MSC: Mesenchymal stem cell.

appear in the interstitial space to induce sex differentiation just after the formation of the testis cord. Adult Leydig cells, which originate from mesenchymal precursor cells present in the testicular interstitium, appear to induce puberty. During the postnatal period, fetal Leydig cells are replaced by adult Leydig cells in prepubertal testis. Therefore, it should be possible to use transplanted BM-MSCs in such conditions *in vivo*. Indeed, after 3 wk, transplanted GFP-positive cells were located in the interstitium and expressed various steroidogenic enzymes for androgen production (P450scc/Cyp11a1, 3 β -HSD I and

Cyp17). These results indicate that MSCs have the capacity to differentiate into steroidogenic Leydig cells *in vivo*. Although these data suggest that the injected stem cells differentiated into Leydig cells, the apparent stem cell plasticity may also be explained by possible cell-nuclear fusion between donor and recipient cells. However, purified murine BM-MSC lines spontaneously differentiate into steroidogenic cells *in vitro*^[57]. A human *CYP11A1* promoter-driven GFP reporter, which consisted of a 2.3-kb fragment that drives reporter gene expression selectively in adrenal and gonadal steroidogenic cells^[77],

Table 2 Properties of steroidogenic cells derived from mesenchymal stem cells induced by steroidogenic factor-1/liver receptor homolog-1 and cAMP

Cells	Origin	SF-1/LRH-1	Produced	Properties of differentiated cells
KUM9	Mouse Bone marrow	Plasmid	Testosterone	Testicular leydig cells
hMSC- TERT-E6/7	Human Bone marrow	Plasmid Retrovirus	Cortisol Cortisol	Adrenal fasciculata cells
UE7T-13	Human Bone marrow	Retrovirus	Testosterone, cortisol	Fetal adrenal-like cells
UE6E7T-12		Retrovirus	Testosterone, cortisol	
UE6E7T-11		Retrovirus	Testosterone, cortisol	
UCB408E6E7T-33	Human Umbilical cord blood	Retrovirus	Progesterone cells	Ovarian granulosa-luteal cells

has been transfected into BM-MSCs to detect cell populations committed to the steroidogenic lineage. In some transfected cell lines, GFP fluorescence was detected in very small populations that were also positive for Cyp11a1. Further analysis showed that these cells expressed several Leydig cell markers, including 3β -HSD type I and VI and luteinizing hormone (LH) receptor. These observations further support the *in vivo* findings that MSCs have the capacity to differentiate into steroidogenic cells, even under the isolated condition. Therefore, part of population of MSCs can spontaneously differentiate into steroidogenic cells *in vitro*. Interestingly, SF-1 expression was also detected in the GFP-positive cells.

Differentiation of MSCs into steroidogenic cells induced by SF-1 and LRH-1

The above mentioned results strongly suggest that SF-1 can effectively direct the differentiation of MSCs into the steroidogenic lineage. Indeed, MSCs completely differentiate into steroidogenic cells and show their phenotype after stable expression of SF-1 (using plasmids or retroviruses) and cAMP treatment (Figure 1B)^[36,37,44,57,78,79]. SF-1 by itself induces morphological changes in BM-MSCs, such as the accumulation of numerous lipid droplets, although these cells hardly express steroidogenic enzyme genes or produce steroid hormones at detectable levels. However, SF-1 expressing cells strongly become positive for CYP11A1/Cyp11a1 after cAMP treatment (Figure 1C). These cells express many other steroidogenesis-related genes (*SR-BI*, *SLAR*, *3β-HSD* and other P450 steroid hydroxylases) and autonomously produce steroid hormones, including androgen, estrogen, progesterone, glucocorticoid and aldosterone. Notably, this approach differentiates human BM-MSCs into high cortisol-producing cells in response to ACTH, which are very similar to fasciculata cells in the adrenal cortex (Figure 1B). Adenovirus-mediated transient expression of SF-1 also differentiates BM-MSCs into steroidogenic cells with the capacity of *de novo* synthesis of various steroid hormones^[80-84]. After transplantation into animal models, these MSC-derived steroidogenic cells can improve symptoms of steroid hormone deficiencies caused by adrenalectomy. However, as mentioned above, these methods are not applicable to ESCs, embryonal carcinoma cells and terminally differentiated cells, such as fibroblasts and adi-

pocytes^[37,57,81]. These results indicate that MSCs are suitable stem cells for differentiation of steroidogenic cells. This hypothesis is supported by the fact that after pre-differentiation into MSCs, ESCs can also be subsequently differentiated into steroidogenic cells using SF-1^[37].

As in the case of SF-1, introduction of LRH-1 (using retroviruses) into BM-MSCs with the aid of cAMP induced the expression of steroidogenic enzymes and differentiation into steroid hormone-producing cells^[44]. Expression of SF-1 was never induced in LRH-1-transduced cells and vice versa. Therefore, LRH-1 could act as another master regulator for determining the MSC fate to the steroidogenic lineage. This phenomenon is likely to represent a situation of active progesterone production in human corpus luteum; LRH-1 is highly expressed, whereas SF-1 is expressed at very low levels^[36,42].

MOLECULAR MECHANISMS OF DIFFERENTIATION

Steroidogenic cells derived from various MSCs and their properties

In addition to BM-MSCs, various MSC types have been differentiated into steroidogenic cells by the above mentioned methods. However, their steroidogenic properties markedly vary and depend on the derivation tissues and species (Table 2)^[36,42,57,83,84]. For example, hBM-MSCs differentiated into cortisol-producing adrenocortical-like cells and umbilical cord blood (UCB)-derived MSCs differentiated into granulosa luteal-like cells, which produced high levels of progesterone^[36,57]. Gondo *et al.*^[83] also reported that steroidogenic profiles of adipose tissue-derived MSCs were markedly different from those of BM-MSCs prepared from the same mouse. However, the cell differentiation fate was consistent in each MSC. These findings suggest that the steroidogenic properties of the differentiated cells depend on the characteristics of the originating MSCs.

To determine the difference between BM-MSCs and UBC-MSCs, the fluctuations in gene expression were investigated by a DNA microarray^[36,85]. Among the identified genes, peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) was expressed only in UBC-MSCs at relatively high levels. Consistent with these re-

sults, the expression of PGC-1 α was observed in ovarian granulosa cells. Overexpression of PGC-1 α in granulosa cells induced the genes essential for progesterone synthesis, whereas knockdown of PGC-1 α in granulosa cells attenuated the expression of these genes. These results demonstrate that PGC-1 α represents one of the important factors for progesterone production in luteinized granulosa cells.

Epigenetic regulation during differentiation

Differentiation of stem cells into specialized cells can be viewed as a process in which epigenetic changes result in alterations in genes expressed by the cell as it becomes more specialized^[86,87]. Thus, stem cell differentiation is a process that involves a series of epigenetic changes in the genome: histone and DNA modifications cause chromatin structural changes and affect the profiles of gene expression. In fact, such epigenetic modifications contribute to the induction of steroidogenesis-related genes when MSCs differentiate into steroidogenic cells^[44,88-90].

The histone code hypothesis predicts that post-translational modifications of histone tails, alone or in combination, function to direct specific and distinct DNA-templated programs^[91]. Histone acetylation is a positive marker of transcription, while histone methylation correlates with transcriptional activation (H3K4, H3K36) and repression (H3K9, H3K27) that are dependent on their amino acid residues^[92]. In hMSCs-derived steroidogenic cells, H3K27 acetylation and H3K4 dimethylation (active enhancer markers) increased in the regulatory regions of some steroidogenesis-related genes (glutathione S-transferase A and ferredoxin reductase) after the introduction of SF-1^[89,90]. Conversely, histone eviction, which has been reported in actively transcribed genes^[93], took place on the promoter and the enhancer regions of the *SLAR* gene^[88]. Because these modifications occurred around the SF-1 binding sites, recruitment of SF-1 to the regulatory regions is likely to induce recruitment of various transcriptional regulators and histone modifiers, which in turn alter chromatin structure and lead to the expression of steroidogenesis-related genes.

In addition to histone modifications, DNA methylation at cytosine residues of the dinucleotide sequence CpG, which induces gene silencing, is essential for differentiation and development^[94,95]. In MSC-derived steroidogenic cells, the DNA methylation status changes in the promoter regions of some steroidogenic genes during differentiation^[44]. In undifferentiated hBM-MSCs, the *CYP11A1* promoter region is hypomethylated, whereas the *CYP17A1* promoter region is highly methylated. In SF-1/LRH-1-introduced MSCs during cAMP treatment, this condition was almost completely unchanged in the *CYP11A1* promoter region, whereas the *CYP17A1* promoter region was progressively demethylated. These methylation patterns of the *CYP11A1* and *CYP17A1* promoters closely paralleled the induction patterns of both genes by cAMP. There is a time lag associated with the induction of steroidogenic enzymes by

cAMP treatment in SF-1/LRH-1-introduced MSCs^[44,57]. The order of induction of the enzymes is similar to the sequential order of the steroid hormone synthesis pathway; upstream enzymes (CYP11A1 and 3 β -HSD) were rapidly induced at earlier time points (6-12 h), whereas downstream enzymes (CYP17A1 and CYP11B1) were induced at later time points (24-48 h). Because this time lag disappeared by treatment with a demethylating agent, the status of DNA methylation in the promoter regions could be important for regulating the expression of steroidogenic enzymes in MSCs.

CONCLUSION

It is clear that SF-1 represents a master regulator, not only for the development of steroidogenic organs, but also for steroidogenesis following organogenesis. LRH-1 is also important for steroidogenesis in gonads. In addition, SF-1 and LRH-1 direct differentiation of non-steroidogenic stem cells into steroidogenic cells. Among the various stem cell types, MSCs are suitable stem cells for the differentiation of steroidogenic cells. After pre-differentiation into MSCs, pluripotent stem cells can also be subsequently differentiated into steroidogenic cells using SF-1. These cells may provide a source for regenerative and gene therapies, although various problems should be resolved in future studies. It is essential to delineate the conditions that allow the directed differentiation into specific steroidogenic lineages with the characteristics of testicular Leydig cells, ovarian granulosa and theca cells, as well as various types of adrenocortical cells (reticularis, fasciculata and glomerulosa). In addition, it is necessary to establish methods for inducing SF-1 and LRH-1 expression in stem cells without gene transfer. Further studies are required for the realization of regeneration of steroidogenic tissues.

MSC-derived steroidogenic cells also provide opportunities for investigating various phenomena involved in differentiation of steroidogenic cells and steroidogenesis. In addition to the molecular mechanisms of differentiation described herein, the conservation and evolution of the androgen metabolic pathway (11-ketotestosterone production) between teleost fish and mammals has been revealed^[78,96]. Genome-wide analyses of differentiated cells identified novel target genes regulated by SF-1 and LRH-1^[89,90,97,98]. In addition, they contributed to the elucidation of one of the causes of steroidogenesis disorders^[99-101]. Thus, progression of these studies is also important for the understanding of steroidogenesis and its related disorders.

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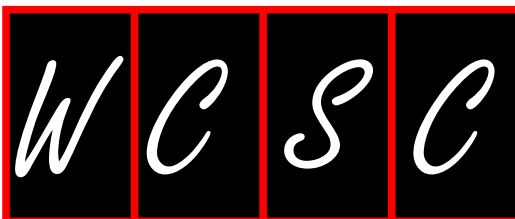
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WCSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Adipose-derived mesenchymal stromal/stem cells: An update on their phenotype *in vivo* and *in vitro*

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Abstract

Adipose tissue is a rich, ubiquitous and easily accessible source for multipotent stromal/stem cells and has, therefore, several advantages compared to other sources of mesenchymal stromal/stem cells. Several studies have tried to identify the origin of the stromal/stem cell population within adipose tissue *in situ*. This is a complicated attempt because no marker has currently been described which unambiguously identifies native adipose-derived stromal/stem cells (ASCs). Isolated and cultured ASCs are a non-uniform preparation consisting of several subsets of stem and precursor cells. Cultured ASCs are characterized by their expression of a panel of markers (and the absence of others), whereas their *in vitro* phenotype is dynamic. Some markers were expressed *de novo* during culture, the expression of some markers is lost. For a long time, CD34 expression was solely used to characterize haematopoietic stem and progenitor cells, but now it has become evident that it is also a potential marker to identify an ASC subpopulation *in situ* and after a short culture time. Nevertheless, long-term cultured ASCs do not express CD34, perhaps due to the artificial environment. This review gives an update of the recently published data on the origin and phenotype of ASCs both *in vivo* and *in vitro*. In addition, the composition of ASCs (or their subpopulations) seems to vary between different laboratories and

preparations. This heterogeneity of ASC preparations may result from different reasons. One of the main problems in comparing results from different laboratories is the lack of a standardized isolation and culture protocol for ASCs. Since many aspects of ASCs, such as the differential potential or the current use in clinical trials, are fully described in other recent reviews, this review further updates the more basic research issues concerning ASCs' subpopulations, heterogeneity and culture standardization.

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Key words: Adipose-derived stromal/stem cells; Adipose tissue; Subpopulation; Heterogeneity; Phenotype; CD34; Mesenchymal stem cells

Core tip: Adipose tissue is a rich, ubiquitously available and easily accessible source for multipotent stromal/stem cells. This review gives an update of the recently published data on the origin and phenotype of adipose-derived stromal/stem cells (ASCs) both *in vivo* and *in vitro*. Furthermore, since many aspects of ASCs, such as the differential potential or the current use in clinical trials, are fully described in other recent reviews, this review also updates the more basic research issues concerning ASCs' subpopulations, heterogeneity and culture standardization.

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INTRODUCTION

The isolation and culture of mesenchymal multipotent

stromal/stem cells (MSCs), beginning with the pioneering work of Friedenstein *et al*^[1,2] nearly 50 years ago, and the introduction of the nomenclature “marrow stromal stem cells^[3]” and “mesenchymal stem cells^[4]” (MSCs), opened up a new field of stem cell research. In order to address the discrepancy between the nomenclature and biologic characteristics of MSCs, the International Society for Cellular Therapy recommended that MSCs, regardless of their tissue origin, be termed multipotent mesenchymal stromal cells, while the term mesenchymal stem cells should only be used for the subset (or subsets) that meets specified stem cell criteria^[5-7]. On the other hand, Arnold Caplan, who coined the term MSC, has recently further proposed naming these cells “Medicinal Signalling Cells” to preserve the MSC acronym and, in his opinion, correctly explain their function^[8]. Obviously, this term only describes MSCs’ function in pathological situations and, therefore, ignores their physiological functions as structural cells in the haematopoietic stem cell niche of the bone-marrow. Furthermore, whereas MSC differentiation may not be the main regenerative mechanisms in cell therapy, the multipotent character of these preparations has been shown *in vitro* and this is the main intention for their usage in tissue engineering approaches.

In general, MSCs are described as immature cells within the bone-marrow, peripheral blood, menstrual blood, and nearly all adult tissues (*e.g.*, adipose tissue, synovium, dermis, periosteum, deciduous teeth) and solid organs (*e.g.*, liver, spleen, lung)^[9-11]. MSCs are a rare and quiescent population (or populations) within the perivascular niche (or are derived from perivascular cells or pericytes^[12]) within fully specialized tissues. MSCs derived from different tissues not only share many similarities, but also seem to have many differences in terms of their marker expression and their biological properties (*e.g.*, differentiation potential). It has been shown, for example, that MSCs from different tissue origin of the same donor differ in some features^[13]. Whereas MSCs isolated from the bone-marrow and cardiac tissue (cMSCs) shared a common stromal surface phenotype, their gene, microRNA and protein expression profiles were remarkably different. cMSCs were less competent in acquiring the adipogenic and osteogenic phenotype, but showed a higher cardiovascular differentiation potential.

There is a large number of studies showing that cultured MSC preparations are heterogeneous and consist of different populations of stem and progenitor cells with self-renewal properties and established multipotent differentiation profiles^[14]. In general, MSCs are isolated by their capacity to adhere to cell culture plastic surfaces. The cells can be expanded in culture while maintaining their multipotency in standard culture conditions, and are phenotypically characterized *in vitro* by a specific panel of markers. In this context, it should be mentioned that the clear characterization of MSCs remains difficult due to the lack of a unique cellular marker^[7]. In 2006, the International Society for Cellular Therapy proposed minimal phenotypic criteria for the definition of cultured MSCs: expression of CD73, CD90, and CD105, and lack of

CD11b or CD14, CD19 or CD79, CD45, and HLA-DR expression^[5,7]. [It should be noted that the main criteria for MSCs are (1) plastic adhesion; (2) the above described phenotype; and (3) their tri-lineage differentiation potential^[5]]. In this position statement, the society also specified CD34 as a negative marker for MSCs^[5], but recent reports have shown that this marker must be highlighted separately due to the tissue from which the MSCs were isolated (discussed later in this review).

Nevertheless, independent from the term used for MSCs and independent from its mechanism of action during repair or regeneration (*e.g.*, paracrine stimulation, immunomodulation, angiogenic effect, differentiation), it should be noted that MSCs have been proven to be beneficial in different medical treatments and exert positive therapeutic effects and proregenerative activities. It has been shown that MSCs secrete cytokines, growth factors and bioactive molecules with trophic, paracrine effects at variable concentrations in response to local microenvironmental cues, which seems to be the main (but maybe not the only) mechanism for their regenerative and repair potential^[15]. MSCs have also been shown to possess immunogenic properties and a powerful immunosuppressive potential, which also make them attractive for allogeneic cell therapy^[16-19]. MSCs are attractive cells for clinical applications to repair or regenerate damaged tissues, especially because they hold no ethical concerns (in contrast to embryonic stem cells). Furthermore, MSCs from an autologous origin seem to be a safe source for cell-based regenerative approaches. However, ideal MSCs for use in therapeutic approaches need to be isolated with minimal harm to the patient, must be available in high cell numbers, proliferate in culture, and differentiate into a broad spectrum of lineages^[7].

MESENCHYMAL STROMAL/STEM CELLS FROM FAT

It has been shown over the past few decades that adipose tissue is in addition to his main function as an energy reservoir also a abundant resource for multipotent stromal cells. The dissociation method and the biological characterization of these stromal cells from adipose tissue was first shown nearly 40 years ago^[20,21], but their multipotent character was first confirmed only at the beginning of the recent millennium^[22,23]. Adipose tissue seems to be the ideal source for multipotent stromal/stem cells as it has several advantages over other sources^[7]. Subcutaneous fat is omnipresent in humans and is easily accessible in large quantities by liposuction aspiration. Liposuction is a well-tolerated and almost safe procedure yielding large quantities of tissue aspirate^[7]. Furthermore, the lipoaspirate is finally discarded as medical waste, qualifying this as a good starting material for adipose-derived stromal/stem cell (ASC) isolation. In addition, the tissue contains a large number of multipotent cells which can easily be isolated and proliferated in culture. Stem and precursor cells in the freshly isolated stromal vascular fraction (SVF)

usually account for up to 3%, and this is approximately 2,500-fold more than the frequency of stem cells in bone-marrow (up to 0.002%)^[24].

However, it is important to mention that many different names and abbreviations for these cultured adipose-derived cells can be found in the literature generating a confusing discrepancy. The terms “adipose-derived adult stem cells”, “adipose-derived adult stromal cells”, “adipose-derived stromal cells”, “adipose stromal cells (ASC)”, “adipose mesenchymal stem cells”, “preadipocytes”, “processed lipoaspirate cells”, “vascular stromal/stem cells” and “adipose-derived stromal/stem cells (ASCs)” for cells isolated by an almost similar isolation procedure have been used in the last ten years. It should also be noted that others use the abbreviation ASC for adult stem cells in general. In order to eliminate this discrepancy, the International Fat Applied Technology Society (IFATS) reached a consensus to use the term “adipose-derived stromal/stem cells” (ASCs) to name the plastic-adherent, cultured and serially passaged, and multipotent cell population from adipose tissue^[7,9,25]. In 2013, the IFATS published a revised statement to point out the minimal phenotypic criteria to characterize the uncultured SVF and the adherent stromal/stem cell population from adipose tissue^[26]. In the SVF, native ASCs are now characterized as CD45⁺/CD235a⁺/CD31⁺/CD34⁺ cells, which represent approximately 20% of the whole SVF^[26]. The authors proposed the inclusion of CD235a (glycophorin A) to monitor any contaminating erythroid lineage cells. The leukocyte common antigen CD45 should be used as a classic marker to identify cells of haematopoietic origin (except for red blood cells) and CD31 (PECAM-1) to detect endothelial cells and their progenitors (and also platelets and leukocytes)^[26]. The authors further state that cultured ASCs are characterized as CD73⁺/CD90⁺/CD105⁺/CD44⁺/CD45⁺/CD31⁺ cells^[26]. Furthermore, cultured ASCs can be distinguished from BM-MSCs by their expression of CD36 and their negativity for CD106^[26]. Nevertheless, more characterization studies are needed to identify the *in vivo* counterpart(s) of the ASC population(s).

IS THE *IN SITU* LOCALIZATION AND PHENOTYPE OF ASCS SHOWN CONVINCINGLY?

Several studies have tried to identify the origin of the stromal/stem cell population within adipose tissue *in situ*. This is a complicated attempt because no marker has been described recently which unambiguously identifies native ASCs. Traktuev *et al*^[27] demonstrated that ASCs are rarely distributed among adipocytes, but are predominantly associated with vascular structures in the walls of adipose microvasculature (with a CD34⁺/CD31⁺ phenotype). They detected a portion of CD34⁺ cells co-expressing CD31 (capillary endothelial cells), and a separate and predominant population of CD34⁺/CD31⁺

cells (ASCs) in a perivascular location using immunofluorescence staining.

Corselli *et al*^[28,29] proposed that blood vessels in virtually all tissues house MSCs in a perivascular niche. The group also described a perivascular cell subset (including pericytes in small vessels and adventitial cells around larger vessels), which natively expresses MSC markers and displays a multilineage differentiation potential *in vitro*^[28,30-32]. The cells were extensively branched and are located in non-muscular vessels, capillaries and venules^[30]. They demonstrated that these perivascular cells (or pericytes) express CD146, neuro-gial proteoglycan 2 (NG2), CD140β, and also co-express MSC-specific markers (CD44, CD73, CD90, CD105)^[31]. However, this subset was shown to be negative for CD34 expression. Due to the phenotype with the expression of CD146 shown, the authors hypothesized that pericytes are an *in vivo* counterpart of cultured MSCs, but questioned whether all MSCs are derived from pericytes^[29]. Zannettino *et al*^[33] also described CD146⁺ (co-localized with the mesenchymal marker Stro-1 and the pericyte marker 3G5) cells within adipose tissue, which reside perivascularly and show the biological characteristics of MSCs *in vitro*. Nonetheless, as shown by their CD146 expression, this cell population is clearly distinct from the population described by others^[27,34,35] and it seems likely that these cells are a different subset of ASCs or pericytes.

In context with many other studies, it seems likely that the *in vivo* counterparts of ASCs express CD34. Maumus *et al*^[36] have shown that ASCs are scattered *in situ* in the fat stroma, express CD34⁺ and do not express pericyte markers such as NG2, CD140α, and α-smooth muscle actin (SMA) *in situ*. They identified ASCs *in situ* by their CD34 expression and discriminated them from endothelial, pericytes and other perivascular cells by immunofluorescence staining of human native adipose tissue^[36]. Unfortunately, the authors did not further characterize these CD34⁺ cells *in situ* by additional staining for other markers of ASCs. It has also been speculated that ASCs (and MSCs in general) are localized within blood vessels as a subset of pericytes or vascular precursor (stem) cells at various stages of differentiation located in the wall surrounding the vasculature^[34,37]. The same group demonstrated in a newer publication that ASCs exist as CD34⁺/CD31⁺/CD140α⁺/SMA⁺ cells in capillaries and in the adventitia of the vasculature^[37]. They speculated that ASCs in capillaries coexist with pericytes and endothelial cells (and that both are progenies of ASCs), whereas ASCs exist in the adventitia of larger vessels as specialized fibroblasts with stem cell properties^[37]. Zimmerlin *et al*^[35] also encouraged the hypothesis of a perivascular localization of ASCs. This study has shown CD90⁺/CD34⁺/CD31⁺/CD146⁺/SMA⁺ cells in the outer adventitia of blood vessels, and postulated this population as supra adventitial ASCs. Furthermore, the authors detected cell populations which may represent transitional stages between undifferentiated stromal cells (ASCs) and perivascular cells (pericytes). These “transitional” cells were

characterized by their marker expression and their adipogenic differentiation potential, and clearly discriminated against endothelial cells. These perivascular cells are organized in two discrete layers (CD146⁺/CD34⁺ pericytes and CD146⁺/CD34⁺ supra adventitial ASC), whereas a CD146⁺/CD34⁺ subset suggests a population transitional between pericytes and ASCs.

In summary, the results from recent histological studies using immunological staining techniques suggest that ASCs reside in a (peri-)vascular location, where they co-exist with pericytes and endothelial cells. Nevertheless, the exact location within the vascular niche (adventitia, inner intima, subendothelial) has not been precisely determined. It seems clear that there is a close relationship between tissue-resident stem/progenitor cells (MSCs/ASCs) and vascular pericytes. With regard to a subendothelial location, some authors concluded that pericytes are the *de facto* MSCs^[9,12]. These authors suggest “MSCs (or even pericytes) stabilize blood vessels and contribute to tissue and immune system homeostasis under physiological conditions and assume an active role in the repair of focal tissue injury^[9]”. However, many studies demonstrated a phenotypic difference between ASCs and pericytes. Recent studies provided much evidence that native ASCs *in situ* express a CD34⁺/CD90⁺/CD31⁺/CD45⁺/CD146⁺ phenotype. However, a definite phenotype of ASCs *in situ* has not been convincingly shown. Several studies contradict the expression of some markers (especially CD146 and CD34). Therefore, it is important to mention that the results of many studies suggest that ASCs (and MSCs in general) may be comprised of subsets or subpopulations at various stages, perhaps with varying differentiation potentials.

ASCs CHANGE THEIR PHENOTYPE EARLY IN CULTURE AND CONSIST OF SUBPOPULATIONS

In general, ASCs are isolated by plastic adherence from adipose tissue using the so-called SVF, regardless if isolated from a subcutaneous or a perirenal fat source or any other fat tissue. The percental cellular composition of the stroma vascular fraction has been described with large lab-to-lab variability, whereas it seems unquestionable that the SVF consists of adipose stromal (stem and progenitor) cells, and also endothelial cells, fibroblasts, lymphocytes, monocyte/macrophages, and pericytes, among others (*e.g.*, haematopoietic stem cells, erythrocytes)^[19,26]. Several subpopulations in the SVF have been cytometrically identified, including potential ASCs (CD31⁺/CD34⁺/CD45⁺/CD90⁺/CD105⁺/CD146⁺), endothelial (progenitor) cells (CD31⁺/CD34⁺/CD45⁺/CD90⁺/CD105^{low}/CD146⁺), pericytes (CD31⁺/CD34⁺/CD45⁺/CD90⁺/CD105⁺/CD146⁺), and blood-derived cells (CD45⁺)^[7,38]. However, native ASCs could not be clearly separated from the whole heterogeneous mixture as they share membrane antigens with other cells found

in the SVF. This is indeed based on the fact that the term ASCs is related to the plastic adherent and cultured population, which dramatically changes the phenotype very early during cell culture.

After adherence to cell culture plastic, these ASC preparations are less heterogeneous than the SVF, but they are not a homogeneous culture. However, more than 85% of the initially adhering cells are shown to express CD34 and do not express CD31, CD45, and CD146^[39]. The cells of this fraction are characterized early during primary culture by a slightly heterogeneous morphology indicating different stem and progenitor cell subsets, and (perhaps) more differentiated cells (dedifferentiated endothelial cells, smooth muscle cells and pericytes)^[7,39]. Many researchers have described and compared the expression profile of the cultured ASC. They have shown the alterations during culture passaging, and described a dynamic phenotype which changes during cell culture^[17,22,39-42]. Immediately after the cell isolation procedure, ASCs do not consistently express all characteristic MSC markers which are supposed to be expressed no matter from where the ASCs are derived. It has been shown that some specific surface markers (*e.g.*, CD105, CD166) increase during culture, while the expression of others decreases (*e.g.*, CD34)^[17,39]. Later on during culture, the heterogeneity of ASCs decreases, leading to the finding that the characteristic marker expression of ASCs (and MSCs in general) depends on the culture conditions (or environment) and time in culture. ASCs in passage 2 or 3 are morphologically a homogeneous population of fibroblastoid cells. These cells uniformly express the characteristic MSC markers: CD29, CD44, CD73, CD90, CD105, and CD166, and lack expression of CD11b, CD14, CD31, and HLA-DR. Nevertheless, different subpopulations can also be detected in these cultures^[43]. In a recent study, we analysed subsets/subpopulations of cultured ASCs by multicolour flow cytometry. In this study, we also characterized the overall phenotype of cultured ASCs using a high throughput technology with a screening panel of 242 antibodies and assessed the donor-dependent variations of the ASC phenotype^[43]. Unfortunately, due to the high number of cells which are needed to perform this assay, we were not able to analyse ASCs very early after isolation to further investigate phenotypic alterations during time in culture. However, we analysed specific subsets of ASCs in culture (Passage 2-4, median culture time 45 d), demonstrating CD34⁺, CD36⁺, CD200⁺ and CD201⁺ subsets. All of them co-expressed the MSC-characteristic antigens CD73, CD90 and CD105^[43]. Several other studies analysed the subpopulations of ASC preparations or the properties of the subsets. These subsets were characterized either in the SVF or whole ASC cultures or isolated using flow cytometric or immunomagnetic sorting. Kawamoto *et al*^[44] sorted murine ASCs due to a different expression of CD90, and demonstrated CD90^{high} and CD90^{low} subpopulations. CD90^{high} ASCs had a greater reprogramming capacity, and also showed increased numbers of alkaline phosphatase-positive colonies compared

to the CD90^{low} subpopulation. It has also been shown that sorted human CD90^{high} ASCs are more potent for osteogenic differentiation compared to CD90^{low}, CD105^{high} and CD105^{low} subpopulations^[45].

Others demonstrated a small subpopulation of pluripotent stem cell-like cells, termed adipose-multilineage differentiating stress enduring (adipose-Muse) cells, which can be identified as CD90⁺/CD105⁺/SSEA-3⁺ cells with ASC preparations^[46]. Importantly, this subpopulation of ASCs was shown to be able to cross the boundaries from mesodermal to ectodermal or endodermal lineages even under cytokine induction^[46]. The existence of an ASC subpopulation that expresses SSEA-4, a marker usually associated with pluripotency, has also been shown and isolated by immunomagnetic cell sorting^[47,48]. The cells have been shown to exhibit a higher potential for endothelial, osteogenic and adipogenic differentiation compared to whole ASCs. On the other hand, it has been shown that MSC preparations from adipose tissue lack a CD106⁺ subpopulation, whereas this subset is present in MSC preparations from bone-marrow and umbilical cord blood, and most prominent from placental chorionic villi^[49].

AND WHAT ABOUT CD34?

CD34 was specified as a negative marker for MSCs in the position statement of the International Society for Cellular Therapy from 2006^[5]. For several years, many researchers have followed this statement. It was generally accepted that ASCs do not express CD34 either, even though first reports about CD34 expression early after primary isolation were published^[17,38]. One explanation is that many studies which showed the absence of CD34 used plastic-adherent cultured ASCs in higher passages, and did not investigate the expression of CD34 earlier in their cultures. For a long time, CD34 was solely used as a marker for haematopoietic stem and progenitor cells, but endothelial progenitor cells, skeletal muscle satellite cells and other precursors were also shown to express CD34^[50,51]. Moreover, there are studies that provided convincing evidence that BM-MSCs also express CD34^[52-54]. Nevertheless, expression of CD34 decreases in the following passages and gets totally lost during cell culture^[17,38]. This cell culture-related loss of CD34 expression has also been described in other cells *in vitro* (e.g., endothelial cells, haematopoietic stem cells)^[52].

The percentage of SVF cells expressing CD34 has been reported with great variability among authors^[35,39,42,55]. It has been shown that up to 85% of the cells in the stroma vascular fraction express CD34^[39,50,56]. Two days after plastic adherence, more than 95% express CD34, co-express mesenchymal (CD10/CD13/CD90) and pericytic markers (CD140a and -b), and are CD31⁺/CD45⁻^[27]. Furthermore, different CD34⁺ subpopulations were described^[39,42,57]. Astori and co-workers identified two CD34⁺ populations (CD34^{dim} and CD34^{bright}) in the SVF with a marked difference in the intensity of antigen expression,

the majority of the cells expressing CD34 at low intensity^[42]. Nevertheless, they found that only approximately 7% of the SVF cells expressed CD34. Others also described several CD34⁺ subpopulations in the SVF^[57]. Beside the assumed ASCs (CD34⁺/CD31⁺/CD146⁻), they identified endothelial cells (CD34⁺/CD31⁺/CD146⁺), haematopoietic stem-like cells (CD34⁺/CD45⁺) and vascular smooth muscle cells/pericytes (CD34⁺/CD31⁺/CD146⁺)^[57]. An adipose tissue resident macrophage population expressing CD34 (and co-expressing the macrophage marker CD206) within the SVF has also been described^[58]. Furthermore, several haematopoietic CD34⁺ subpopulations (co-expressing CD45) were also described, but these were eliminated by the following plastic adhesion.

Others isolated the CD34⁺ subpopulations from the SVF by an immunomagnetic method and characterized the native and cultured cells^[56]. In this study, about 40% of the whole SVF cells were described as CD34⁺, with an increase of CD34-expressing cells in the adhered and cultured cell population (to 80%). They also described that the proliferation rate of the isolated CD34⁺ population(s) was negatively correlated with the decrease of the antigen in the following passages. A study by Suga *et al*^[59] opposed the sorted CD34⁺ to the CD34⁻ subpopulation of shortly cultured ASCs. They described that CD34⁺ cells proliferate faster and formed more colonies, whereas the CD34⁻ cells differentiate better into adipo- or osteocytes. In addition, the CD34⁺ subpopulation expressed some endothelial markers and, therefore, correlates with endothelial characteristics (progenitors?). On the other hand, the CD34⁻ cells expressed pericyte markers CD146 and NG2^[59]. The authors further speculated that CD34 expression in human ASCs correlates with replicative capacity, differentiation potential, expression profiles of angiogenesis-related genes, and immaturity or stemness of the cells. The loss of CD34 expression may be related to the physiological process of commitment or differentiation. Others concluded that the decrease of CD34 expression depends on the environment because of the cultured cells' lack of their specific *in vivo* microenvironment^[52]. Furthermore, the kinetics of decrease seems to vary strongly between different studies, depending on the culture conditions (e.g., plating density, culture medium)^[38,50]. In a recent study, we showed a specific subset in cultured ASCs (passage 2-4) which is positive for CD34 and co-expressed the MSC characteristic antigens CD73, CD90, and CD105^[43]. We also detected other subsets (e.g., CD36⁺, CD200⁺, CD201⁺), but detected no double positive subpopulation for these markers (e.g., a CD34⁺-CD36⁺ subpopulation). However, it should be mentioned that the percentages of these subsets varied between isolations from different donors. It has also been described that the culture medium influences the decrease of CD34^[38]. CD34 expression was maintained for at least 10-20 wk by using a cell culture medium supplemented with acidic fibroblast growth factor^[38]. Scherberich *et al*^[50] described that the CD34 expression in ASCs is maintained when the cells were cultured in a model recapitu-

lating the complex microenvironment of their niche. In this three-dimensional physiological environment, ASCs persist in a CD34⁺/CD31⁻/CD105⁻ phenotype for up to six weeks in culture.

Finally, there are some technical difficulties concerning the verification of CD34 expression which should also be pointed out. Firstly, we were only able to detect CD34⁺ cells using a PE-labelled antibody. If we stained the same cell population with a FITC-labelled antibody, we were not able to detect these cells in our multicolour cytometric analyses. Furthermore, it has been described that there are multiple classes of CD34 antibodies recognizing unique immunogens and influencing the signal^[60]. Bourin *et al*^[26], therefore, recommended the use of class III CD34 antibodies (*i.e.*, clone 581 or 4H11) for SVF cell characterization^[60].

According to current published data, CD34 is a potential marker that can be used to identify an ASC subpopulation *in situ* and after a short culture time^[26]. Nevertheless, it should be noted that there is the possibility that CD34⁺ ASC subsets *in vivo* also exist. As the proof of CD34 expression in ASCs has only quite recently been accepted, it is not surprising that little is known about the functional role of CD34 in ASCs. Recently, it has become more and more accepted that MSCs (or subsets) *in vivo* also express CD34^[52]. Two recent reviews excellently summarized the current knowledge of the expression of CD34 by MSCs in general^[52] and ASCs^[50].

DO WE COMPARE “THE SAME ASCs”? THE PROBLEM WITH DONOR-SPECIFIC DIFFERENCES AND CULTURE STANDARDIZATION

One of the main problems in comparing results from different laboratories is the lack of standardized methodologies to culture ASCs. The composition of ASC subpopulations varies between different isolations^[61] and the phenotype of ASCs display a dynamic phenotype during cell culture also due to culture conditions. Heterogeneity of ASC and MSC preparations and cultures has been discussed in many publications^[7,62-64]. It has been described, for example, that single-cell-derived clonal MSC populations are also highly heterogeneous and contain undifferentiated stem/progenitors and lineage-restricted precursors with varying capacities to proliferate and differentiate^[65,66]. Therefore, the resulting variability limits the standardization of MSC-based repair strategies and impedes the comparison of clinical study outcomes^[65].

The heterogeneity of ASC preparations may result from different reasons; some of them can be influenced by the researchers, some not. First of all, the donors from which ASCs are isolated. These donors differ in age, body mass index, gender, ethnicity, and existing diseases^[7]. The negative correlation of the body mass index, for example, and the number of stromal cells per gram and their differentiation capacity has been shown^[67]. Our

recent data also revealed donor-specific differences in the composition of ASC subpopulations^[43]. A total of forty-nine cellular surface markers in a comprehensive phenotyping study showed a high variability in their expression between the donors. Anyway, all cells expressed the main characteristic markers (CD73, CD90, CD105). Expression of CD36 and CD34 from different donors, for instance, varied highly from no expression, scattering of fluorescence intensity to highly expressed. In summary, albeit positive for the main characteristic markers, the cells also differ in their expression of some other markers^[43]. In conclusion, it is extremely difficult, if not impossible, to standardize these donor-related variables^[7].

On the other hand, further points to consider are the liposuction procedure, which may differ between different clinics, the time lapse until cell isolation procedure starts, or the temperature at which the lipoaspirate is stored until cell isolation. It has been reported that liposuction side and liposuction procedure influences the cell yield, proliferation capacity and frequency of isolated stromal cells^[57,67-70], but it is unclear whether this promotes different subpopulations in the isolates.

Next, a standardization of the isolation and culture conditions may increase the comparability of the results from different laboratories^[7]. The first critical step is the point in time after which the initial cultures are washed (*i.e.*, the initial time for adherence). It has been shown that ASCs' heterogeneity can be reduced by a washing procedure early after plating the SVF^[71], indicating that several subsets require different time points to adhere to the cell culture plastic. Another effort to reduce the heterogeneity of ASCs was carried out by using flow cytometric sorting or immunomagnetic separation, either by positive or by negative selection for a specific marker^[7,71-74]. Nevertheless, usage of such techniques only select specific subpopulations which must be evaluated separately, and studies using such isolation cannot be compared with results from ASCs.

In addition, no unique and standardized culture protocol for ASCs has been accepted overall. There are different variables that may impair ASCs (and their subsets) in their undifferentiated state: density of initially plated cells, surface-coating, culture medium composition, supplements (bovine serum, human serum, platelet lysate, or growth factors), oxygen partial pressure, antibiotics, method of subculturing, and method of cryopreservation. Only limited information is available about which medium optimally expands ASCs by maintaining the undifferentiated character *in vitro*^[59,75,76]. In MSC cultures, it has been shown that basal medium, glucose concentration, quality of fetal calf serum, cell plating, and cell density highly affects the final outcome resulting in the expansion of populations with totally different potential^[7,77]. Culture medium composition also affects the expression stem cell-related transcription factors NANOG, Oct-4, Sox-2, and Rex-1^[7,76]. Many investigators use Dulbecco's modified Eagle medium (DMEM) as a standard medium for ASCs, but no further description of the DMEM

used in the study is given. We always utilize low-glucose DMEM (physiological glucose content, 100 mg/dL), whereas others use DMEM with a higher glucose content because ASCs cultured in this medium show a much better proliferation rate. Nevertheless, the glucose content is one variable which must be considered to be near to the *in vivo* situation. Using high glucose medium raises the question about the effects of such a “diabetic” environment on the cells. In most cases, ASCs were cultured with foetal calf or bovine serum as a proliferation supplement. Related to a possible use of ASCs in human therapeutic approaches, there are many concerns about the usability of foetal calf or bovine serum (infectious complications, host immune reactions)^[78]. The usage of low-serum containing culture media supplemented with recombinant growth factors [e.g., epidermal growth factor, platelet-derived growth factor and/or basic fibroblast growth factor] has been described^[79-82]. Low doses of bone morphogenic protein 4 have also been shown to stimulate ASC proliferation^[83]. Nevertheless, the gold standard for culturing ASCs will be a medium absolutely free from animal serum or factors, with well-known ingredients^[7].

In summary, modifications in the isolation and/or culture conditions might select for the expansion of subpopulations and have a huge impact on the differentiation potential of the cells cultured, albeit the primary cells could be phenotypically identical if characterized with a standard marker panel^[64]. Therefore, standardization of the isolation and culture procedure is highly necessary for a good reproducibility of results from different laboratories and studies^[7].

WHAT NEEDS TO BE RESOLVED?

Although ASC preparations are already used in different clinical trials, many questions concerning their counterparts and biology *in situ*, differentiation potential *in vitro* and *in vivo*, and also the mechanism of repair or regeneration (paracrine effects, differentiation, immunomodulation) are not completely understood or still unsolved. Research goes on and therapy approaches are also possible without the exact knowledge of the mechanisms as long as they are safe and beneficial for the patient.

However, what are the main questions in basic ASC research which need to be resolved? Many recent histological studies have tried to identify native ASCs (or ASC subsets) *in situ* due to their expression of some markers, but the exact phenotypic definition of the cells/cell populations we call ASCs relies solely on the analysis of a culture-expanded preparation. Therefore, there is the possibility that the phenotype and potential of ASCs/ MSCs varies between *in vivo* and *in vitro* settings provoked by the isolation technique and culture conditions. Despite intense investigation, the physiological role of the native ASCs in adipose tissue (and MSCs in general) *in vivo* is not fully understood. Therefore, it is extremely important to overcome the lack of standardization in order to abolish the variability in cell quality (if not solely based

on donor-specific variabilities). In addition, alternative culture methods should be developed to avoid the loss of CD34 expression and to preserve a physiological phenotype^[50]. Research progress has also been hampered by the limited knowledge of the subsets/subpopulations of ASCs, due to the lack of unique subset markers for their characterization^[7]. A lot of work still remains.

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WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Periosteum derived stem cells for regenerative medicine proposals: Boosting current knowledge

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Abstract

Periosteum is a thin fibrous layer that covers most bones. It resides in a dynamic mechanically loaded environment and provides a niche for pluripotent cells and a source for molecular factors that modulate cell behaviour. Elucidating periosteum regenerative potential has become a hot topic in orthopaedics. This review discusses the state of the art of osteochondral tissue engineering rested on periosteum derived progenitor cells (PDPCs) and suggests upcoming research directions. Periosteal cells isolation, characterization and migration in the site of injury, as well as their differentiation, are analysed. Moreover, the role of cell mechanosensing and its contribution to matrix organization, bone microarchitecture and bone strength is examined. In this regard the role of periostin and its upregulation under mechanical stress in order to preserve PDPC survival and bone tissue integrity is contemplated. The review also summarized the role of the periosteum in the field of dentistry and maxillofacial reconstruction. The involvement of microRNAs in osteoblast differentiation and in endogenous tissue repair is explored as well. Fi-

nally the novel concept of a guided bone regeneration based on the use of periosteum itself as a smart material and the realization of constructs able to mimic the extracellular matrix features is talked out. Additionally, since periosteum can differentiate into insulin producing cells it could be a suitable source in allogenic transplantations. That innovative applications would take advantage from investigations aimed to assess PDPC immune privilege.

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Key words: Periosteum; Mesenchymal stem cells; MicroRNA; Bone tissue engineering; Bone turn-over

Core tip: Periosteum provides a niche for pluripotent cells. Elucidating periosteum regenerative potential is a hot topic in orthopaedics. This review discusses the state of the art of osteochondral tissue engineering rested on periosteum derived cells and suggests upcoming research directions aimed to the development of new standards of care for the maintenance of bone mass both in post-trauma healing process and in physiological turn-over.

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INTRODUCTION

The field of Tissue Engineering and Regenerative Medicine (TERM) has burgeoned in the last decade. The term "Regenerative Medicine" was first found in a 1992 Kaiser *et al*^[1] paper as "a new branch of medicine that

attempts to change the course of chronic disease and in many instances will regenerate tired and failing organ systems”.

Products for regenerative medicine can consist in proteins, able to stimulate endogenous repair, living cells or even organs. Advances in regenerative medicine applications have been useful to develop new standards of care for the treatment of several diseases such as neurological, cardiovascular, metabolic (*e.g.*, diabetes), oncologic and orthopaedic disorders.

The idea of using cells to restore damaged tissue is intuitively based on their native role in tissue development and homeostasis. Cells could be delivered to the patient alone or combined with a natural or synthetic biomaterial. The interactive “diamond” concept of TERM suggests that in addition to cell type, 3D dimensional structure/architecture, mechanical/physical signals, and bioactive factors in the environment are critical and act in concert to direct tissue repair and regeneration^[2]. Each of those areas is currently under dynamic investigation. In this review we will focus on cell-based therapeutic applications in skeletal tissue repair.

Mesenchymal stem cells (MSCs) represent the leading cell type for regenerative medicine purposes. They are multipotent stromal cells capable of both self-renewal and differentiation into lineages of mesenchymal tissue, including cartilage, bone, adipose tissue and skeletal muscle^[3]. MSCs were originally identified in the bone marrow stroma, where they regulate key stages of haematopoiesis. Ever since, they have been isolated from other anatomical sites, such as amniotic fluid^[4], Wharton’s jelly^[5], umbilical cord blood^[6], adipose tissue^[7], skin^[8], synovial membrane^[9], articular cartilage^[10] and compact bone^[11].

The main challenge in osteochondral tissue repair is the healing of critical-size defects that don’t bridge on their own. They result from pathological events (*e.g.*, tumour, trauma, inflammation or congenital malformation) and can lead to a delayed union or non-union fracture^[12]. Surgical procedures employed for bone gaps treatment may be time-consuming, expensive and exposing patients to high risk of complications and discomfort^[13]. To overcome these issues regenerative medicine is working to restore structure and function of damaged tissues by TERM approaches.

Since bone marrow contains osteogenic progenitors, its use was proposed to lead efficient bone regeneration and, effectively, preclinical and clinical investigations corroborated this speculation^[14]. Periosteum has been identified as an intriguing niche for cells of the osteoblastic lineage as well.

Periosteum is a specialized highly vascularized connective tissue that envelopes bone surfaces (Figure 1). It is composed of an external fibrous layer containing elastic fibres and microvessels and an inner cambium layer where reside periosteum derived progenitor cells (PDPCs) that act as major players in bone development and fracture healing^[13,15].

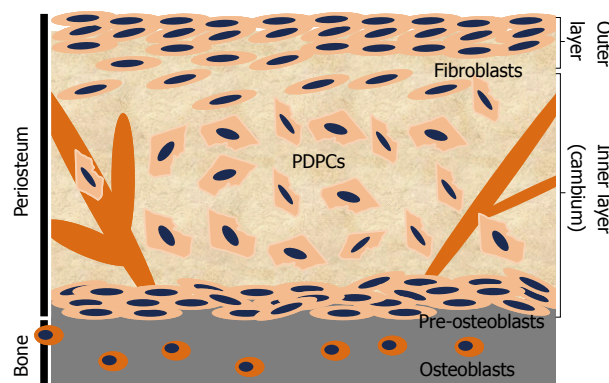


Figure 1 Schematic representation of periosteum as well as the distribution of cell populations and extracellular matrix that contribute to its biological and mechanical properties. PDPCs: Periosteum-derived precursor cells.

REGENERATIVE POTENTIAL OF PERIOSTEUM

The paramount importance of the periosteum in bone healing process was suggested since 1800 s when de Mourgues^[16] discovered that transplanted periosteal tissue induced new bone growth. In 1932, Fell^[17] was the first to successfully culture periosteum and in 1990s Nakahara *et al.*^[18] explored the osteogenic potential of PDPCs in bone tissue engineering. At the same time O’Driscoll *et al.*^[19] underlined the possibility to regenerate cartilage in damaged joints by periosteum transplantation.

The use of autologous periosteum graft has long been known in orthopaedic surgery. However, it’s only after recent progresses that the contribution of the different sources of MSCs in bone repair, as well as their response to growth factors favouring specific differentiation processes has been examined in depth.

Periosteum as a whole have been used in thousands of orthopaedic surgeries as covering layer in autologous chondrocyte transplantation^[20], in the treatment of non-union fractures^[21], as a graft for reconstruction of the patellar articulation^[22], or as tissue engineered bone transplant for maxillary sinus floor augmentation^[23].

However, only in 2009 Colnot^[24] provided direct evidence that periosteum, endosteum, and bone marrow are the major sources of skeletal stem/progenitors cells and that they differently contribute to osteogenesis and chondrogenesis. In bone healing, periosteum and endosteum both give rise to osteoblasts, whereas periosteum is the only source of chondrocytes. The distinct cellular contributions of periosteum, endosteum, and bone marrow suggested the presence of both intrinsic dissimilarities within these residing stem cell populations and differences in the tissue environment. The correct identification of *in vivo* adult skeletal progenitor sources as well as their response to nutrients, metabolites and growth factors will therefore have profound implications in cell-based therapies for the treatment of recalcitrant fractures or bone and cartilage diseases. Exploring and optimising

Table 1 Surface markers of periosteum-derived cells

		Ref.
Minimal criteria for MSCs		
CD73	+	[13,35,79,94]
CD90	+	[13,35,79,94]
CD105	+	[13,35,79,94]
CD45	-	[13,35,79,94]
HLA-DR	-	[13,35,79,94]
CD14	-	[13,35,79,94]
CD34	-	[13,35,79,94]
Integrins		
CD29	+	[13,94]
CD49e	+	[13,94]
Adhesion molecules		
CD31	-	[13,94]
CD44	+	[13,94]
CD166	+	[13,36,94]
CD54	+	[13,94]
CD146	+	[37,38]
MHC class		
HLA-ABC	+	[13,94]
Hematopoietic markers		
CD14	-	[13,94]
CD33	-	[13,94]
CD34	-	[13,94]
CD45	-	[13,94]
CD133	-	[13,94]
Additional markers		
MSCA-1	+	[93]
CD9	+/-	[13,36,94]
CD13	+	[37,38]
STRO-1	+	[37,38]
SSEA-4	+	[37,38]
ScaI	+	[37,38]
Sox2	+	[39]
Oct4	+	[39]
Nanog	+	[39]

CD: Cluster of differentiation; HLA: Human leucocyte antigen; MSCA-1: Mesenchymal stem cell antigen 1; STRO-1: Stromal cell antigen -1; SSEA-4: Stage specific embryonic antigens 4; ScaI: Stem cell antigen I; Sox2: Sex determining region Y-box 2; Oct4: Octamer-binding 4.

the governing factors that controls PDPCs osteogenesis and chondrogenesis will be a considerable benefit. It is worth noting that periosteum meets the three primary requirements for tissue engineering: cell font, scaffold for cell retaining and delivery, as well as source of local growth factors. These peculiar features endorse its use as a whole, in autologous grafts. The injection of cell suspensions and the transplantation of cells within scaffolds have been largely employed as well^[20,23,25].

PERIOSTEUM AS CELL SOURCE

PDPCs hold promise in osteochondral repair applications due to their ease of isolation and expansion potential. Several studies reveal periosteum as a better cell source for bone regeneration than either bone marrow or other mesenchymal cell origins. This is due to the fact that PDPCs display multipotency at single cell level^[3] and a higher proliferation rate while retaining their ability to differentiate *in vitro*^[26]. Furthermore, PDPCs from elderly show performances comparable to that of cells from

younger subjects^[3,27,28]. This may be related to telomeres stability, since *in vitro* analysis showed that after 24 population doublings telomere lengths and telomerase activity are similar to those of the parental population^[3].

Harvest site, donor conditions and technical factors could affect periosteum regenerative potential: load-bearing bones have a more osteogenic periosteum than flat bones, and also inter-individual differences influence periosteum biology^[29,30]. Moreover, resection methods and cell isolation procedure could affect periosteum regenerative properties as well. To this end, the use of instruments (like forceps) that can disrupt the inner cambium layer should be avoided^[13]. After dissection, cells are typically obtained by egression or enzymatic digestion. Despite of isolation method, culture expanded cells retain their osteochondral potential^[31,32]. Even though both techniques are commonly used, cell egression from their native environment may maintains their physiological state, without artefacts^[33]. The choice of basal medium is equally important to preserve MSC characteristics and multipotent properties, even after prolonged culture *in vitro*.

Despite there is still a lack of consensus on the ideal method of culturing MSCs, it has been demonstrated that the use of DMEM-F12 preserves MSC stemness and ability to differentiate for more than 25 sub-culture passages^[34].

A long-debated issue is the obtainment of a pure PDPC population, since no exhaustive markers to identify MSC populations are established. PDPCs were commonly characterized by the classic MSC antigenic profile in agreement with the minimal criteria of the International Society for Cellular Therapy (Table 1)^[35]. Yet, additional efforts are required to circumvent the isolation of contaminant cells, such as fibroblasts. The use of two additive surface markers, CD166 and CD9 and the comparison of their expression levels on MSCs and fibroblasts, could address this item (Table 1). The expression of CD166 is generally higher on MSCs than on fibroblasts, while CD9 expression has the opposite pattern^[36]. Moreover, MSCs with a “fibroblast-like” expression pattern (*i.e.*, low CD166 and high CD9) display a poor osteogenic differentiation^[36].

Further markers enable to identify periosteum mesenchymal progenitors (Table 1) could be STRO-1, stage-specific embryonic antigen-4, ScaI and CD146, also known as melanoma cell adhesion molecule^[37,38].

In addition, it could be helpful to evaluate the gene expression profile of transcription factors, such as sex determining region Y-box 2 (Sox2), octamer-binding 4 and Homeobox protein Nanog, associate to pluripotency and stemness^[39].

Population enrichment for a cell-type specific surface markers by cell-sorting is recommended, too. At last, novel isolation and characterization strategies, from a heterogeneous population, are currently developing. One example is an innovative droplet-based microfluidic device as a platform for the identification and quantification of distinct cell phenotypes^[30].

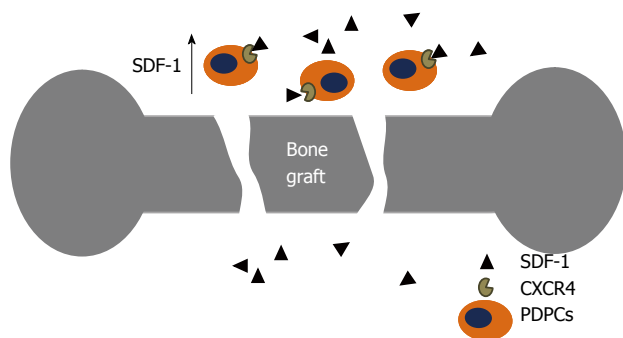


Figure 2 Stromal cell-derived factor 1/chemokine receptor 4 can recruit mesenchymal stem cells to induce fracture repair in skeletal healing. Stromal cell-derived factor 1 (SDF-1) is expressed on the periosteum of the bone graft and recruited chemokine (C-X-C motif) receptor 4 (CXCR4) expressing mesenchymal stem cells in the acute phase of bone repair. PDPCs: Periosteum-derived precursor cells.

MOLECULAR PATHWAYS IN PERIOSTEUM

The potential use of mesenchymal cells for *in situ* repair of osteochondral defects is related to their migration and homing. Understanding how MSCs migrate into tissue injured sites is therefore useful to augment cell transplantation efficiency by enhancing cell targeting.

PDPCs show a dose-dependent migratory effect under chemokine receptor ligands stimulation^[40]. Interestingly, PDPCs express chemokine (C-X-C motif) receptor 4 and chemokine (C-X-C motif) receptor 5 that respectively respond to the stromal cell-derived factor 1 (SDF-1) and B cell-attractive chemokine 1 (BCA1). Osteoblasts derived from post-traumatic or osteoarthritis patients express SDF-1 and BCA1 in the bone remodelling area, indicating the potential role of these chemokines not only as chemo-attractant but also as a signaling molecule for *in situ* bone regeneration. Additional studies showed that the expression of SDF-1 is up-regulated in periosteal cells at the sites of injury and it serves as a potent chemo-attractant to recruit circulating or residing CXCR4 expressing MSCs^[41], to promote their proliferation (Figure 2). Apparently, the involvement in PDPCs of the SDF-1/CXCR4 axis during bone repair has not been fully elucidated. However, SDF-1 or CXCR4 blocking clearly inhibits BMP2-induced osteogenic differentiation, probably interfering with Smads and MAP-kinase activation^[40].

Bone graft integration depends on the orchestrated activation of growth factors and cytokines in both host and graft. Activation, expansion and differentiation of periosteal progenitor cells act as an essential step for successful bone remodelling. Understanding the molecular events that initiate these actions (*e.g.*, BMP2 signaling) provides insights into endogenous regeneration of periosteum and offers information for optimizing tissue engineering constructs^[42].

BMP2 is a bone morphogenic protein that belongs to the transforming growth factor-beta (TGFβ) superfamily. TGFβ/BMPs signaling have widely recognized role

in bone formation during mammalian development. Signaling TGFβ/BMPs transduction is performed by both canonical Smad-dependent and non-canonical Smad-independent [*e.g.*, p38 mitogen-activated protein kinase pathway (MAPK)] pathways. Smad and p38 MAPK pathways converge to *Runx2* gene and control mesenchymal precursor cells differentiation^[43].

BMP2 is at the apex of the signaling cascade that starts periosteal progenitor proliferation and differentiation during repair and regeneration. *In vivo* studies highlight that in the absence of BMP2, periosteal progenitors remain quiescent and healing does not initiate^[44]. In addition, the expression of Sox9, a chondrogenic marker is reduced as well. Thus, BMP2 is essential for the activation of periosteal progenitor cells and their subsequent differentiation along the osteo-chondrogenic lineage^[44]. The relevance of BMP2 in triggering osteochondral tissue remodelling is related to its involvement in all crucial osteogenic pathways: Wnt/β-catenin cascade, Fibroblast growth factor-2 (FGF2) and Hedgehog (Hh) signaling^[43]. Multiple Wnt proteins and their modulators are expressed in periosteum. Their cross-talk with Hh intermediates enhances fracture healing^[42]. The role of Hh pathway in the promotion of osteogenic and chondrogenic differentiation of PDPCs in adult bone repair has been recently confirmed by *in vivo* investigations^[45]. FGF2 signaling has a critical function at the early stage of fracture repair, it improves new bone volume and mineral content and it also takes part in angiogenesis^[45].

BMP2 also functions as focal point for the interaction of Smad and Notch signaling during osteoblast differentiation. The latter enhances BMP-induced Alkaline Phosphatase (ALP) activity and formation of calcified nodules *in vitro*^[43,44].

In-depth knowledge on BMP2 and its related signaling-pathways, hence, would provide interesting targets to promote osteochondral repair.

It is also emerging that cartilage and bone regenerative techniques are related to nuclear factor kappa β (NF-κβ)/p65 signaling, which determines the early expression of Sox9 and facilitates the subsequent chondrogenic differentiation^[46,47].

MECHANOSENSING IN PERIOSTEUM

It is now well accepted that MSC differentiation and phenotypic expression can be influenced by cues from surrounding environment, both soluble (*e.g.*, cytokines and growth factors) and insoluble (*e.g.*, ECM density and stiffness). Due to its external localization on bone, periosteum is particularly sensitive to mechanical stimuli and, even in absence of other stimulations, mechanical load induces new bone formation from periosteum^[48], suggesting that this is a highly specialized mechanosensitive tissue^[13].

Several studies show that substrate stiffness affects cell shape thus controlling MSCs fate, including self-renewal and lineage commitment^[13]. The native environment of PDPCs is mechanically regulated by a com-

bination of tension and shear. PDPCs ability to carry intracellular tension through their microfilament network controls a signaling cascade that, in turn, is responsible for the expression of soluble factors that modulate bone and cartilage growth^[13].

In critical size defects, applying tensions in periosteum after surgery leads to rapid *de novo* bone healing. Therefore, mechanical signaling at the tissue level may be responsible for the start of bone regeneration at cell level^[13].

Periosteum mechanobiology is probably related to its local microstructure and collagen content^[13]. Some studies evidence the emerging role of periostin in the correct collagen fibrillogenesis. Periostin belongs to the matricellular proteins family and regulate cell functions and cell-matrix interaction. Periostin is expressed at high level in the periosteum during embryogenesis and it is re-expressed after mechanical stress and fracture^[48]. It is also present in connective tissues subjected to mechanical stress, such as periodontal ligament, heart valves and tendons. Periostin preferential expression in collagen-rich tissues submitted to mechanical stresses (*i.e.*, periosteum) suggests it may play an essential role in bone maintenance and regeneration^[48].

As matter of fact, the regulation of the periostin expression occurs by Wnt pathways; BMP2, TGF β and retinoic acid stimulate periostin expression as well^[49-51].

Through interaction with several integrins, periostin recruits and attaches osteoblasts to bone matrix and activates pro-survival signaling, by caspases inactivation, resulting in increasing bone formation^[48]. In addition, periostin interacts with BMP1 to augment its deposition in the fibronectin matrix, in close proximity of lysyl oxydase, an enzyme that catalyses the collagen cross-linking^[48]. At last, periostin has a binding site for glycoproteins, glycosaminoglycans and proteoglycans, suggesting a role of this protein in supporting mechanical strength in periosteum^[48]. Taken together these data suggest that periostin, contributing to matrix organization, bone microarchitecture and bone strength^[48], may acts as a support, thus playing a clear role in the intrinsic mechanobiology of periosteal tissue.

These insights in understanding and harnessing the innate mechanosensing of both periosteum and its cells provide a unique opportunity to induce differentiation without perturbing the biochemical environment^[14].

MICRORNAS AND PERIOSTEUM

MicroRNAs (miRs) are small noncoding RNAs that have emerged as crucial post-transcriptional regulators of gene expression by either inhibiting mRNA translation or inducing mRNA degradation^[52,53]. MiRs can be transcribed individually or in clusters and are encoded by introns or intergenic regions. After being transcribed, primary miRs are processed by protein complexes containing the endonuclease Droscha into the precursor miR (pre-miR), which is approximately 70 nucleotides. Pre-miR is subsequently exported to the cytoplasm^[52,53]. Next, the endonuclease

Dicer further cleaves the pre-miR, resulting in the generation of the approximately 22-bp miR duplexes, which are incorporated in the RNA-induced silencing complex. One strand is then retained in the complex and becomes the mature miR, which binds to the 3' untranslated region of the target mRNA.

Hundreds of miRs have been described and currently approximately 1500 miRs are considered to be expressed in humans. Each miR binds up to several hundred complementary mRNAs, thereby modulating gene expression patterns rather than single genes. In the past decade, miRs were extensively investigated and were shown to act as key players in various critical cellular processes such as proliferation, cell cycle progression, apoptosis and differentiation.

As far as stem and progenitor cells are concerned, distinct miRs regulate their functions, modulating cell survival and homing or controlling differentiation and maturation. Additionally, experimental studies shown that miRs regulate endogenous tissue repair and might potentially be useful to enhance bone regeneration^[54].

The switch between self-renewal and differentiation requires rapid widespread changes in gene expression. Since miRs can repress the translation of many mRNA targets, they are good candidates to regulate cell fate^[55]. Throughout recent years extensive molecular studies have unraveled genetic and epigenetic mechanisms involved in osteoblasts differentiation and functions^[54].

As mentioned above, differentiation of MSCs into the osteogenic lineage is tightly regulated by local growth factors (*e.g.*, BMPs, FGFs) that activate specific intracellular pathways, thus triggering the expression of crucial transcription factors such as Runx2 and Osterix (Osx)^[54]. miRs regulate each differentiation step by targeting multiple proteins and various signaling pathways, exerting a positive or a negative effect on osteogenesis.

MiR-29b, miR148b, miR196a, miR-210, miR-2861 and miR-3960 have been reported to cause down-regulation of various inhibitors of osteoblasts differentiation, thus exerting stimulatory effects. For instance, miR-29a potentiates osteoblastogenesis by modulating Wnt signaling through a positive feedback loop^[56].

On the contrary, miR138, miR-133 and miR-204 are associated with a low bone mineral density. Particularly, miR138 was shown to attenuate the ERK-dependent pathway, phosphorylation of Runx2, and Osx expression, being able to inhibit osteoblasts differentiation and bone formation by human MSCs both *in vitro* and *in vivo*^[57].

Elucidating the molecular mechanisms that regulate MSC differentiation is important not only for the treatment for orthopaedic trauma, but also for regenerative medicine purposes in case of the loss of functions that naturally occurs with age. Bone homeostasis is in fact strictly related to the balance between bone deposition and resorption as well as to the correct response to mechanical forces.

MiRs act as key regulators of both bone formation and remodelling and degeneration, as well. Deregulation of miRs-mediated mechanisms is pathologically linked

to bone-related diseases, such as osteoporosis^[58]. Indeed, since miRs control differentiation of osteoblast from stem cells and differentiation of osteoclasts from hematopoietic precursors^[58], deregulation at these levels could affect osteoclast-related bone remodelling^[58].

At present, no data are available on miRNA expression in periosteum. Therefore, profiling of miRs in PDPCs could be useful in elucidating crucial mechanisms governing pre-osteoblasts differentiation during bone development and remodelling. Moreover, advances in miR expression knowledge could also provide information on bone tissue metabolism during lifespan, with particular attention to changes related to inflammation and/or ageing.

PERIOSTEUM AND CARTILAGE REGENERATION

The chondrogenic potential of periosteum is well documented both *in vitro* and *in vivo*^[19,59], in fact free autogenous periosteal grafts restore cartilage defects^[60].

Immediately following cortical bone injury, periosteum undergoes a series of changes to initiate bone formation at the fracture site. Cells at the periphery of the cortex adopt an osteogenic fate whereas cells near the cortical bone junction differentiate into chondroprogenitors^[42]. Chondrocytes within the fracture callus are primarily derived from the periosteum inner cambium-layer as indicates the presence of Sox-9 expressing chondroprogenitor cells in the periosteum adjacent to the fracture site^[61].

The development and maturation of neochondrocytes involves several growth factors, encompassing insulin growth factor 1, TGFβ1, TGFβ3, growth differentiation factor 5 and BMP2^[62]. In addition the expression of adhesion molecules, such as N-cadherin, play a role in the regulation of chondrocytic phenotype^[63]. At last, for resurfacing arthroplasty in humans, periosteum has been used alone or in combination with continue passive motion to stimulate joint neochondrogenesis^[62].

With aging the chondrogenic potential of periosteum decreases, as the number of chondrocytes precursors decline in the cambium layer^[62]. However sub-periosteal injection of both TGFβ1^[64,65] and TGFβ3 has been shown to stimulate the proliferation of PDPCs and to induce their chondrogenic differentiation^[63]. Yet, a recent study showed that a subperiosteal injection of a chondroinductive growth factor mixture do not stimulate tissue differentiation of an autologous osteoperiosteal graft^[66]. This suggests that the repair of cartilage defects could benefit from an *in vitro* pre-treatment of micromass PDPCs cultures with TGFβ3, which improves periosteum ability to undergo chondrogenesis and produce hyaline cartilage^[66]. Quality of tissue harvest, choice and amount of appropriate stimulating molecule, time of exposure, as well as intervals between injections, may influence healing. Mechanical stimulations could affect the clinical outcome as well.

Tissue engineering approaches in cartilage tissue re-

generation could be also useful to potentiate the *in vivo* outcomes. Recently, Casper *et al*^[67] showed the potential of PDPCs to infiltrate poly-epsilon caprolactone (PCL) nanofiber scaffolds in a rabbit model and the possibility to produce engineered cartilage *in vitro*. The same group has also demonstrated that the application of a directional fluid flow to periosteal explants seeded onto PCL scaffolds enhances cell proliferation, chondrogenic differentiation and organization, thus modifying the biomechanical properties of the engineered cartilage^[68].

In order to generate 3D artificial cartilage resembling native articular one, a recirculating flow-perfusion bioreactor, which simultaneously offer shear stress and hydrodynamic pressure, was also developed and, in presence of periosteum/PCL constructs, good ECM composition, cell distribution and mechanical properties were obtained^[59].

PERIOSTEUM AND BONE HEALING

In fracture healing, periosteum is the major responsible for bridging the callus formation and participating to endochondral and intramembranous ossifications.

Steps of fracture bone repair have been well summarized by Shapiro^[69]. After fracture, cells from the inner cambium layer of periosteum proliferate and differentiate: at the periphery of the fracture the inner layer arranges a collar of bone by intramembranous ossification; nearer to the fracture site the cambium layer produces a mass of cartilage around the fracture location that, subsequently, undergoes to endochondral ossification^[69]. Osteoblastic potential of periosteum differs not just with age but also by location: calvaria periosteum showed less osteogenic potential than tibia ones^[29,70].

Even though the use of periosteal autografts for the treatment of bone fractures is a well-established procedure^[21,51], only recently it was demonstrated that autologous periosteal precursor cells cultured on a 3D matrix are responsible to promote the healing of a distal femur atrophic non-union^[71]. Unfortunately, autografts are not always feasible, also due to donor-site morbidity, and alternatives have to be sought. Indeed, the use of allografts for the treatment of critical sized bone defects remains a challenge. Allografts avoid donor site pain and morbidity and fill the need for large volumes of graft materials^[72]. Yet, clinical evidences showed that where periosteum orchestrates bone remodelling, allograft healing ability is lower if compared to autograft^[73]: allografts exhibit minimal engraftment and a 60% failure rate 10-years-post-transplantation^[74,75].

Alternatives to the use of native periosteum for critical size defects healing could be hence hypothesized. For instance, when periosteum contains too few PDPCs or has been damaged, it is possible to create a tissue engineered periosteum (TEP)^[13]. At present, few studies have well characterized TEP mechanical properties. Therefore, this approach is currently intended only for use in oral applications, where TEP would experience less mechani-

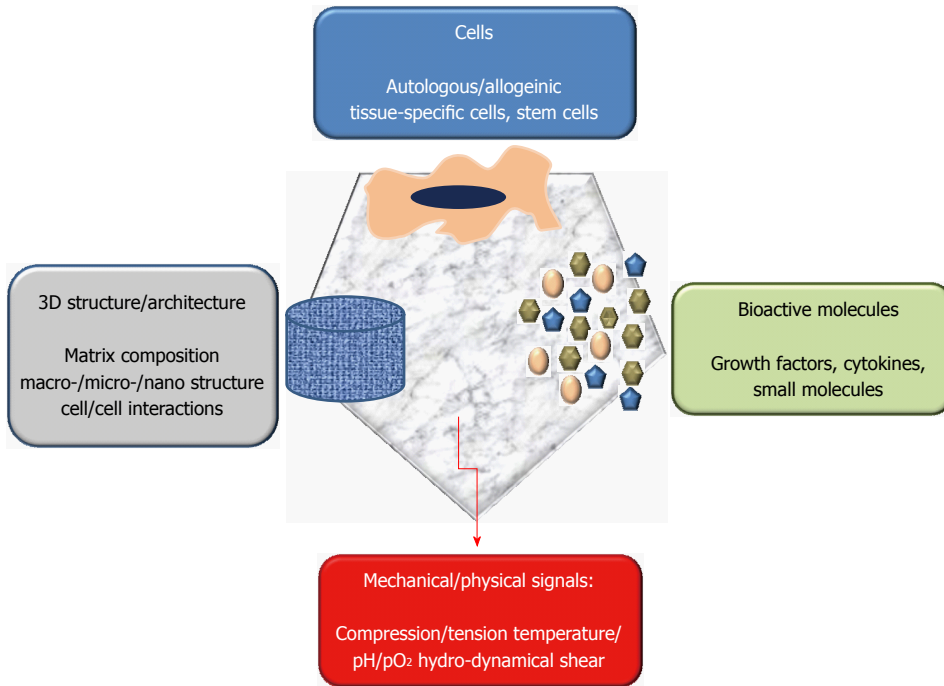


Figure 3 Interactive “diamond” concept of Tissue Engineering and Regenerative Medicine suggests that in addition to cell type, 3D dimensional structure/architecture, mechanical/physical signals, and bioactive factors in the environment are critical and act in concert to direct tissue repair and regeneration. Cell activity is dynamically regulated by the other key cornerstones of the diamond.

cal strain than in a dynamically loaded environment (*i.e.*, femur)^[13].

It has been a long time since the need to realize constructs that reproduce the intrinsic properties of autogenous bone, by culturing PDPCs *ex-vivo* and subsequently seeding into a natural or synthetic scaffold, has emerged^[33]. The success of this approach is strictly related to the use of an appropriate material able to improve PDPC differentiation, with a corrected structure/topography and able to provide adequate support for nutrients and growth factors^[2] (Figure 3).

For the development of an engineered tissue, elucidating the steps that can enhance PDPC osteogenic differentiation is advantageous as well^[76]. In mesenchymal stromal cells this involves the following processes: cell proliferation, cell migration-aggregation and cell differentiation with the dynamic expression of osteogenic transcription and growth factors^[77]. Moreover, early MSC osteogenic differentiation is characterized firstly by a proliferative burst, including the formation of nodule-like structures, accompanied by the expression of ALP.

To replicate this differentiation profile, PDPC culture conditions reproducing these key events are required. It has been widely demonstrated that under osteogenic conditions, PDPCs express mRNAs for bone markers (*e.g.*, collagen type I, osteopontin and osteocalcin), whilst in a chondrogenic environment they display chondrogenic markers such as collagen type II and aggrecan^[76]. Moreover, the addition of foetal bovine serum (FBS) and dexamethasone (Dex) to the culture media has a positive effects on osteocalcin and ALP expression, in the early differentiation stages^[78]. For the expression of the main transcription factors governing osteogenesis and

hence differentiation towards a mature osteoblast, the subsequent combination of trans-retinoic acid (atRA), FBS, Dex and BMP2 is required^[78]. At last, also vascular endothelial growth factor (VEGF) plays a role in osteogenesis and it is expressed in human normal periosteum as well as in periosteum after fracture healing: the addition of VEGF to a basal culture medium enhance PDPC osteoblastic differentiation. That was corroborated by our results as well^[79].

In bone tissue engineering approach, scaffolds are generally used as temporary substitutes of the original tissue after injury. As well-known, 3D scaffolds should be tolerated by the body, provide cell attachment, migration and proliferation, allow for biochemical signaling and possess a bone-like stiffness and degradation rate commensurate to bone healing^[2,80]. Canonical classification includes natural and synthetic scaffolds. Natural scaffolds such as chitosan, collagen, gelatine, fibrin glue and hyaluronic acid show several advantages, such as an ECM-like chemistry and structure, the presence of cell-adhesive sequences and a resorbability driven by enzymes, with the production of non-toxic easily excreted molecules. Natural materials are also often used as drug carriers for their aptitude to retain growth factors that encourage cellular migration and proliferation^[81]. Drawbacks in their use include limited availability, low mechanical resistance and potential immunogenicity^[80]. In this respect synthetic scaffolds display many advantages, encompassing easy modulation of chemical and mechanical properties, biodegradability and avoidance of infections or immunogenicity.

Hydroxyapatite or its analogues (including natural bone matrix) are the most popular inorganic components

for bone replacement, due to their chemical similarity to the mineral component of mammalian bone^[80]. Collagen/demineralised bone powder scaffold combined with PDPCs has been proposed as a potential tool for bone tissue engineering^[82]. In our experience scaffolds with an increased amount of inorganic phase were able to modulate stem cells behaviour^[11] as well as periosteal-derived stem cells osteogenic properties^[83]. The rationale for the use of Calcium Phosphate biomaterials and the evaluation of their bone forming capacity in the presence of PDPCs has been recently summarized by Roberts *et al*^[84].

Modern bone regenerative medicine strategies aim to “take lesson from Nature” in scaffold development. To this respect a chitosan-heparin coating acting as a synthetic periosteum was recently proposed for the improvement of bone allografts outcomes^[85]. Several biomaterials, such as naturally derived acellular matrices, commercially available collagen-based sponges and synthetic polymers^[86-88] have also been investigated as periosteum mimicking. These materials improve cell localization but show an inadequate cell survival^[86-88]. Instead, the use of hydrogels, which emulate mechanical properties and hydration of the native periosteum ECM, seems a promising approach. Hydrogels may be properly tailored for correct degradation, inclusion of biomolecules and cell-adhesion ligands in order to elicit a specific cell functions^[85]. It has been shown that hydrogel-based tissue engineered periosteum enhance osteoblast progenitor cells infiltration, bone callus formation and allograft biomechanical stability^[72,73].

Besides, peculiar surgical techniques have been used as a tool for mimicking periosteum. Since 1986 Masquelet^[89] developed a simple method to reconstruct long bone defects based on the insertion of a cement spacer that maintains the space for bone reconstruction and promotes the formation of a synovium-like membrane. This induced membrane (IM) prevents the graft resorption and favours its re-vascularization. Moreover, the membrane acts as an *in situ* growth factors delivery system, which is capable of enhancing bone graft healing^[89].

Recently, Cuthbert *et al*^[90] investigated the morphology, molecular properties and gene expression pattern of IMs from patients undergoing large bone defects surgery, showing that IMs share strong architectural similarities, vascular features and growth factor expression of periosteum^[90]. Moreover, cells expanded from IMs revealed a mRNA profile similar to PDPCs^[90]. Cuthbert *et al*^[90] thus, provided evidences that the IM technique generates a dynamic periosteum-like structure, offering important insights into new bone regeneration approaches. Nevertheless, further studies are required to establish if this surgical technique could be suitable for all bone regeneration applications despite of the nature of disease, the lesion site and the patient-related features.

PDPCS IN ORAL AND MAXILLOFACIAL TISSUE ENGINEERING

Periosteum has found great use in enhancing bone for-

mation in the field of dentistry and maxillofacial reconstruction^[91,92]. Even though human jaw periosteal cells (JPC) are a promising source for the engineering of cell-based osseointegrative grafts in oral surgery^[93], their harvesting and subsequent characterization is not particularly easy. Specific surface markers can facilitate the isolation of a cell pure population, while an accurate analysis of the gene expression profile can allow a detailed comprehension of the JPCs.

In the last years, several markers have been suggested to enrich the osteogenic progenitor cell fraction from the entire JPCs population. Among these, particular attention has received mesenchymal stem cell antigen-1 (MSCA-1) and CD166. MSCA-1⁺ enriched JPCs have an higher osteogenic potential compared with MSCA-1, as well as CD166⁺ respect to CD166⁻^[93]. Magnetic-activated cell sorting isolation technology was also recommended for increasing recovery and purity of rare MSCA-1⁺ cells from jaw periosteum^[93].

The high osteogenic potential of MSCA-1⁺ cell fraction is strictly related to the expression of specific markers, such as lipoprotein receptor-related protein 6 (LRP-6), a key component of the WNT receptor complex. MSCA-1⁺/LRP-6⁺ also induce an high expression of stanniocalcin 1 (STC-1) and of tissue inhibitor of metalloproteinases-4 (TIMP-4)^[93]. STC-1 is involved in endochondral and intramembranous bone formation while TIMP-4 is tangled in ECM remodelling during JPCs osteogenesis^[93].

In spite of PDPCs derived from periosteum, other sources of stem cells such as dental pulp^[94,95] and periodontal ligament^[95] have been proposed for dentistry applications. Harvest morbidity and patient acceptance should affect the final choice of the appropriate cell source for regenerative medicine purposes.

Cutting-edge applications

The great plasticity of mesenchymal stromal cells, due to their ability to differentiate into multiple lineages, makes them good candidates for *in vivo* regeneration innovative procedures. The use of allogeneic MSCs in regenerative medicine is also encouraged by their immunosuppressive and immunomodulatory features.

MSCs derived from different sources have been studied for the generation of Insulin-Producing Cells (IPCs) in the treatment of type 1 diabetes. Kim *et al*^[96] examined the differentiation in IPCs of MSCs isolated from different sources: bone marrow, adipose tissue, Wharton's jelly and periosteum. Even though cultured under similar conditions, only IPCs derived from PDPCs showed a significant increase in insulin secretion under glucose stimulation^[96].

These results indicate the periosteum as a suitable source of multipotent progenitor cells that could be employed in allogenic transplantations.

However, even if MSC immune privilege is well known for cells derived from bone marrow, umbilical cord blood and adipose tissue, no studies confirm that

PDPCs have similar properties. Therefore, this aspect needs to be further investigated in order to accomplish PDPCs innovative applications^[96].

CONCLUSION

Small bone defects can be bridged with conventional grafting^[97], whilst bone regeneration in large bone defects is challenging and several factors (*i.e.*, defect site and patient related factors) may affect treatment outcomes. The healing of large size defects, hence, looks into tissue engineering strategies, including the use of exogenous stem cells, growth factors and bioactive scaffolds^[2,98]. Recently relevant breakthroughs in designing and creating bone substitutes have been achieved. After the sophisticated approaches combining biomaterials/stem cell constructs the concept of a guided bone regeneration has received attention: the use of smart, bioactive-induced membranes started gaining momentum.

Scientific word is therefore going on with investigations understanding molecular basis of cell/tissue endogenous repair, as well as, improving scaffold design. To this end, periosteum will offer new intriguing cues for further investigation.

Periosteum plays a key role in ECM architecture and cell cytoskeletal reorganization under mechanical stress, by the activation of the mechanosensing signaling. The comprehension of cell molecular mechanisms associated with mechanosensing and cell intrinsic repair abilities has underlined a critical role of periostin. Its expression is up-regulated in the presence of mechanical stress in order to preserve bone tissue integrity and function. Periostin up-regulation leads to the activation of specific pathways that support cell survival. It also ensures a correct collagen fibrillogenesis and matrix organization, opening intriguing perspective in designing future strategies for bone tissue regeneration.

In addition, a further characterization of cellular epigenetic mechanisms miRs related is encouraged: directing the mRNAs expression, miRs affect pivotal differentiation pathways and could therefore represent important targets in promoting osteochondral regeneration.

Finally, considering periosteum dynamic response to environmental and mechanical stimuli, two strategies have been pursued: the use of periosteum itself as a “smart material” (*i.e.*, TEP) and the realization of constructs (*e.g.*, chitosan-heparin coating and PEG-hydrogels) able to mimic the ECM features of this tissue. At present TEP constructs are not tuned for the repair of a dynamically loaded environment such as long bones.

Taken together, the data highlight periosteum involvement in bone anabolic pathways and suggest novel TERM approaches in osteochondral tissue repair. Moreover, a deeper understanding of the molecular basis of cell mechanosensing, as well as of microRNA involvement in PDPC differentiation responses, could be useful for the development of new procedures for the maintenance of bone mass both in post-trauma healing process and in physiological turn-over (therefore preventing

osteoporosis).

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WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Mesenchymal stem cells for treatment of aortic aneurysms

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Abstract

An aortic aneurysm (AA) is a silent but life-threatening disease that involves rupture. It occurs mainly in aging and severe atherosclerotic damage of the aortic wall. Even though surgical intervention is effective to prevent rupture, surgery for the thoracic and thoraco-abdominal aorta is an invasive procedure with high mortality and morbidity. Therefore, an alternative strategy for treatment of AA is required. Recently, the molecular pathology of AA has been clarified. AA is caused by an imbalance between the synthesis and degradation of extracellular matrices in the aortic wall. Chronic inflammation enhances the degradation of matrices directly and indirectly, making control of the chronic inflammation crucial for aneurysmal development. Meanwhile, mesenchymal stem cells (MSCs) are known to be obtained from an adult population and to differentiate into various types of cells. In addition, MSCs have not only the potential anti-inflammatory and immunosuppressive properties but also can be recruited into damaged

tissue. MSCs have been widely used as a source for cell therapy to treat various diseases involving graft-versus-host disease, stroke, myocardial infarction, and chronic inflammatory disease such as Crohn's disease clinically. Therefore, administration of MSCs might be available to treat AA using anti-inflammatory and immunosuppressive properties. This review provides a summary of several studies on "Cell Therapy for Aortic Aneurysm" including our recent data, and we also discuss the possibility of this kind of treatment.

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Key words: Aortic aneurysm; Mesenchymal stem cells; Cell therapy; Elastin; Chronic inflammation; Extracellular matrices; Macrophages; Matrix metalloproteinases

Core tip: Aortic aneurysm (AA) is caused by an imbalance between synthesis and degradation of extracellular matrices (ECMs) such as collagen and elastin in the aortic wall. The chronic inflammation enhances the degradation of ECMs directly and indirectly. We hypothesized that administration of mesenchymal stem cells (MSCs) might be able to treat AA given the anti-inflammatory and immune-suppressive potential of MSC. In this article, we review papers that attempt to treat AA using MSCs with our recent results, as well as review the molecular pathogenesis of AA and characteristics of MSC.

Original sources: Yamawaki-Ogata A, Hashizume R, Fu XM, Usui A, Narita Y. Mesenchymal stem cells for treatment of aortic aneurysms. *World J Stem Cells* 2014; 6(3): 278-287 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i3/278.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i3.278>

INTRODUCTION

Trend of aortic aneurysm

An aortic aneurysm (AA) occurs mainly in aging and

chronic inflammation associated with atherosclerosis. It is a common and silent disease but also a life-threatening one involving rupture. AA has an incidence of 6%-9% in men over the age of 65 in abdominal aorta^[1,2]. AA larger than 55 mm in diameter in the abdominal aorta and 60 mm in diameter in the thoracic aorta increase the risk of rupture. Therefore, patients of the kind require surgical intervention such as prosthetic graft replacement to prevent rupture^[3]. However, surgery for thoracic and thoraco-abdominal aorta is a highly invasive procedure with high mortality and morbidity rate. On the other hand, abdominal or thoracic endovascular aneurysm repair (EVAR, TEVAR), which are catheter-based interventions, called internal aortic stent grafting, might be used for conventional surgically inapplicable patients with a high risk for surgical repair. However, EVAR and TEVAR have drawbacks such as limitations of anatomic and clinical criteria, complications of endoleaks, and graft migrations^[4]. Thus, an alternative less invasive strategy is required for treatment of AA.

Development of medical treatment for aortic aneurysm

Recently, the molecular pathology of AA has been clarified, and control of chronic inflammation is crucial for AA progression. AA is caused by an imbalance between synthesis and degradation of the extracellular matrices (ECMs) such as collagen and elastin in the aortic wall. Chronic inflammation enhances the degradation of ECMs directly and indirectly. Therefore, control of inflammation may be an alternative strategy for treatment of AA. A number of experimental investigations and clinical studies have attempted to treat AA using various drugs and factors to control the inflammation; for example, angiotensin converting enzyme inhibitor and statin associated with reduced abdominal AA (AAA) rupture in a case-control study^[5,6], doxycycline decrease in aneurysmal expansion rate in an experimental model^[7] and in a randomized double-blinded clinical trial^[8], nonsteroidal anti-inflammatory drugs decrease AAA expansion rate in a case control study^[9], and c-jun N-terminal kinase inhibitor regresses AAA in a CaCl₂-treated mice model^[10]. However, these pharmacotherapies have still not been established for clinical application because of their array of side effects caused by systemic administration of these agents. Another disadvantage of using these agents is that special equipment might be required to deliver them locally for treatment of AA.

Mesenchymal stem cell therapy

Meanwhile, the recent progress in stem cell research in regenerative medicine is remarkable. Stem cell is one of the most important cell sources for treatment of damaged organs using regenerative technology. Mesenchymal stem cells (MSCs) can be obtained from adult tissue such as bone marrow^[11,12], adipose tissue^[13,14] and others. MSCs can be differentiated into various types of cells such as osteoblast, adipocyte and chondrocyte. In addition, MSCs have anti-inflammatory and immunosuppressive proper-

ties as well that can be recruited into damaged tissue^[15,16]. By utilizing their unique potential, MSCs have been widely used as a cell source for cell therapy to treat various diseases involving graft-versus-host disease, stroke, myocardial infarction (MI), and chronic inflammatory disease such as Crohn's disease clinically^[17-21].

In this article, we review papers that attempt to treat AA using MSCs with our recent results, and we also discussed the update status of the molecular pathogenesis of AA and characteristics of MSC.

MOLECULAR PATHOGENESIS OF AORTIC ANEURYSMS

The molecular pathology of AA is a failure in the balance between synthesis and degradation of ECMs in the aortic wall. These phenomena are induced by chronic inflammation associated with atherosclerosis. Aortic ECMs are mainly composed of elastin and collagen and play an important role in the aortic strength and flexibility to withstand arterial blood pressure. Especially, elastin is a major fibrillar component in the arterial wall, and destruction of elastin fiber directly leads to expansion of AA^[22]. Elastin polypeptide is known to be synthesized by vascular smooth muscle cells (VSMCs)^[23], and its gene expression is modulated by transforming growth factor (TGF)- β 1 and insulin-like growth factor (IGF)-1^[24,25]. On the other hand, degradation of ECMs is caused by mainly secretion and activation of matrix metalloproteinases (MMPs), leading to the weakening of the aortic wall. In particular, MMP-2 and MMP-9 are known as a powerful proteinase that degrades elastin fiber, and they are secreted from macrophages which have infiltrated the inflammatory site^[26,27]. Macrophage plays a major role of inflammatory cells in the development and progression of AA, and also secretes various cytokines, chemokines and proteinases. Many studies have been reported that interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α and monocyte chemotactic protein (MCP)-1 were up-regulated in the AA wall of human or experimental animal aortic aneurysm^[28-30]. These cytokines and chemokines induce recruitment of monocytes^[31], apoptosis of VSMCs^[32] and regulation of MMP secretion^[33]. On the other hand, failure of ECM synthesis is reportedly due to a disability of ECM synthesis and decrease of cell number by apoptosis of VSMCs in the AA wall^[34]. Therefore, the inhibition of excessive inflammation and the recovery of ECM synthesis are key factors for treatment of AA.

DETERMINATION OF MSCS

Surface maker of the MSCs

MSC is one of the adult somatic stem cells which can be isolated from adult organs including bone marrow and adipose tissue^[13,35]. Early in culture, the spindle-shaped plastic-adherent cells do not appear uniformly by contamination of hematopoietic cells, but this heterogeneity gradually decreases influenced by culture conditions and

Table 1 Mesenchymal stem cells phenotypic characteristics

		Positive marker	Negative marker	Pluripotency	Ref.
ISCT criteria	Human MSC	CD73, CD90, CD105	CD34, CD45, CD11b or CD14, CD19 or CD79 α , HLA-DR	Osteogenic Chondrogenic Adipogenic	[38]
In AA experimental studies	Mouse BM-MSC	CD44, CD106, Sca-1	CD11b, CD31, CD34, CD45, CD86, CD117	Osteogenic Chondrogenic Adipogenic	[51,53]
	Human placental-MSC	CD29, CD44, CD73, CD90, CD105	CD14, CD19, CD34, CD45, HLA-DR	Data not shown	[54]
	Rat BM-MSC	CD44, CD73, CD90, CD105	CD11b, CD45	Data not shown	[56]
	Pig ASC	CD73, CD90, CD105	CD14, CD11b	Osteogenic Chondrogenic Adipogenic	[57]
	Pig BM-MSC	CD13, CD29	CD31, CD34, CD45	Data not shown	[58]

ISCT: International society of cell therapy; MSC: Mesenchymal stem cell; AA: Aortic aneurysm; BM-MSC: Bone marrow-derived MSC; HLA-DR: Human leukocyte antigen-DR; ASC: Adipose tissue-derived MSC.

consecutive passages^[36,37]. The International Society of Cell Therapy criteria propose (ISCT) that human MSCs should be positive for the expression of CD73, CD90 and CD105 ($\geq 95\%$ positive), and lack expression of CD34, CD45, CD11b or CD14, CD19 or CD79 α , and HLA-DR ($\leq 2\%$ positive). Also, MSC should differentiate into osteogenic, adipogenic and chondrogenic lineage (Table 1)^[38]. However, CD73 and CD105 are also expressed on fibroblast and endothelial lineage cells and CD90 is also expressed on haematopoietic stem cells^[39,40]. To improve purity of the human MSC population, several studies have been performed using a combination such as Stro-1, CD271, CD146 and PDGFR- α , not only CD73, CD90 or CD105^[41-43].

Migration mechanism of MSCs

Through a CXCR4 signaling pathway of damaged tissue stimuli migration and activation of MSC *via* stromal cell-derived factor-1, MSCs are known to accumulate in damaged tissue sites^[44]. In addition, it also has been reported that the migration of MSCs is accelerated through up-regulation of pro-MMP-2 and membrane-type 1-MMP complex by stimulation of the inflammatory cytokines IL-1 β ^[45,46].

Immunosuppression and anti-inflammation properties of MSCs

MSCs have the capability of immunosuppression and anti-inflammation properties. Several investigations were reported regarding the mechanisms of immunosuppression and anti-inflammation of MSCs. MSCs do not express the costimulatory molecules CD80, CD86 and CD40, which have been identified to play a role in the initiation of immune responses by T and B lymphocytes^[47,48]. Also, MSCs can inhibit activation of T-cells immune response and proliferation by expression of indoleamine 2,3-dioxygenase, which degrades tryptophan and suppresses T-cell proliferation. Moreover, MSCs reduce the secretion of interferon (IFN)- γ , which regulates several aspects of the immune response, from T-helper 1 (Th1) cells, and conversely increase secretion of IL-4, which plays a cen-

tral role in the inhibitory regulation of immune response, from Th2 cells. In addition, MSCs inhibit proliferation of natural killer cells through soluble factor prostaglandin E₂ (PGE₂), which inhibits actions on T cells depending on their maturation and activation state, and TGF- β which were secreted from MSC, and reduce the proinflammatory potential of dendritic cell-1 (DC1) by inhibition of their secretion TNF- α , IFN- γ and IL-12 and conversely increase IL-10 secretion from DC-2^[49,50].

TREATMENT OF AORTIC ANEURYSMS USING MSCs

Recently, several studies using MSCs as a cell source for treatment of AA have been reported including our own studies. Published experimental studies were summarized in Table 2.

Implantation of bone marrow-derived MSC cell-sheet for aortic aneurysm

We earlier reported that AA formation and growth were attenuated by intraperitoneal implantation of bone marrow-derived MSC (BM-MSC) cell-sheet using an angiotensin II (AT II)-infused apolipoprotein E-deficient (apoE^{-/-}) mouse model^[51]. The BM-MSC cell-sheet was prepared using an Upcell[®] which is a thermoresponsive polymer-grafted dish surface, and the BM-MSC cell-sheet was implanted into the nearby abdominal aortic adventitia at the time of implantation of Alzet osmotic minipump to infuse the AT II (Figure 1). Four weeks after implantation of BM-MSC cell-sheet, the aortic diameter of the BM-MSC cell-sheet implanted group was significantly lower than that of the apoE^{-/-} + AT II group at the infrarenal aorta (Figure 2A). The enzymatic activities of MMP-2 and MMP-9 were suppressed in the BM-MSC cell-sheet implanted mice group. The downregulation of MMP enzymatic activity may be influenced *via* the paracrine effect of soluble factors secreted from BM-MSC because we showed that gene expression of MMPs in macrophages was decreased by indirect co-culture

Table 2 Animal studies for treatment of aortic aneurysm using mesenchymal stem cells

Experimental AA model	Cell source	Number of cells	Injection time	Delivery	Efficiency	Ref.
AT II -infusion mouse model	BM-MSc	Cell-sheet	Same time as AT II -infusion	Implantation of MSC-sheet around infrarenal aorta	4 wk after implantation, inhibition of AA development and growth, and elastin degradation downregulation of IL-1 β , IL-6, MCP-1 and TNF- α protein expression, and MMP-2 and -9 enzymatic activity Up-regulation of IGF-1 and TIMP-1 protein expression Positive for MSC specific surface marker	[51]
AT II -infusion mouse model	BM-MSc	1 \times 10 ⁶ /every week, 4 times	Same time as AT II -infusion	<i>iv</i> -administration	4 wk after injection, inhibition of AA development and growth, elastin degradation, M ϕ infiltration downregulation of IL-1 β , IL-6 and MCP-1 protein expression, and MMP-2 and -9 enzymatic activity Up-regulation of IGF-1 and TIMP-1 protein expression	[53]
Elastase-perfusion mouse model	Placental-MSc	1 \times 10 ⁶	1 d after elastase-perfusion	<i>iv</i> injection	Detection of MSC in the aortic wall 2 wk after injection, inhibition of AA expansion, inflammatory cell infiltration, and elastin degradation, downregulation of IL-17, IL-23, INF- γ , TNF- α , RANTES and MCP-1 protein expression Increase of α -SMA expression	[54]
Xenograft rat model	BM-MSc	1 \times 10 ⁶	Same time as surgical intervention	Catheter	1 wk after surgical intervention inhibition of inflammatory cells infiltration and MMP-9 gene expression, and increase of TIMP-1 gene expression, after 4 wk, inhibition of AA expansion, increase of α -SMA expression, elastin and collagen content	[56]
Dacron-patch pig model	ASC	1 \times 10 ⁶	Same time as surgical intervention	Catheter	Attenuation of inflammation reaction, detection of ASC 3 wk after surgical intervention	[57]
Balloon injury with type 1 collagen and elastase-perfusion porcine model	BM-MSc	1 \times 10 ⁶	Same time as balloon-injury	Direct injection into aortic wall	72 h after injection, Increase of VEGF-A mRNA expression level 1 wk after injection, detection of GFP-labeled MSC at aortic wall and vWF positive cells formed tubuloluminal structures within outer layer of media and throughout the adventitia	[54]

AA: Aortic aneurysm; AT II: Angiotensin II; MSC: Mesenchymal stem cell; BM-MSc: Bone marrow-derived MSC; *iv*: Intravenous; VEGF: Vascular endothelial growth factor; GFP: Green fluorescent protein; TIMP-1: Tissue inhibitor of metalloproteinase 1; MMP: Matrix metalloproteinases; MCP-1: Monocyte chemotactic protein 1; IL: Interleukin; TNF: Tumor necrosis factor; IGF: Insulin-like growth factor; INF: Interferon; ASC: Adipose tissue-derived MSC; vWF: von Willebrand factor.

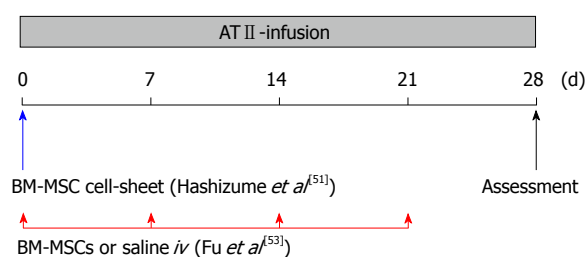


Figure 1 Diagram of bone marrow-derived mesenchymal stem cell cell-sheet implantation or bone marrow-derived mesenchymal stem cell intravenous-administration protocol. At the time of Alzet osmotic minipump implantation, the BM-MSc cell-sheet was implanted into the nearby abdominal aortic adventitia^[51], and 1 \times 10⁶ BM-MSCs (in 0.2 mL saline) or 0.2 mL saline were injected intravenously via the tail vein every week^[53]. Mice were sacrificed and assessed on day 28. AT II: Angiotensin II; *iv*: Intravenous; BM-MSc: Bone marrow-derived mesenchymal stem cell.

with BM-MSCs *in vitro* in this paper. In addition, the protein expression of tissue inhibitor of metalloproteinase

(TIMP)-1 was increased in the BM-MSc cell-sheet implanted group. The BM-MSc cell-sheet implanted group also showed decreased inflammatory cytokines including IL-6, MCP-1 and TNF- α . These results suggested that BM-MSc cell-sheet might suppress the excess inflammatory reaction which caused AT II-induced AA. On the other hand, degradation of elastin was inhibited by implantation of the BM-MSc cell-sheet compared with control. This result could be supported by the increase of the gene expression of elastin in VSMCs co-cultured with BM-MSCs *in vitro*. Moreover, the protein expression of IGF-1 and TIMP-1 in AA tissue with BM-MSc cell-sheet implantation was deemed to be in a paracrine manner, because the IGF-1 and TIMP-1 are identified to be present in the condition medium of MSCs^[46,52]. Our study showed a new approach by treating AA through implantation of BM-MSc cell-sheet. However, such implantation using laparotomy is a relatively invasive procedure, even less invasive than prosthetic graft replacement

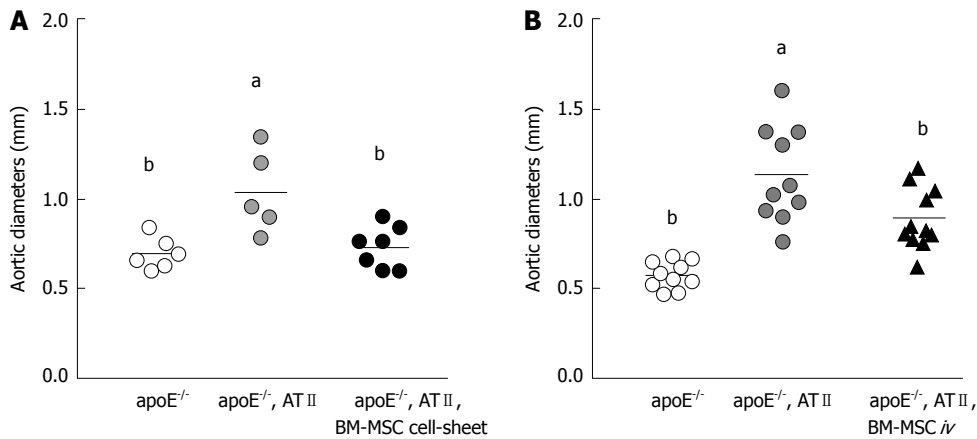


Figure 2 Bone marrow-derived mesenchymal stem cell cell-sheet implantation or bone marrow-derived mesenchymal stem cell IV-administration attenuates aortic aneurysm progression and expansion. Aortic diameter was measured at the infrarenal aorta in the bone marrow-derived mesenchymal stem cell (BM-MSC) cell-sheet (A) or the BM-MSC IV-administration. Data are assessed by one-way ANOVA with Bonferroni correction. ^a $P < 0.05$ vs apoE^{-/-} group, ^b $P < 0.05$ vs apoE^{-/-} + AT II group. Data are from Hashizume *et al.*^[51] and Fu *et al.*^[53]. AT II: Angiotensin II; *iv*: Intravenous.

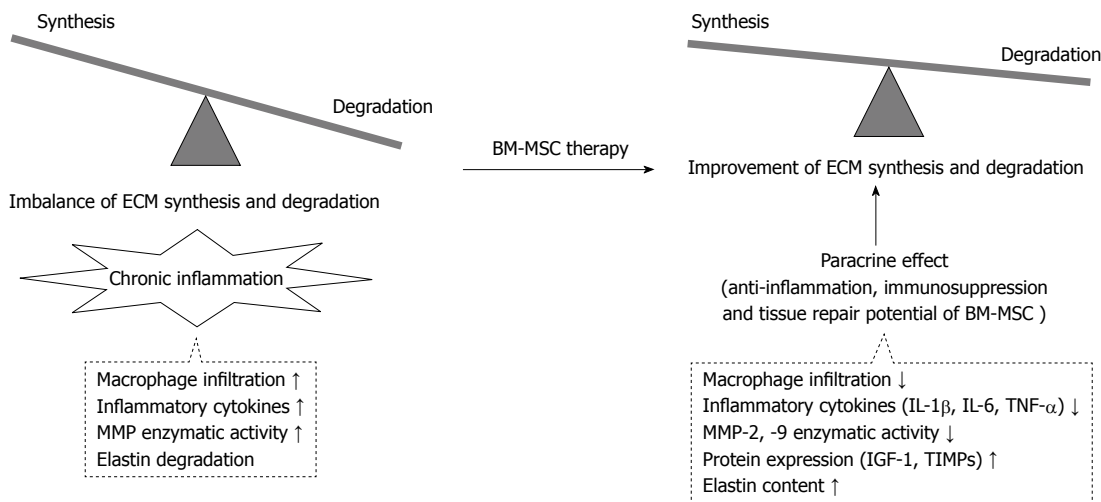


Figure 3 Attenuation of aortic aneurysm development and growth is associated with improvement of the imbalance between degradation and synthesis of extracellular matrices by bone marrow-derived mesenchymal stem cell therapy. ECM: Extracellular matrices; BM-MSC: Bone marrow-derived mesenchymal stem cell; TIMP: Tissue inhibitor of metalloproteinase; MMP: Matrix metalloproteinase; IL: Interleukin; IGF: Insulin-like growth factor.

for AA.

Intravenous administration for aortic aneurysm

To treat AA by a less-invasive BM-MSC delivery, we demonstrated multiple intravenous (*iv*) administration of BM-MSC for an AT II-infusion AA mouse model^[53]. At the time of Alzet osmotic minipump implantation, 1×10^6 BM-MSCs (in 0.2 mL saline) or 0.2 mL saline were injected intravenously *via* the tail vein every week (Figure 1). After the treatment (4 wk later), the BM-MSC (*iv*)-administration group reduced the incidence of AA compared with that of the saline group, and attenuated the progression and expansion at the infrarenal levels of the aorta (Figure 2B). The BM-MSC IV-administration group also suppressed MMP-2 and MMP-9 enzymatic activity and protein expression of inflammatory cytokines including IL-1β, IL-6 and MCP-1 in the aortic tissue. In addition, the infiltration of macrophages was suppressed by BM-MSC IV-administration. Moreover, the BM-MSC

IV-administration group showed inhibition of elastin degradation, which might have been affected by the up-regulation of IGF-1 and TIMP-2 protein expression. This study showed that the multiple IV-administration of BM-MSC inhibits AA development and progression as a less-invasive procedure. Our studies suggest that the attenuation of AA development and growth is associated with improvement of the imbalance between degradation and synthesis of ECMs due to the anti-inflammation, immunosuppression and tissue repair potential of BM-MSC (Figure 3).

Sharma *et al.*^[54] reported the role of IL-17 in the elastase-perfused mouse AAA model and the effectiveness of *iv* injection of human placental-derived MSC for experimental AAA. T-cell-produced IL-17, which is known as a regulator of inflammation and VSMC apoptosis, induced the expression of various cytokines, chemokines, and MMPs^[55]. On day 1 after elastase-perfused wild-type (WT) mice, 1×10^6 placental-derived MSCs were

injected intravenously *via* the tail vein. After 2 wk, the aortic diameter was attenuated in the placental-derived MSC-treated mice group compared with untreated elastase-perfused WT mice group. In histological analysis, infiltration of inflammatory cells including CD3⁺ T cells, macrophages and neutrophils was attenuated and elastic fiber disruption decreased in placental-derived MSC-treated mice group. In addition, the placental-derived MSC-treated mice group suppressed the protein production of IL-17, IL-23, IFN- γ , TNF- α , RANTES and MCP-1 in aortic tissue. The same investigators suggested that placental-derived MSC treatment attenuated AAA formation and inflammatory cytokine production including IL-17 *via* paracrine effect of soluble factors secreted from MSCs such as TGF- β , hepatocyte growth factor, or PGE₂. This suggestion was supported by co-culture of placental-derived MSCs and mononuclear cells (MNCs) in an *in vitro* experiment. The placental-derived MSCs co-cultured with MNCs suppressed the proliferation of activated MNCs and attenuated IL-17 production from MNCs.

Catheter-based MSC therapy for aortic aneurysm

Schneider *et al.*^[56] also reported that an already-formed tentative AA was stabilized by BM-MSCs using a xenograft rat AAA model. To obtain xenograft, guinea pig infrarenal aortas were decellularized using 1% sodium dodecyl sulfate. Then, the male Fischer 344 rat aorta was replaced by a decellularized xenograft. Fourteen days after xenograft implantation, 1×10^6 BM-MSCs were injected into the lumen of clamped xenograft aorta through a PE10 catheter, and allowed to attach for 8 min. The results showed down-regulation of MMP-9 mRNA, up-regulation of TIMP-1 mRNA and decrease of macrophages at the xenograft site at 1 wk, and a decrease in aortic diameter at the xenograft site 4 wk after BM-MSC injection. These results suggest that BM-MSCs inhibit xenograft aneurysmal wall injury and heal through paracrine mechanisms and induction of collagen production rather than direct differentiation. This endovascular seeding of BM-MSCs may support the development of catheter-based intervention for AA treatment in the future.

The possibility of catheter-based delivery of MSCs has also been reported by Riera Del Moral *et al.*^[57] who demonstrated adjuvant treatment with MSCs in EVAR based on clinical current treatment. They injected 1×10^7 adipose tissue-derived MSCs (ASCs) (in 1 mL fibrin sealant) inside the aneurysmal sac through a second 5F introducer using a Dacron-patched AAA pig model. This study investigated whether the MSCs induced local immunosuppression, prevention of excessive fibrosis, prevention of apoptosis and induction of intrinsic progenitor cell. The results showed that the ASC-treated group was a lower infiltration of inflammatory cells compared with the non-treated group, and green fluorescent protein (GFP)-linked ASCs were detected 3 wk after. They suggested that ASC endovascular administration into aneurysmal sac assuming common clinical treatment might stabilize AAA.

Direct Injection of MSCs to the aneurysmal aortic wall

Turnbull *et al.*^[58] reported the success of implantation of autologous BM-MSC by direct injection into the aortic wall using a porcine AAA model and the potential of cell-based therapies. The aneurysm was created by injection of type 1 collagenase and elastase solution into the aortic lumen, following dilation of the infrarenal aorta using a 12 mm noncompliant angioplasty balloon. After that, 1×10^7 BM-MSCs were directly injected into the aortic wall immediately after the injury. The GFP-labeled BM-MSCs were identified in the aortic wall 1 wk after injection. And, von Willebrand factor positive cells formed tubuloluminal structures were detected within the outer layer of the media and throughout the adventitia. In addition, the mRNA level of vascular endothelial growth factor (VEGF)-A was increased at 72 h in BM-MSC-injected aortic tissue compared with non-treated control aorta. Thus, they suggested BM-MSC-enhanced wound healing and angiogenic response through paracrine factor, such as VEGF.

In these studies, MSC phenotypic characteristics have been identified by surface marker and pluripotency. Although these different positive and negative immunophenotypes are concerned with differences in animal species, they resemble human MSC immunophenotypic characteristics.

FUTURE PERSPECTIVE OF MSC THERAPY FOR AA

Unresolved issues

The efficacy of MSC for treatment of AA has been suggested in current experimental studies showing the advantages of inhibition of excess inflammation, decrease of inflammatory cells, suppression of elastic fiber disruption and increase of elastin content by MSC administration. However, some unresolved issues remain in the treatment with MSCs. First, it remains unclear whether the cell numbers, frequency and administration timing of MSCs are required for AA treatment. Thus, these optimizations warrant further investigation. Second, the delivery methods of MSC in these studies are respectively different. Investigators have performed administration using several methods involving implantation of cell-sheet, IV-administration, direct injection into aortic wall, and catheter delivery (Table 3). Among them, although IV-administration is the least invasive and simple procedure, the targeting ability is lower and injected MSCs are trapped in other tissues such as lung, spleen, liver and kidney. In contrast, the implantation of cell-sheet and the direct injection into aortic wall make it possible to target AA. However, these are relatively invasive procedures. On the other hand, endovascular delivery using a catheter is less invasive and has a high targeting ability. Third, the long-term follow-up administration of MSCs has not been reported yet. The injected MSCs may differentiate into adipocyte, osteocyte or other differentiated cells at the aortic wall site. These cells might promote harmful

Table 3 Methodology of delivery system

Delivery system	Administration site	Localization, timing	Delivery system		Ref.
			Merits	Demerits	
Cell-sheet	Adventitia of abdominal aorta	Adventitia, 4 wk after implantation	High targeting ability	Invasive procedure by laparotomy	[51]
<i>iv</i>	Tail vein	Media and/or adventitia, at 4 wk	Least invasive	Low targeting ability and trapping in other tissue	[53]
<i>iv</i>	Tail vein	Data not shown	Least invasive	Low targeting ability and trapping in other tissue	[54]
Catheter	Clamped endovascular	Intima 1 wk after injection	Less invasive and high targeting ability	Requirement of a surgical procedure or advanced catheter intervention	[56]
Catheter	Clamped endovascular	Media 3 wk after injection	Less invasive and high targeting ability	Requirement of a surgical procedure or advanced catheter intervention	[57]
Direct injection	Injured aortic wall	Aortic wall, 1 wk after injection	High targeting ability	Risk of rupture	[58]

iv: Intravenous.

effects to the aorta such as deposition of lipid or calcification. Fourth, the isolation and expansion of MSCs might become difficult with aging. Therefore, we must investigate the therapeutic effect of AA using allogeneic MSCs, not only autologous MSCs. Finally, further investigation using a large animal will be ultimately required to confirm the repeatability.

Future perspective

Meanwhile, it is important to elucidate the mechanisms by which MSCs induced negative effects for progression of AA. One of the mechanisms was suggested to be the paracrine effect of MSCs. Recently, trophic factors of MSC-conditioned medium (MSC-CM) were profiled by proteomic analysis using mass spectrometry, protein microarrays and bioinformatics; as a result, many candidates such as TGF- β , IGF-1, epidermal growth factor, fibroblast growth factor, interleukins, MMPs, or TIMPs were identified^[59]. TGF- β is an important signal that induces smooth muscle cell (SMC) differentiation and increases serum response factor (SRF) expression through an increase in transcription of the *SRF* gene^[60]. Moreover, SRF controls vasoconstriction *via* SMC phenotypic modulation^[61]. This fact might be supported by cellular activities in the treatment of AA using MSC therapy. Timmers *et al.*^[62,63] demonstrated that *iv* injection of human MSC-CM for treatment of MI in porcine model, resulted in reduced myocardial apoptosis, oxidative stress, myocardial infarct size, preserved systolic and diastolic function through reduction of TGF- β signaling including phospho-Smad2 and apoptosis including active caspase 3 following MSC-CM treatment. These studies also revealed that the fraction of the MSC-CM containing products > 1000 kDa improved cardiac function rather than the fraction of products < 1000 kDa. This result indicates that a large complex protein such as a combination of angiogenic factors rather than a single protein may be the responsible paracrine factor. Regarding MSC for AA, it is not clear which factors can induce a better effect. Although *iv* injection of MSC-CM provides easy delivery compared to direct MSC injection, the effects would be of short-term duration by degradation of molecules im-

mediately.

Study of MSC therapy for AA has only just begun, and MSCs are indeed a promising tool for AA treatment. Some studies have suggested that inflammation and ECM degradation at the AA wall site were inhibited by various anti-inflammatory cytokines, inhibitor of proteases and stimulator of ECMs synthesis which were induced by various growth factors secreted from MSCs. In addition, MSC therapy has been demonstrated to have an efficacy not only for prevention of AA development and progression but also regression of already-formed AA. These healing mechanisms remain unknown, and so further research will be warranted in the future.

CONCLUSION

Treatment of AA using MSCs has been demonstrated to be effective, and promises to be a new non-surgical therapeutic strategy. These effects might be promoted in a paracrine manner from MSCs as one possible mechanism.

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WCSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Osteogenic potential: Comparison between bone marrow and adipose-derived mesenchymal stem cells

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Abstract

Bone tissue engineering (BTE) is now a promising research issue to improve the drawbacks from traditional bone grafting procedure such as limited donor sources and possible complications. Stem cells are one of the major factors in BTE due to the capability of self renewal and multi-lineage differentiation. Unlike embryonic stem cells, which are more controversial in ethical problem, adult mesenchymal stem cells are considered to be a more appropriate cell source for BTE. Bone marrow mesenchymal stem cells (BMSCs) are the earliest-discovered and well-known stem cell source using in BTE. However, the low stem cell yield requiring long expansion time *in vitro*, pain and possible morbidities during bone marrow aspiration and poor proliferation and osteogenic ability at old age impede its' clinical application. Afterwards, a new stem cell source coming from adipose tissue, so-called adipose-derived stem

cells (ASCs), is found to be more suitable in clinical application because of high stem cells yield from lipoaspirates, faster cell proliferation and less discomfort and morbidities during harvesting procedure. However, the osteogenic capacity of ASCs is now still debated because most papers described the inferior osteogenesis of ASCs than BMSCs. A better understanding of the osteogenic differences between ASCs and BMSCs is crucial for future selection of cells in clinical application for BTE. In this review, we describe the commonality and difference between BMSCs and ASCs by cell yield, cell surface markers and multiple-differentiation potential. Then we compare the osteogenic capacity *in vitro* and bone regeneration ability *in vivo* between BMSCs and ASCs based on the literatures which utilized both BMSCs and ASCs simultaneously in their articles. The outcome indicated both BMSCs and ASCs exhibited the osteogenic ability to a certain extent both *in-vitro* and *in-vivo*. However, most *in-vitro* study papers verified the inferior osteogenesis of ASCs; conversely, *in-vivo* research reviews revealed more controversies in this issue. We expect the new researchers can have a quick understanding of the progress in this filed and design a more comprehensive research based on this review.

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Key words: Bone marrow mesenchymal stem cell; Adipose-derived stem cell; Osteogenesis

Core tip: Both bone marrow stem cells (BMSC) and adipose-derived stem cells (ASC) have been reported to have the osteogenic capacity *in vitro* and *in vivo*. ASCs possess some attractive characters for clinical application compared to BMSCs, such as abundant stem cells from lipoaspirates, faster growth and less discomfort and morbidity during surgery. Nevertheless, the arising question is that "Is the osteogenic capacity of ASCs the same or far better than BMSCs?". The purpose of this review paper is to compare the osteogenic capacity between BMSCs and ASCs based on the literatures

which using both BMSCs and ASCs simultaneously in their articles.

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INTRODUCTION

Bone defect reconstruction after tumor ablation, trauma injury and infection is still a challenge issue for orthopedic surgeon and cranio-facial surgeon. Traditionally, autogenous bone grafting is the first choice for bridging the bone defect. The merits are free of immunogenic problem and absolutely biocompatible because it comes from patient himself. The disadvantages are limited donor source, donor site morbidities and variable bone graft survival^[1,2]. Xenograft and Allograft are alternative choices for the treatment. However, immunogenic reaction and inadequate bone regeneration due to incomplete resorption sometimes result in non-union or pathologic fracture, respectively. Recently, bone tissue engineering (BTE) becomes a promising issue to improve bone defect repair.

The basic concepts of BTE comprise of three components: scaffold, cytokines and cells. Scaffold provides support for cell attachment, void space for cell proliferation and guides the surrounding tissue to grow into. Usually the scaffold using in BTE should have three-dimensional porous structure with interconnected tunnel between pores and good mechanical strength to replace the loading bearing function before new bone regeneration. Cytokines can enhance cell proliferation, the homing of circulating or regional mesenchymal stem cells and differentiation of cells into osteoblast lineage. The function of cells in BTE is to be differentiated into osteoblasts which can produce the extracellular matrix, secret bone-specific proteins and cytokines to enhancing new bone formation, angiogenesis, *etc.*

Stem cells are the first choice in BTE due to the ability of selfrenewal and multi-lineage differentiation. Although the use of embryonic stem cells is attractive due to their pluripotency, their clinical applications are limited owing to ethic issues and difficulties in controlling single-lineage differentiation, which usually result in teratoma formation. Alternatively, scientists find out the mesenchymal stem cells from adult residing in wide range of tissue, which own the function to repair damage or diseased tissue. The adult stem cells possess the multipotent ability of differentiation at least into osteoblasts, chondrocytes and adipocytes. Bone marrow mesenchymal stromal/stem cells (BMSCs) are the most well-known and-characterized source of adult stem cells. It was first described by Friedenstein *et al*^[3] that the stem

cell could be isolated from bone marrow with the character of adherence to plastic surface and fibroblast-like appearance in culture. Since then, papers based on BMSCs were published in application of BTE both *in-vitro* and *in-vivo* study. The disadvantages of BMSCs are the low stem cell yield from bone marrow aspirates, painful procedure, potential complications derived from the procedure and poor mutlipotent ability after extensive passage or at aged people. Therefore, scientists are urged to search a better alternative cell source for BTE.

In 2001, Zuk *et al*^[4] described a new mesenchymal stromal/stem cell isolated from adipose tissue after liposuction procedure. Briefly, the lipoaspirate tissue is digested with collagenase first, followed by centrifugation to obtain a cell pellet at the bottom of tube. The cell pellet is so-called stromal vascular fraction.(SVF) Actually, the SVF is a heterogeneous cell population of red blood cells, fibroblasts, endothelial cells, smooth muscle cells, pericytes and adipose tissue-derived stromal/stem cells (ASCs) which have the plastic-adherent character. After culturing SVF *in vitro* overtime, the cell population becomes homogenous to primarily plastic-adherent ASCs. The ASCs also display the ability of multilineage differentiation into adipocytes, osteoblasts, chondrocytes and myocytes. In addition, the liposuction procedure is simple, easy and repeatable with less discomfort and complications. The cell yield of ASCs from adipose tissue is higher than BMSCs from bone marrow aspirates. Hence, the ASCs have been suggested as a better cell sources in BTE than BMSCs. Since then, many researches demonstrated the osteogenic potential of ASCs both *in vitro* and *in vivo*.

Although the ASCs are considered to have the opportunity to replace the role of BMSCs in BTE, the arising question is that "Is the osteogenic capacity of ASCs the same or far better than BMSCs?". The issue is still controversial now because the majorities of papers describe the osteogenic potential or bone regeneration capacity only by either BMSCs or ASCs. A lot of variations in culturing and analytic methods, selection of scaffolds and animal models result in difficulties to make a convinced conclusion to prove the best stem cells for BTE by comparing these papers. The purpose of this review paper is first to describe the commonality and difference between BMSCs and ASCs, followed by comparing the osteogenic capacity *in vitro* and bone regeneration ability *in vivo* between BMSCs and ASCs based on the literature which utilized both BMSCs and ASCs simultaneously in their articles.

COMMONALITY AND DIFFERENCE OF BMSCS AND ASCS

Before the comparison of osteogenesis between BMSCs and ASCs, we should clarify whether both BMSCs and ASCs fit the criteria of mesenchymal stromal/stem cell and realize the commonality and differences between them. The Mesenchymal and Tissue Stem Cell committee

of the International Society for Cellular Therapy provided the minimal criteria for defining the human mesenchymal stem cells (MSCs): (1) Plastic-adherence when maintained under standard culture conditions; (2) Multi-lineage differentiation into osteogenic, adipogenic and chondrogenic cells; (3) Expressing stromal surface markers of CD73, CD90 and CD105; and (4) Not expressing hematopoietic lineage markers c-kit, CD14, CD11b, CD34, CD45, CD19, CD79- α and human leukocyte antigen-DR^[5].

As we know, both MSCs are plastic-adherent under standard culture conditions with the fibroblastic, spindle-shape appearance. Both cells also are clonogenic, formed colonies in culture conditions. However, ASCs have been found that they can be maintained *in vitro* for extended periods with stable population doubling, higher proliferative capacity and low levels of senescence compared with BMSCs^[6,7]. Furthermore, the osteogenic potential and cell proliferation of BMSCs seems to be reduced by age. In contrast, the decline in osteogenic potential of ASCs is not so prominent by aging^[8-10]. Chen *et al.*^[9] compared the osteogenic differentiation of ASCs and BMSCs between young group (36.4 ± 11.8 years old) and elderly patients (71.4 ± 3.6 years old). They found the level of matrix mineralization of ADSCs from aged patient was comparable to that of ADSCs from young patient, whereas BMSCs from aged patient produced least amount of mineral deposits and had a lower expression level of osteogenic genes^[9]. Wu *et al.*^[10] described the effect of age on human adipose stem cells by comparing the osteogenic potential among infant (< 1 year), adult (22-54 years) and old (> 55 years). They concluded the infant adipose-derived stem cells exhibited elongated spindle morphology and increased telomere length compared with older cells. Angiogenic factors were more highly expressed by infant cells, whereas osteogenic expression was similar among all ages^[10]. Except the minimal criteria of trilineage differentiation into bone, cartilage, and adipose tissues *in vitro*, both stem cells are able to differentiate into other mesodermal tissues such as skeletal muscle, tendon, and myocardium^[11,12]. Furthermore, both stem cells have also been demonstrated to cross germinal boundaries and differentiate into ectodermic origin and endodermic origin^[11,12].

Although there is no single specific surface marker that is unique to stem cells, some known surface markers are found on them. Both stem cells have the stromal cell markers such as CD13, CD73 and CD90. Both stem cells have negative expression of hemopoietic markers CD11b and CD45. However, the CD34 is generally expressed on ASCs during the early phase of culture with decreasing after extensive passage^[13]. In contrast, BMSCs do not express CD34. The alternative surface markers to distinguish ASCs from BMSCs are CD36 and CD106 because the ASC, in contrast to BMSC, is positive for CD36 and negative for CD106^[13].

Another difference between BMSCs and ASCs is the cell yield from the bone marrow- and lipo-aspirates, respectively. The bone marrow aspirates yield 6×10^6

nucleated cells per mL in average, only 0.001% to 0.01% are stem cells^[12]. In contrast, 2×10^6 cells can be isolated from 1 gm adipose tissue, and 10% are thought to be stem cells^[14,15]. The feature makes the ASCs to be a good cell source for clinical application. For example, if we draw 100 mL bone marrow aspirates from an adult patient, in which there are only 6×10^3 to 6×10^4 stem cells. The cell population is usually insufficient for clinical applications. However, we can usually draw 1000-2000 cc lipoaspirates from patient without any discomfort or complication; there may be 2×10^8 to 4×10^8 stem cells, which are already enough for repairing a small bone defect. Namely, extensive *in-vitro* passaging to obtain adequate cell numbers usually is required in BMSCs, not in ASCs. The disadvantages of long-term *in-vitro* passaging are the possible contamination, time-consuming, labor-dependent and possible gene mutation during passaging.

IN-VITRO OSTEOGENESIS POTENTIAL BETWEEN HUMAN BMSCS AND ASCS

Although the ASCs possess so many better features than BMSCs for future clinical application in BTE, the determinant factor relies on “do ASCs have equal or far better osteogenic ability than BMSCs”. If the answer is yes, then the ASCs can replace the role of BMSCs in BTE without any doubt. In this section, we search in the literatures to find the articles that comparing the osteogenic potential between BMSCs and ASCs *in vitro* simultaneously to avoid the bias from different culturing and analytic methods among different papers. Zuk *et al.*^[3] was first describing the isolation of ASCs from adipose tissue and executed some experiments to characterize their phenotype and multipotency. In their study, they found the alkaline phosphatase (AP) activity was significantly higher in osteo-induced human ASCs than in BMSCs at 3 wk induction. However, the matrix calcification was 35-fold and 68-fold increase in induction of ASCs and BMSCs over the 6 wk induction period, respectively. Although they performed the gene expression of specific osteogenic gene such as osteocalcin (OCN), core-binding factor subunit alpha-1 (Cbfa-1) also known as Runt-related transcription factor 2 (Runx II), AP, osteonectin (ON), osteopontin (OPN), and bone morphogenic protein-2 (BMP-2) on both osteo-induced ASCs and BMSCs, no quantitative data (qPCR) was shown to compare the relative expression between two cells. No conclusions were made from their results to prove which cells had superior osteogenic potential. Afterwards, more and more papers began to compare the osteogenic potential by quantitative methods such as biochemical analysis (AP activity, calcium assay), qPCR of osteogenic gene expression and microarray (Table 1).

De Ugarte *et al.*^[16] in 2003 showed no significant difference of osteogenesis between human ASCs and BMSCs by AP activity and calcium content assay. The AP activity was 0.10 ± 0.12 and 0.08 ± 0.07 nmol p-nitrophenol produced/min per μ g protein; and total calcium

Table 1 *In-vitro* osteogenesis potential between human bone marrow mesenchymal stem cells and adipose-derived stem cells

Ref.	Osteogenic medium	Culturing condition	Analytic methods	Outcome of osteogenic ability
Park <i>et al</i> ^[22]	0.1 mmol/L nonessential amino acids, 50 µg/mL ascorbic acid-2-phosphate, 100 nmol/L dexamethasone and 10 mmol/L β-glycerolphosphate	Mechanical stimulation by dynamic hydraulic compression	AP, Alizarin red stain, qPCR, immunofluorescence	BMSC > ASC under mechanical stimulation
Vishnubalaji <i>et al</i> ^[20]	10 nmol/L calcitriol, 10 nmol/L β-glycerolphosphate, 50 µg/mL L-ascorbic acid, 10 nmol/L dexamethasone	2-D static culture	AP, Alizarin red, Von Kossa stain, Calcium concentration, qPCR	BMSC > ASC
De Ugarte <i>et al</i> ^[16]	50 µg/mL ascorbic acid-2-phosphate, 100 nmol/L dexamethasone and 10 mmol/L β-glycerolphosphate	2-D static culture	AP activity, calcium assay	BMSC = ASC
Im <i>et al</i> ^[17]	100 nmol/L dexamethasone, 50 µmol/L ascorbate-2-phosphate, 10 mmol/L dexamethasone β-glycerolphosphate	2D static culture	AP activity, Von Kossa staining	BMSC > ASC
Shafiee <i>et al</i> ^[19]	10 nmol/L dexamethasone, 0.2 mmol/L ascorbic acid-2-phosphate, 10 mmol/L β-glycerolphosphate	2D cell culture	AP activity, Alizarin red staining, qPCR	BMSC > ASC
Liu <i>et al</i> ^[18]	0.1 µmol/L dexamethasone, 50 µmol/L ascorbic acid-2-phosphate and 10 mmol/L β-glycerolphosphate	2D cell culture	Alizarin red stain and microarray	BMSC > ASC
Zhang <i>et al</i> ^[21]	10 mmol/L β-glycerolphosphate, 10 ⁻⁸ mol/L dexamethasone, and 0.2 mmol/L ascorbic acid	2D cell culture 3D cell culture	Calcium assay, qPCR, SEM	BMSC > ASC

AP: Alkaline phosphatase; BMSC: Bone marrow mesenchymal stem cell; ASC: Adipose-derived stem cell; 2D: Two dimensional; SEM: Scanning electronic microscope.

was 33 ± 38 vs 42 ± 55 mmol/L Ca/µg per protein in osteo-induced ASCs and BMSCs, respectively. Im *et al*^[17] demonstrated the ASCs may have an inferior potential for osteogenesis compared with BMSCs due to the much less AP staining and amount of matrix mineralization by Von Kossa staining in osteo-induced ASCs. In addition to alizarin red staining, Liu *et al*^[18] in 2007 further used microarray to screen the different gene expressions in multilineage differentiation between BMSCs and ASCs, followed by qPCR assay to confirm the differences. The outcome in osteogenesis showed the BMSCs had more calcium depositions than ASCs under 14-d osteogenic induction; and the extracellular matrix genes [osteomodulin (*OMD*) and tissue inhibitor of metalloproteinase-4 (*TIMP4*)] were progressively increasing expressions in BMSCs, in contrast, no changes or decreasing in ASCs. Their conclusion suggested that BMSCs differentiate more efficiently into bone and cartilage, whereas ASCs differentiate better into adipocytes. Shafiee *et al*^[19] demonstrated ASCs had lower AP activity and mineralization than BMSCs during osteogenic differentiation on days 7 and 14. Although ASCs expressed higher levels of collagen type I, ON and BMP-2 in undifferentiated state, these were expressed higher in BMSCs during osteogenic differentiation. BMSCs also expressed higher levels of AP, OCN and Runx II during induction. Their conclusions supported the BMSCs had the best capacity for osteogenic differentiation and hold promising potential for BTE. Vishnubalaji *et al*^[20] also verified the superior osteogenic capacity of BMSCs than ASCs by cytochemical qualitative analysis, calcium mineralization and qPCR of

AP, osteocalcin and osteopontin.

Generally speaking, the osteoblasts and osteocytes are living in a three-dimensional (3D) bone tissue environment, which is different from *in-vitro* 2D culture dish. Thus, the outcome of comparing the osteogenic ability between BMSC and ASC on bio-mimetic 3D scaffold seems to be more convincing than in 2D dish. Zhang *et al*^[21] seeded both stem cells on the three-dimensional Polycaprolactone/tricalcium phosphate (PCL/TCP) scaffold and cultured them under osteo-induced medium to distinguish which cells had superior osteogenic capacity on 3D environment. They found human BMSC exhibited superior osteogenic potential by more calcium production per scaffold, higher expression of osteogenic gene (Runx II, AP, ON, collagen type I) compared to human ASC. In addition, SEM demonstrated trabecular-like networks with minerals (calcium/phosphate) deposits within the scaffold construct only on BMSC group, in contrast, none on ASC group.

Cells, especially the osteocytes, are apt to sense, adapt and respond to mechanical stimuli for maintaining the physiological and mechanical properties of mature bone. Mechanical stimuli also were known to regulate the osteogenesis of stem cells. Park *et al*^[22] compared the osteogenesis between ASCs and BMSCs under the mechanical stimulus of dynamic hydraulic compression (1 Hz, 1 psi) by a micro cell chip. They found the dynamic hydraulic compression increased production of extracellular matrix [bone sialoprotein (BSP), OPN, collagen type I]. In addition, the osteogenic specific genes (BSP, OPN and Runx II) were also upregulated on both cells. However,

the AP and alizarin red staining showed significant increases in BMSCs, whereas no significant in ASCs under the mechanical stimulus. They concluded the BMSCs were more sensitive to mechanical stimulation and more effective towards osteogenic differentiation than ASCs under dynamic hydraulic compression. Taken together, most evidences support human BMSCs have superior osteogenic potential than human ASCs under static or dynamic compression culture.

IN-VITRO OSTEOGENESIS POTENTIAL BETWEEN ANIMAL BMSCS AND ASCS

Before the BTE can be translated from bench study to clinical application, pre-clinical animal studies are required. Hence, there are some papers discussing about the *in-vitro* osteogenic differentiation between ASCs and BMSCs in other species in order to set up the *in-vivo* animal model. Kang *et al.*^[23] found the osteogenesis ability of canine ASCs was better than BMSCs due to the higher AP activity and mineralization in osteo-induced ASCs. They concluded the canine ASC can potentially be used in place of BMSCs for clinical BTE. Chung *et al.*^[24] showed similar osteogenic potential between canine ASCs and BMSCs with the similar alizarin red stain and pattern of gene expression (Osterix, Runx II, and OCN). They also found the hypoxia environment would inhibit the osteogenesis on both cells, which should be considered when using ASCs or BMSCs in clinically hypoxic environment (e.g., fracture or infection). Vidal *et al.*^[25] showed the better osteogenesis of equine BMSCs than ASCs due to early formation of positive Alizarin red nodule and macroscopic AP staining in BMSC group. Toupadakis *et al.*^[26] detailed the osteogenic gene expression between osteo-induced equine BMSCs and ASCs and revealed that BMSCs had the highest overall expression of the osteogenic genes *Cbfa1*, *Osteorix*, and *OMD*. Hayashi *et al.*^[27] found the rat BMSCs appeared superior osteogenic ability than rat ASCs by mineralization, AP activity and osteocalcin secretion. Monaco *et al.*^[28] conducted an interesting functional analysis by transcriptomics which might indicate the differences in therapeutic application between osteogenic porcine BMSC and ASC. They found ASC appeared to be more myogenic and BMSC appeared to be more prone to migration, induce to bone and neurons prior differentiation. Both cells expressed in large amount of collagen formation, immune suppression and angiogenesis prior differentiation. During early osteogenic differentiation, ASC seemed to have higher lipid metabolism, migration, immunomodulation, while the BMSC had larger induction of inflammation, cell growth and steroid biosynthesis. During late osteogenic differentiation, the ASCs have better angiogenesis capacity than BMSCs. Although they did not make a conclusion of which cells have superior osteogenic ability, the gene expression characters prior or during differentiation gave the scientists a basis to select or design the suitable cells for their BTE component. For example, the

angiogenesis is a crucial factor for successful bone tissue engineering. The early vascularization can enhance the survival of stem cells within the scaffolds. Although the ASCs and BMSCs both showed good angiogenesis prior differentiation, the ASCs were reported to exhibit higher angiogenic efficacy than BMSCs by either the ability of differentiation into endothelial cells or angiogenic factors secretion^[29]. The endothelial cells induced by angiogenic factors secreted by MSCs could further produce BMP-2 to enhance the osteogenesis of MSCs^[30]. In addition, the expression and activity of the early osteoblastic marker, AP, was found to be elevated when the MSCs were co-cultured with human umbilical vein endothelium cells^[31]. In summary, the angiogenesis not only enhance the survival of stem cells but also the osteogenesis.

OSTEOGENESIS POTENTIAL BETWEEN BMSCS AND ASCS IN ANIMAL MODEL

Although both BMSCs and ASCs have been reported to have the capacity of orthotopic and ectopic bone formation *in vivo*, direct comparison between both cells are scarce in human study and few in animal study (Table 2). Hayashi *et al.*^[27] implanted the composite of rat stem cells/hydroxyapatite in the rat subcutaneous pocket. After 6 wk implantation, they found more new bone formation in BMSC groups than ASC groups by micro-CT and histological analysis. Niemeyer *et al.*^[32] created a critical size defect in sheep tibia as the animal model. They implanted the collagen sponge/BMSCs or ASCs into the defect and collagen sponge/ASCs mixed with platelet rich plasma (PRP) as another group. After 26 wk implantation, radiographic evaluation revealed a higher new bone formation in BMSC group than ASC group. However, surprisingly the PRP-plus ASC group showed similar bone formation as the BMSC group. Their conclusion addressed the inferior osteogenic ability of ASCs compared to BMSCs but it can partially be compensated by addition of PRP. Kang *et al.*^[23] used the canine radius defect as animal model. They implanted the β -tricalcium phosphate/BMSCs or ASCs into the defect. After 20 wk implantation, they found the similar healing rate and new bone formation area between BMSCs and ASCs. They considered the ASCs can replace the role of BMSC in future clinical bone reconstruction. Stockmann *et al.*^[33] studied the pig monocortical calvarial bone defect model. They filled the defect with a collagen scaffold seeded either by ASCs or BMSCs. They found the healing rate and new bone formation were not significantly different between ASCs and BMSCs group. Wen *et al.*^[34] compared the bone regeneration capacity on 5 mm cranial defect of SD rat between human ASCs and BMSCs which combined with collagen gel. They found no significant difference of new bone regeneration between two groups by X-ray and histology analysis. They also transfected the human mesenchymal stem cell with *GFP* gene by lentivirus to further confirm the cell sources in the regenerated bone tissue. The expression of *GFP* *via* immunohistochemistry

Table 2 Osteogenesis potential between bone marrow mesenchymal stem cells and adipose-derived stem cells in animal model

Ref.	Animal model	Scaffold	Analytic methods	Outcome of osteogenic ability
Hayashi <i>et al</i> ^[27]	Subcutaneous implantation in rat	Hydroxyapatite	Micro-CT	New bone volume BMSC ($6.85 \pm 1.89 \text{ mm}^3$) > ASC ($0.05 \pm 0.05 \text{ mm}^3$)
Niemeyer <i>et al</i> ^[32]	3-cm tibial defect in sheep	Collagen sponge	Radiographic and histologic analysis	BMSC > ASC BMSC = ASC + PRP No original data for bone volume
Kang <i>et al</i> ^[23]	1.5-cm radial bone defect in dog	Tricalcium phosphate	Radiographic, histological and histomorphometric analysis	New bone percentage: BMSC (33.56%) = ASC (33.9%)
Stockmann <i>et al</i> ^[33]	1-cm calvarial bone defect in pig	Bovine collagen type I	Microradiography, histomorphometric evaluation	BMSC = ASC No original data for bone volume
Wen <i>et al</i> ^[34]	5-mm calvarial defect in SD rat	Collagen gel	X-ray and histology	BMSC = ASC No original data for bone volume
Zhang <i>et al</i> ^[21]	Subcutaneous pocket of nude mice	PCL/TCP	Micro-CT	New bone volume: BMSC ($16.6 \pm 3.0 \text{ mm}^3$) > ASC ($9.1 \pm 1.1 \text{ mm}^3$)

BMSC: Bone marrow mesenchymal stem cell; ASC: Adipose-derived stem cell; PRP: Platelet rich plasma; PCL/TCP: Polycaprolactone/tricalcium phosphate; CT: Computed tomography.

addressed the implanted human mesenchymal stem cells participated in new bone formation on the defect. Zhang *et al*^[21] implanted the composites of stem cells and PCL/TCP scaffold under the subcutaneous pocket of nude mice. They found the human BMSC had more ectopic bone regeneration than ASC by micro-computed tomography analysis and Von Kossa stain. Taken together, it is still controversial to conclude which cells had better osteogenic potential by animal study.

FUTURE DIRECTION AND SUMMARY

Although most papers support the inferior osteogenic capacity of ASCs than BMSCs, many issues remain to be elucidated in comparing the osteogenic potential between BMSCs and ASCs. For example, most papers only compare the two stem cells in the normal, healthy environment. However, in clinical situation there are many scenes that may be encountered in bone defect reconstruction such as fracture location and tissue condition around the bone defect such as non-union, osteomyelitis and osteoradionecrosis. In these circumstances, the surrounding tissue of the bone defect is relative non-vascularized with scarring tissue and inflammation or infection. In addition, the systemic illness or healthy conditions such as diabetes mellitus, heavy smoking, old age, osteoporosis, bone marrow disease, obesity and so on also influence the selection of stem cells for osteogenesis by clinical physicians.

Besides, most papers in the literature only use the same osteogenic medium containing dexamethasone, β -glycerophosphate and ascorbic acid to compare the osteogenic potential between BMSC and ASC. However, some chemical additives or growth factors such as 1,25-dihydroxyvitamin D₃, BMP-2 and retinoic acid, tumor necrosis factor- α , and histone deacetylase inhibitor valproic acid were reported to be an osteogenic enhancer^[35-37]. Mechanical stimulation and electric stimulation were proved to enhance bone regeneration^[38-40]. Combining different strategies to enhance ASCs' osteogenic capacity to or

above the level of BMSCs may be one of future directions to improve their future clinical application.

In summary, this review provides readers the current progress in comparison the osteogenesis between BMSCs and ASCs both *in vitro* and *in vivo* study. We expect the new researchers can have a quick understanding of the progress in this filed and design a more comprehensive research based on this review.

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WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Mesenchymal stem cells: Potential role in corneal wound repair and transplantation

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Key words: Mesenchymal stem cells; Corneal injury; Wound repair; Immune modulation; Transplantation

Core tip: Mesenchymal stem cell (MSC)-based therapy has been proposed as a possible treatment strategy for tissue wound repair, autoimmune diseases, and solid organ transplantation. MSCs are a promising stem cell population because of their self-renewal, pluripotential capability, immunomodulatory, and anti-inflammatory properties. Recent studies have suggested that application of MSCs may be a new alternative method for wound healing after severe corneal damage and for immune rejection after corneal transplantation. In this review, we discuss the potential functions of MSCs in protecting corneal tissue and their possible mechanisms in corneal wound healing and corneal transplantation.

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Abstract

Corneal diseases are a major cause of blindness in the world. Although great progress has been achieved in the treatment of corneal diseases, wound healing after severe corneal damage and immunosuppressive therapy after corneal transplantation remain problematic. Mesenchymal stem cells (MSCs) derived from bone marrow or other adult tissues can differentiate into various types of mesenchymal lineages, such as osteocytes, adipocytes, and chondrocytes, both *in vivo* and *in vitro*. These cells can further differentiate into specific cell types under specific conditions. MSCs migrate to injury sites and promote wound healing by secreting anti-inflammatory and growth factors. In addition, MSCs interact with innate and acquired immune cells and modulate the immune response through their powerful paracrine function. Over the last decade, MSCs have drawn considerable attention because of their beneficial properties and promising therapeutic prospective. Furthermore, MSCs have been applied to various studies related to wound healing, autoimmune diseases, and organ transplantation. This review discusses the potential functions of MSCs in protecting corneal tissue and their possible mechanisms in corneal wound healing and corneal transplantation.

INTRODUCTION

Severe corneal injury caused by chemical or thermal burns, mechanical injury, and immune or hereditary disorders results in corneal inflammation, ulceration, neo-vascularization, conjunctivalization, limbal stem cell deficiency (LSCD), and stromal scarring, all of which may lead to blindness. Current therapeutic strategies include anti-inflammatory drug administration, limbal stem cell (LSC) transplantation, and corneal transplantation. However, these treatments have certain clinical limitations. Anti-inflammatory drugs are not sufficient to suppress

angiogenesis, conjunctivalization, and corneal scarring. LSC transplantation has a high risk of immune rejection^[1,2]. Corneal transplantation remains the main and effective method for visual rehabilitation once a disease has affected corneal clarity^[3]. Despite corneal transplantation is the most successful solid organ transplantation, immune rejection is still the major cause of graft failure. Over the last decade, mesenchymal stem cell (MSC) therapy has been proposed and used as a possible treatment strategy for cardiovascular diseases, renal wound repair, diabetes, systemic lupus erythematosus, and solid organ transplantation^[4-7]. MSCs are a promising stem cell population because of their self-renewal, pluripotential capability, low immunogenicity properties, and notable immunomodulatory and anti-inflammatory activities^[8]. Methods for the isolation and proliferation of MSCs are also simple. MSCs are mainly derived from bone marrow tissue. MSCs can also be isolated from niche of other tissues, including corneal limbal stroma. This review summarizes the therapeutic potential of MSCs in corneal wound repair and keratoplasty.

CHARACTERIZATION OF MSCS

Aside from hematopoietic stem cells, bone marrow tissue also contains non-hematopoietic stem cells^[9]. These stem cells, given their multi-lineage differentiation potential and hematopoiesis-supporting function^[10], are called marrow stromal stem cells or MSCs. Friedenstein *et al.*^[11] first described MSCs as spindle shaped cells derived from bone marrow that were able to adhere to plastic and form fibroblast colonies, which were defined colony-forming unit fibroblasts. MSCs derived from the mesodermal germ layer can differentiate into mesenchymal cell lineages (*e.g.*, adipocytes, osteocytes and chondrocytes) and non-mesenchymal cell lineages (*e.g.*, cardiomyocytes, hepatocytes-like cells, neurons, astrocytes, and endothelial cells) both *in vivo* and *in vitro*^[12-15]. MSCs have been isolated from several adult tissues, including the liver, dental pulp, adipose tissue, endometrium, muscle, amniotic fluid, placenta, and umbilical cord blood^[12-14]. MSCs isolated from bone marrow^[16], umbilical cord^[17], and adipose tissue^[18] promote regeneration and corneal wound repair. In addition, MSCs exist in the perivascular niche of several tissues, including the skeletal muscle and pancreas^[19]. These perivascular MSCs, usually pericytes, are regarded as a subset of MSCs that surround the blood vessel wall^[19-21]. However, identifying the authentic MSCs is difficult because of the lack of specific markers for these cells. The International Society for Cellular Therapy (ISCT) has provided the following minimum criteria defining multipotent MSCs: plastic-adherent under standard culture conditions; positive for the expression of CD105, CD73, and CD90 surface markers; negative for the expression of CD11b, CD14, CD19, CD34, CD45, CD79a, and HLA-DR surface markers; and capable of differentiating into osteocytes, adipocytes, and chondrocytes under a specific stimulus *in vitro*^[22]. Studies have demonstrated that human bone marrow-derived MSCs exhibit hetero-

geneity, which is related to proliferation potential and differentiation potency^[23,24]. Heterogeneity also partially effects the inconsistency of therapy in different laboratories^[25,26]. Heterogeneous bone marrow-derived MSCs are involved with mixed MSC subtypes, and their phenotypes remain poorly described.

NICHE OF MSCS IN LIMBAL STROMA

A population of cells isolated from the human corneal limbal stroma express MSC markers, including stem cell protein ATP-binding cassette transporter subtype G-2 (ABCG-2) and ocular development gene *PAX6*, which could not be expressed by adult keratocytes^[27,28]. Human corneal limbal stromal niche cells subjacent to limbal basal epithelial cells^[29] reportedly possess stem cell-like features. Moreover, these cells are similar to bone marrow-derived MSCs in terms of their adherent nature, phenotypic marker expression profile, low immunogenicity, self-renewal capacity, and colony forming efficiency^[30]. Branch *et al.*^[31] confirmed that peripheral and limbal corneal stromal cells are MSCs because they conform to all the ISCT criteria. The limbal stroma is another MSC niche. These cells can support the self-renewal of limbal epithelial progenitor cells^[28] and up-regulate several molecular markers of keratocytes^[27]. They can also differentiate into corneal epithelium^[32], vascular endothelial cells, and pericytes^[33]. Niche stromal cells might provide a specialized microenvironment for the maintenance of LSCs^[30]. In addition, corneal limbal stromal cells possess immune privileged^[34] and immunosuppressive properties. Limbal MSCs can suppress T cell proliferation by constitutively secreting transforming growth factor- β 1 (TGF- β 1)^[35]. However, the immunosuppressive potential of limbal MSCs is considerably weaker than that of bone marrow-derived MSCs. Corneal limbal stroma stem cells, which can differentiate into functional keratocytes, may serve as an excellent candidate for the generation of bio-engineered corneal stroma^[36]. Further studies are required to understand the functions of corneal stromal stem cells in wound healing and corneal tissue regeneration.

FUNCTIONS AND MECHANISMS OF MSCS IN CORNEAL WOUND REPAIR

MSCs contribute to tissue wound repair. Bone MSCs might migrate to the injury sites after tissue damage. The functions of MSCs in corneal wound repair can be attributed to two mechanisms: transdifferentiation and paracrine action.

MSC mobilization and homing

Injury and inflammation induce stem cell mobilization, migration, and colonization^[37]. The mechanisms involved when MSCs home to sites of injury remain unclear. Corneal injury results in the release of specific chemoattractants, which cause bone marrow to mobilize endogenous MSCs into the peripheral blood. Thus, circulating

MSCs increase in number and migrate to the local injured cornea but not the healthy cornea^[16,38]. The chemokine SDF-1 and substance P in the cauterized cornea are involved in regulating the mobilization and recruitment of MSCs to corneal injury sites^[16]. In addition, systemically administered MSCs can migrate toward injured or inflamed tissues because of the presence of chemokines, chemokine receptors, intracellular signals, adhesion molecules, and proteases^[39,40]. Ye *et al.*^[41] showed that systemically transplanted MSCs engraft locally to the cornea with alkali burns. They also suggested that exogenously applied MSCs must be self-renewed, fully activated, and mature in the host bone marrow before reentering the circulation. After systemic intravenous delivery, most cells become trapped in the lungs and other non-target organs, such as the liver, kidney, and spleen^[42]. MSCs have a low frequency in sites with tissue damage. A recent report has shown that systemically administered human MSCs reduced corneal inflammatory damage without engraftment by secreting anti-inflammatory factors in response to injury signals from the cornea^[43]. Alternatively, MSCs may be administered locally to improve the concentration at the injured cornea through subconjunctival administration of MSCs^[44] and through transplantation of MSCs with the amniotic membrane (AM)^[45,46] and a hollow plastic tube onto the cornea^[47].

Differentiation into corneal tissue

LSC transplantation, the currently available treatment for severe corneal epithelium damage, has certain limitations. The renewal and healing of corneal epithelium mainly rely on LSCs. Ocular injury, trauma, congenital diseases, and autoimmune diseases could lead to partial or total deficiency in the number and function of LSCs. These conditions may also contribute to the subsequent development of corneal ulcer, opacity, and corneal neovascularization (CNV), which seriously affect ocular surface function and vision^[48,49]. LSC transplantation is an effective way of treating severe LSC damage. However, this strategy has some limitations. Autologous limbal transplantation is only intended for unilateral lesions and could induce LSCD in the healthy donor eye. Moreover, allograft limbal transplantation has a short supply of donors and has the risk of immune rejection^[2,50].

MSCs have received attention as seed cells for corneal tissue engineering to overcome LSC deficiency and reconstruct ocular surfaces. MSCs can be easily isolated, cultured, and proliferated. They can also retain their pluripotent ability. Gu *et al.*^[51] examined the differentiation ability of MSCs in corneal epithelial cells *in vivo* and *ex vivo*. *In vivo*, rabbit MSCs (Rb-MSCs) were transplanted onto the surface of damaged rabbit corneas using fibrin gels. Results showed that the Rb-MSCs expressed the corneal epithelium specific marker cytokeratin 3 (CK3) and promoted the healing of the injured corneal epithelium. Rb-MSCs were co-cultured *in vitro* with rabbit LSCs (Rb-LSCs) using the Transwell culture system or were cultured in the conditional medium of Rb-LSCs. The

Rb-MSCs rapidly lost their fibroblast morphology, differentiated into cells with a corneal epithelia-like shape, and expressed CK3. Furthermore, the soluble factors secreted by Rb-LSCs were suggested to serve important functions in the differentiation of Rb-MSCs because the two types of cells had no direct contact. In another study, bone MSCs were induced by corneal stromal cells to differentiate into corneal epithelia-like cells and express CK12^[52]. Inducing MSCs in the AM significantly improves the reconstruction of the corneal surface of rats with corneal alkali burn. In a recent *in vitro* experiment, corneal epithelia-like cells were induced from human adipose tissue-derived MSCs by subjecting them to a medium conditioned with corneal epithelial cells^[53]. In addition, MSCs can differentiate into corneal epithelial progenitor cells in Rb-LSC deficiency models^[54]. The expression of limbal epithelial cell markers (*e.g.*, ABCG-2, b1-integrin, and connexin 43) is up-regulated after injecting autologous MSCs under the transplanted AM. The up-regulation of these specific markers indicates the capacity of MSCs to maintain their stem cell-like characteristics or to differentiate into epithelial progenitor cells. However, a previous study showed that MSCs serve functions in wound healing in a rabbit corneal alkali burn model by differentiating not into corneal epithelial cells or limbal progenitor cells but into myofibroblasts, as indicated by the expression of α -smooth muscle actin^[41].

MSCs are promising tissue engineering cells for treating corneal stromal damage and congenital keratocyte dysfunction. Corneal keratocytes are quiescent cells with a flat and dendritic morphology. Keratocytes may become activated by injury, and the syntheses of keratocan and lumican are down-regulated during wound healing^[55]. When cultured in media containing serum *in vitro*, keratocytes change their keratocyte phenotype into activated cells^[56]. In a recent study, human bone marrow-derived MSCs that grow on an AM were cultured in a keratocyte-conditioned medium with cytokines and other growth factors. Results showed that human bone marrow-derived MSCs can directly differentiate into keratocyte-like cells^[45]. The induced MSCs expressed the keratocyte specific markers keratocan, lumican, and aldehyde dehydrogenase 1 family, member A1. They also exhibited a dendritic morphology similar to that of natural keratocytes. Liu *et al.*^[57,58] reported that bone marrow-derived MSCs or umbilical MSCs transplanted into the cornea of keratocyte dysfunction mice significantly increased stromal thickness and improved corneal transparency and host keratocyte functions. These MSCs assumed corneal keratocyte phenotype and expressed the keratocyte-specific markers keratocan and lumican. The results suggest that MSCs can differentiate into keratocyte-like cells and can be influenced by several growth factors and other factors secreted by keratocytes.

MSCs are an ideal candidate for healing damaged corneal endothelium. Corneal endothelial cells mainly provide nutrition to the cornea and maintain corneal transparency by pumping water from the cornea. The cells are

non-renewable after damage and loss. Joyce *et al*^[59] used baseline microarray analysis to show that umbilical cord blood-derived-MSCs (UCB-MSCs) and human corneal endothelial cells (hCECs) have a relative similarity in gene expression. Subsequently, the morphology of MSCs was consistently altered toward a more hCEC-like shape both in tissue culture and in *ex vivo* corneal endothelial wounds when MSCs were grown in a lens epithelial cell-conditioned medium (LECCM). A second microarray analysis showed that UCB-MSCs grown with LECCM had significant changes in the relative expression of genes and differentiated into a more hCEC-like phenotype. These data indicated that UCB-MSCs could be altered toward hCEC-like cells in specific microenvironments and that LECCM influenced the differentiation of UCB-MSCs. However, further research must be conducted to confirm the nature of specific microenvironment.

Paracrine action

MSCs exert therapeutic effects to facilitate tissue wound repair by secreting soluble factors that suppress inflammation and angiogenesis^[60]. Many reports showed that MSCs can improve tissue repair despite exhibiting a small fraction of engraftment in sites of tissue damage^[60]. Ma *et al*^[46] transplanted human MSCs grown and expanded on the AM into chemically burned corneas of rats. After 4 wk, the damaged corneal surface and the vision of the rats were significantly improved. Immunofluorescent analysis showed that epithelial cell markers were not detected in the eyes of rats transplanted with MSCs. However, the expression level of CD45 and interleukin 2 (IL-2) significantly decreased. In addition, matrix metalloproteinase-2 (MMP-2), which is associated with inflammation-related angiogenesis, was not detected in the eyes of the MSC-treated rats^[46]. The subconjunctival injection of MSCs improved the wound healing of corneas with alkali burns. The MSCs remained in the subconjunctival space after 7 d without infiltrating the injured cornea^[44]. These results demonstrate that MSCs exert their therapeutic effects on corneal wound repair by suppressing inflammation and angiogenesis, which serve more important functions than differentiation into corneal epithelial cells. Topically administered MSCs and conditional MSCs medium can obviously attenuate inflammation, reduce CNV, and accelerate corneal wound healing in rats with chemically burned corneas. The soluble factors produced by MSCs are involved in anti-inflammatory and anti-angiogenic effects through paracrine action^[47]. In injured corneas, MSCs transplantation up-regulates the expression levels of the anti-angiogenic factor thrombospondin-1 (TSP-1) and the anti-inflammatory cytokines IL-10, TGF-1, and IL-6 while down-regulates the expression levels of the pro-inflammatory factors IL-2, interferon- γ (IFN- γ), macrophage inflammatory protein-1 α , and vascular endothelial growth factor (VEGF)^[44,47]. Similarly, human MSCs co-cultured with chemically damaged hCECs are associated with changes in the expression level of soluble factors that modulate inflammation and

neovascularization^[61]. Oh *et al*^[62] have recently found that MSCs activated by the signals from injured corneas up-regulate the expression of tumor necrosis factors (TNF)- α -stimulated gene/protein 6 (TSG-6). TSG-6 is an anti-inflammatory protein that can significantly decrease neutrophil infiltration, pro-inflammatory cytokine and chemokine levels in corneas with mechanical injuries, and corneal opacity and neovascularization. In another study, MSCs administered intraperitoneally or intravenously without being engrafted to chemically injured corneas of rats effectively alleviate corneal opacity and inflammation by TSG-6^[43]. MSCs accelerate the neovascularization by secreting VEGF, particularly in ischemic tissues and tumors. By contrast, MSCs are involved in anti-angiogenesis in injured cornea. Such action might be attributed to the high-level of TSP-1, which inhibits VEGF^[47]. The specific mechanism involved remains unclear.

MSCs secrete certain growth factors to facilitate the survival of injured cells and accelerate tissue regeneration in specific microenvironments. Zhang *et al*^[63] cultured MSCs and LSCs that were seeded on a xenogeneic acellular corneal matrix *in vitro*. They observed higher levels of growth factors, including VEGF, epidermal growth factor (EGF), and TGF- β 1, in the MSCs than in the LSCs. The MSCs express beneficial factors for corneal recovery and might permit a potent corneal substitute for healing corneal injury. Aside from up-regulating beneficial factors, MSCs also stimulate the proliferation of LSCs and native corneal cells^[41]. Moreover, MSCs can reduce the severity of LSC loss, suppress inflammation, and improve epithelial regeneration during the acute phase of corneal injury^[16]. The survival and proliferation of rat LECs are markedly promoted and the expression of EGF is up-regulated by co-culturing LECs and rat MSCs or by culturing LECs in a medium pre-conditioned with rat MSCs. The effects of MSCs on LECs might be mediated by paracrine action^[64]. In summary, paracrine mechanisms of MSCs may exert a significant impact in promoting corneal wound repair, which involves the joint participation of different soluble factors that modulate inflammation and angiogenesis as well as improve tissue regeneration. The involved bio-physiological factors and the underlying mechanism in corneal wound healing remain unclear.

MSCS AND CORNEAL TRANSPLANTATION

MSCs, immunity, and solid organ transplantation

Several studies have confirmed that MSCs have potent immune modulatory properties that allow them to exert immunosuppressive effects both *in vivo* and *in vitro*. As immune privileged cells, MSCs reduce the expression of MHC class II and co-stimulatory molecules on the surface of cells (CD80, CD86, and CD40)^[65]. *In vitro*, MSCs influence the innate immune system by suppressing maturation and activation of dendritic cells (DCs) and cytotoxicity of natural killer cells. They also interact with adaptive

immune responses by inhibiting proliferation and cytokine secretion of T cells and maturation of B cells^[7,66]. To effectively influence immunoregulation, the activation of MSCs requires an inflammatory microenvironment and stimulation by pro-inflammatory cytokines, such as IFN- γ and TNF- α , from effector T cells^[67,68]. Several soluble factors produced by MSCs are involved in the immunosuppression of MSCs. These factors include TGF- β , IL-6 and -10, MMP, prostaglandin E2 (PGE2), indoleamine-2, 3-dioxygenase (IDO), human leukocyte antigen-G5 (HLA-G) and nitric oxide (NO)^[69,70]. In addition, MSCs can decrease the expression level of IFN- γ from Th1 cells and increase the expression levels of IL-4 and IL-10 from Th2 cells, thereby promoting immune response of naive CD4⁺ T cells toward the Th2 type response^[71,72]. When co-cultured and placed in contact with naive T cells, human MSCs promote the differentiation and expansion of regulatory T cells (Tregs) in mixed-lymphocyte reactions by secreting PGE2 and TGF- β ^[73]. Tregs, a specialized subset of T cells, retain their capacity to suppress the response of T cells and maintain immune system activation. MSCs were also suggested to maintain tolerance and improve the survival of allografts in solid organ transplantation mainly through the function of Tregs^[74].

Currently, MSCs have been widely used in animal studies that involve solid organ transplantation. However, the *in vivo* effect of MSCs on inhibiting the immune rejection response is controversial. Bartholomew *et al*^[75] first reported that using MSCs can prolong skin graft survival in organ transplantation. In rat and primate models, MSCs can effectively suppress immune rejection; induce immune tolerance; and prolong graft survival in the liver, heart, kidney, pancreas, and other solid organs for transplantation^[76-78]. These results might result from the shifting of the Th1/Th2 cell balance in favor of the latter, thereby increasing the level of Tregs and inhibiting the function of DCs through MSCs. By contrast, other studies suggested that MSCs exert no beneficial effects on organ transplantation. The infusion of donor-derived MSCs accelerated allograft rejection in an immunocompetent rat skin transplantation model^[79]. In addition, Inoue *et al*^[80] reported that MSC therapy with or without cyclosporine A (CsA) is prone to accelerate graft loss in a rat cardiac transplantation model, although donor-derived MSCs inhibit the proliferation of T cells *in vitro*. These results indicate that MSC therapy is not always beneficial and might exacerbate disease under certain circumstances. Interestingly, the combined use of MSCs and mycophenolate mofetil^[81] leads to successfully prolonged graft survival by allowing the IFN- γ stimulation of non-activated MSCs and suppressing the infiltration of antigen presenting cells (APCs) and T cells in the graft of the same model^[82]. The results of this study suggest that the immunomodulation function of MSCs depends on the type of combined immunosuppressive agent. Furthermore, the application of MSCs combined with a subtherapeutic dose of immunomodulator in solid organ transplantation not only potently exerts a synergistic

function in suppressing immune rejection response but also reduces the side effect caused by large doses of immunosuppressive agents alone.

Infusion time is an important factor for MSCs to effectively exert their immunoregulatory effect on organ transplantation^[66,83]. Heart transplants require MSCs to be infused before organ transplantation. An intravenously pre-operative infusion of MSCs can modulate Treg expansion early, and induce immune tolerance before occurrence of inflammation and immune response. By contrast, MSCs infused postoperatively are less effective^[84]. In a mouse kidney transplant model, MSC injection after transplantation failed to prolong graft survival due to complement activation, neutrophil recruitment, and kidney dysfunction. Administering MSCs before kidney transplantation could prevent the deterioration of graft functions^[85]. However, a recent study^[86] that used a rat corneal transplantation model has shown that MSCs prolong corneal allograft survival time only when injected immediately after surgery and that pre-operative infusion exerts no significant effect. The cornea is an immune privileged tissue and is situated in a special immune microenvironment that triggers delayed-type hypersensitivity. Therefore, MSCs injected immediately after the surgery might be more appropriate^[86].

The dose of MSCs is another element that influences their therapeutic effect on solid organ transplantation. During *in vitro* mixed lymphocyte reactions, MSCs could inhibit T lymphocyte proliferation depending on the graded numbers of MSCs^[87]. The best dose and time of MSCs transplantation have yet to be standardized because previous studies used different animal models and infusion methods. In a previous study, patients were intravenously infused with doses of $1.0\text{--}2.0 \times 10^6$ MSC/kg^[88]. Another recent multi-center research has shown that 0.5×10^6 MSC/kg to 9×10^6 MSC/kg is a safe dose range; that is, this range does not elicit adverse side effects^[89]. Results showed that the effects of MSCs are dose-dependent. Therefore, investigating the dose of MSCs is necessary to achieve the best therapeutic results.

MSCs in corneal transplantation *in vivo*

Given their immunomodulatory and anti-inflammatory properties, MSCs are a potential therapeutic tool for corneal allograft transplantation. Corneal allograft has a survival rate that exceeds all other types of solid organ transplantation as a result of immune privilege^[90]. However, corneal graft immune rejection could occur, which remains the leading cause of corneal allograft failure.

Oh *et al*^[91] first examined the immunomodulatory effects of allogeneic recipient-derived MSCs in penetrating keratoplasty in a pig-to-rat model. The topical application of allogeneic rat MSCs caused T cell differentiation in Th2 cells. Subsequently, the balance between Th1 and Th2 cells shifted toward Th2-type. However, the significant increase in Th2-type cytokines induced by MSCs did not effectively prolong the survival of pig corneal xenografts in rats.

Recently, Jia *et al*^[86] have investigated the immunosuppressive function of MSCs in a rat allogeneic corneal transplant model. In this study, donor MSCs were intravenously injected at different times and were administered with different doses of CsA. They found that the postoperative infusion of MSCs inhibits corneal allograft rejection and prolongs corneal graft survival, whereas pre-operative infusion is ineffective. Moreover, the combined effect of MSCs and CsA highly depends on CsA dose. Combining 2 mg/kg CsA with MSCs is the best regimen for achieving a synergistic effect. Further studies on the possible mechanism of MSCs in allogeneic keratoplasty showed that donor-derived MSCs significantly inhibit allogeneic T cell responses both *in vitro* and *in vivo*, reduce Th1 pro-inflammatory cytokines, and increase Th2 anti-inflammatory cytokine secretion in a rat model. Moreover, MSCs up-regulate the number of Tregs, thereby preventing allograft immune rejection and improving allograft survival.

However, a recent study^[92] has suggested that MSCs function in corneal transplantation through a different mode of action. Pre-transplant systemic infusion of human MSCs inhibits the afferent loop of the immune response primarily by reducing inflammation caused by surgery during the early postoperative period, thereby decreasing the immune rejection responses and prolonging the graft survival time in a mouse model of corneal transplantation. MSCs exert their effects by secreting soluble factors, such as TSG-6, rather than engraftment in the cornea allograft. Suppressing inflammation subsequently decreases the activation of APCs in both the cornea and draining lymph nodes. By contrast, human MSCs with TSG-6 knockdown are not effective in reducing the early inflammatory response or prolonging corneal graft survival. This mechanism might also be involved in reducing the immune rejection responses in other organ transplants.

In summary, the application of MSCs may be a new alternative method for prevention and treatment of immune rejection after corneal transplantation. However, the therapeutic effects of MSCs on corneal allograft immune rejection response in clinical and animal models remain unclear. Further studies on the specific molecular mechanism of MSCs must be conducted to develop a novel therapeutic strategy.

CONCLUSION

MSCs promote the healing of corneal wound through their capability of differentiation and paracrine function. In addition, MSCs inhibit the rejection of corneal transplantation and prolong the survival time of corneal allografts. However, the mechanisms of MSCs in corneal injury and keratoplasty are not clear. The time, manner, dose, route of administration, and terminal differentiation of MSCs *in vivo* require further investigation. It is believed that the microenvironment is crucial in modulating the function of MSCs. Further research on microenvironmental factors must be carried out to improve the

therapeutic effect of MSCs. Moreover, the heterogeneity of MSCs leads to treatment discrepancies. Therefore, investigating the characteristics of MSC subpopulation and improving the culture conditions for MSCs may lead to the improvement and predictability of therapeutic effects. The complex features of MSCs contribute to the complexity of their effects. The functions of MSCs and their tremendous potential effects on corneal and other diseases require further exploration.

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Multiple myeloma mesenchymal stromal cells: Contribution to myeloma bone disease and therapeutics

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which clonal plasma cells proliferate and accumulate within the bone marrow. The presence of osteolytic lesions due to increased osteoclast (OC) activity and suppressed osteoblast (OB) function is characteristic of the disease. The bone marrow mesenchymal stromal cells (MSCs) play a critical role in multiple myeloma pathophysiology, greatly promoting the growth, survival, drug resistance and migration of myeloma cells. Here, we specifically discuss on the relative contribution of MSCs to the pathophysiology of osteolytic lesions in light of the current knowledge of the biology of myeloma bone disease (MBD), together with the reported genomic, functional and gene expression differences between MSCs derived from myeloma patients (pMSCs) and their healthy counterparts (dMSCs). Being MSCs the progenitors of OBs, pMSCs primarily contribute to the pathogenesis of MBD because of their reduced osteogenic potential consequence of multiple OB inhibitory factors and direct interactions with myeloma cells in the bone marrow. Importantly, pMSCs also readily contribute to MBD by promoting OC formation and activity at various levels (*i.e.*, increasing RANKL to OPG expression, augmenting secretion of activin A, uncoupling ephrinB2-EphB4 signaling, and through augmented production of Wnt5a), thus further contributing to OB/OC uncoupling in osteolytic lesions. In this review, we also look over main signaling pathways involved in the osteogenic differentiation of MSCs and/or OB activity, highlighting amenable therapeutic targets; in parallel, the reported activity of bone-anabolic agents (at preclinical or clinical stage) targeting those signaling pathways is commented.

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Key words: Mesenchymal stromal cells; Multiple myeloma; Osteolytic lesions; Myeloma bone disease; Bone-directed therapy; Bone-anabolic drugs

Core tip: In multiple myeloma, bone marrow mesenchymal stromal cells (MSCs) primarily contribute to associated osteolytic lesions because of their defective differentiation to mature osteoblasts. Importantly,

Abstract

Multiple myeloma is a hematological malignancy in

these MSCs also contribute to myeloma bone disease by enhancing osteoclast formation and activity through various mechanisms (*i.e.*, increasing the receptor activator of nuclear factor- κ B ligand/osteoprotegerin ratio, augmenting activin A secretion, uncoupling ephrinB2-EphB4 signaling and because of heightened production of Wnt5a). In addition, we overview signaling pathways involved in the osteogenic differentiation of MSCs or osteoblast activity and comment on the reported activity of bone-anabolic agents (preclinical or clinical stage) to restore bone homeostasis in myeloma patients.

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MESENCHYMAL STROMAL CELLS: *IN VITRO* AND *IN VIVO* PROPERTIES

Mesenchymal stromal cells

Bone marrow (BM)-derived mesenchymal stromal cells (MSCs) were initially described by Friedenstein *et al.*^[1] in the late 60-s as adherent cells of fibroblastic morphology with the ability to differentiate into osteogenic cells, although it was later demonstrated that these cells also have chondrogenic and adipogenic differentiation potential^[2]. They were initially named as Colony Forming Unit-Fibroblasts^[3], but soon they were referred to as MSCs, term that gained general acceptance^[4]. Instead, the International Society for Cellular Therapy (ISCT) recommends the term “mesenchymal stromal cells” for MSCs^[5] and published several years ago a number of minimal definition criteria for these cells^[6], which are indicated in Table 1.

MSC isolation, characterization and *in vitro* expansion

BM-derived MSCs may be isolated from mononuclear cells obtained after density-gradient centrifugation of BM aspirates and subsequent adherence to tissue culture plasticware. Since their proportion in a normal BM sample is really low (between 0.01% and 0.0001% of nucleated cells)^[7], for most applications MSCs need to be *in vitro* expanded. The standard culture medium is based on Dulbecco's Modified Eagle Medium or α -Minimum Essential Medium with 10% of fetal bovine serum, although the latter can be replaced by platelet lysate or a commercial concentrate of growth factors^[8]. The expansion medium is replaced twice a week and thus non-adherent cells are removed. After two or three passages, the primary culture contains more than 95% of MSCs, and these cells are then used for most experiments^[9].

According to the ISCT definition criteria^[6] (Table 1), an immunophenotypic study is mandatory to evaluate the

Table 1 Minimal criteria for mesenchymal stromal cell definition (International Society for Cellular Therapy)

Adherence to plastic surfaces in standard culture conditions		
	Positive (> 95% +)	Negative (< 2% +)
Immunophenotype	CD105	CD45
	CD73	CD34
	CD90	CD14 or CD11b
		CD79a or CD19
		HLA-DR
<i>In vitro</i> differentiation to osteoblasts, adipocytes and chondroblasts (demonstrated by appropriate staining of cell cultures)		

positivity for at least CD73, CD105 and CD90 and negativity for HLA-DR and hematopoietic antigens (CD45, CD34, CD19 or CD79 α , CD14 or CD11b).

Differentiation and immunomodulatory properties of MSCs

Upon *in vitro* culture with the appropriate differentiation media, MSCs are able to differentiate into osteogenic, adipogenic and chondrogenic phenotypes^[10]. This multilineage differentiation ability into mesodermal cell types is another definition criteria established by the ISCT^[6] (Table 1), and is the basis for evaluating the therapeutic potential of MSCs in a number of clinical trials, especially for treating musculoskeletal diseases^[11].

Being this property important, the range of diseases in which MSCs are of potential use has widely expanded when these cells demonstrated to display potent immunomodulatory and anti-inflammatory effects both *in vitro* and *in vivo*^[12]. In this regard, MSCs are able to reduce the activation of both T cells^[13] and B cells^[14], and to increase the number of T-regulatory cells^[15]. In addition, MSCs inhibit the maturation of dendritic cells and their capacity to process and present antigens^[16]. MSCs also reduce neutrophil activation and proliferation of natural killer cells^[17,18], thus regulating innate immune system responses. For these reasons, MSCs are currently being evaluated for the treatment of several immune-mediated diseases.

MULTIPLE MYELOMA AND THE BONE MARROW MICROENVIRONMENT. MYELOMA-ASSOCIATED BONE DISEASE

Multiple myeloma and the bone marrow microenvironment

Multiple myeloma (MM) is a hematological malignancy resulting from the clonal expansion of plasma cells in the BM. Diagnostic criteria of symptomatic myeloma include the presence of at least 10% BM myeloma cells and of monoclonal protein in serum and/or urine, together with myeloma-related end-organ or tissue damage (including hypercalcemia, renal dysfunction, anemia, immunodeficiency and bone destruction)^[19]. In fact, almost 80% of myeloma patients develop osteolytic lesions, which are responsible for some of the most devastating characteris-

tics of the disease. In most (if not all) cases, symptomatic myeloma is preceded by sequential asymptomatic stages of monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma^[20], with increasing BM plasmacytosis and monoclonal component as well as augmented risk of progression to active MM (around 1% per year for MGUS patients and 10%-20% for patients with smoldering myeloma)^[21]. MM accounts for more than 1% of all cancers, with an incidence of 33400 new myeloma cases and 20300 deaths in the European Union 27 in 2012^[22].

During last decade, substantial advances both in the knowledge of the biology of the disease and in the use of more effective molecular-targeted novel agents and combinatorial regimens^[23,24] have led to improved responses and survival rates (median survival has increased from 3 to over 6 years^[25]). It is expected that advances in remaining controversies of the pathophysiology of the disease^[25] together with novel therapies currently in development and testing, may further improve myeloma survival in coming years, with future therapeutic aims rather focusing on increasing long-term remission rates and improving the quality of life of myeloma patients.

The biological behavior and clinical outcome in MM is partly dependent on the genomic and epigenetic abnormalities of myeloma cells^[26]. Compelling evidence has accumulated, however, supporting a critical role of the BM microenvironment in the pathogenesis and progression of the disease^[27-31]. MM cells establish complex interactions with other cellular components of the BM milieu [MSCs, osteoclasts (OCs), osteoblasts (OBs) and osteoprogenitor cells, endothelial cells, adipocytes, immune cells-dendritic cells, macrophages, T cells-], with components of the extracellular matrix (ECM) (*e.g.*, laminin, collagen, proteoglycans, glycosaminoglycans), and also with secreted soluble factors (cytokines, chemokines and growth factors). These interactions have bidirectional consequences: on the one hand, interactions of MM cells mainly with MSCs and OCs lead to activation of multiple cellular signaling pathways on myeloma cells [phosphatidylinositol 3-kinase/AKT, Janus kinase/signal transducer and activator of transcription 3, Ras/Raf/mitogen-activated protein kinase kinase/extracellular signal-related kinase, nuclear factor- κ B (NF κ B)] which support their proliferation, survival, migration and even resistance to therapeutic agents (reviewed in^[27,31,32]). On the other hand, myeloma cells perturb the BM homeostasis causing anemia, immunosuppression, and uncoupling of the bone remodeling process leading to the development of osteolytic bone lesions characteristic of the disease.

Myeloma bone disease: Mechanisms of OC activation and OB inhibition

Myeloma bone disease (MBD) is characterized by the presence of osteolytic lesions that result in skeletal-related events (SREs) including severe bone pain, osteopenia, diffuse osteoporosis, focal lytic lesions, pathological fractures and spinal cord compression^[33,34]. Of importance, MBD

not only severely affects the quality of life of MM patients, but is also linked to poor prognosis, shorter overall survival and progression-free survival^[35,36]. This highlights the need of bone-targeted supportive treatments in addition to anti-myeloma therapy, which may reduce the risk of bone complications in MM patients. In addition, accumulating evidences of preclinical and clinical studies support the notion of an intimate relationship between tumor growth and the development of MBD, being one the necessity and consequence of the other^[37]. Since many of the dysregulated factors involved in the pathophysiology of osteolytic lesions also promote MM growth and survival, it is expected that effective interventions on MBD would secondarily lead to myeloma inhibition^[38].

Clinical observations, histomorphometric studies and measurements of serum/urine bone metabolism markers^[39-41] showed that uncoupled bone remodeling in MM was associated to both increased bone resorption (with increased number and activity of OCs) and almost absent bone formation (impaired OB formation and function). Only recently, many of the cellular and molecular interplayers involved in the pathophysiology of MBD have been identified and excellently reviewed^[34,37,38,42,43]. Next, we summarize the main factors and molecular mechanisms leading to the enhanced OC activation and suppressed OB function in MM.

Enhanced OC differentiation and resorptive activity

Enhanced OC formation from myeloid precursors and OC hyperactivation is primarily mediated through increased production of multiple "OC-activating factors (OAFs)" both by MM cells and other cells from the BM microenvironment (*e.g.*, receptor activator of NF- κ B ligand (RANKL), CCL3 [also known as macrophage inflammatory protein-1 α (MIP-1 α)], activin A, interleukin-3 (IL-3), IL-7, IL-1 β , IL-6 and CCL20^[44]; for reviews of cellular origin of OAFs, see^[37,45]; also see Figure 1).

RANKL: RANKL is a member of the tumor necrosis factor (TNF) superfamily expressed as a transmembrane protein by BM MSCs and OBs, and by T lymphocytes as a soluble form; whether MM cells are producers of RANKL is still a controversial issue^[46,47]. Instead, the receptor of RANKL, RANK, is expressed on the surface of OCs and OC precursors. RANK-RANKL signaling has been shown to play an essential role in OC formation, activation and survival preventing OC apoptosis^[48,49]. Osteoprotegerin (OPG) is a soluble glycoprotein secreted by MSCs and OBs which acts as a decoy receptor for RANKL, neutralizing its activity and thus inhibiting osteoclastogenesis and bone resorption^[50]. Myeloma cells induce MSCs and OBs in the BM to upregulate the expression of RANKL and to reduce the expression of OPG, leading to increased RANKL/OPG ratios. In addition, MM cells may also sequester OPG through its binding to syndecan-1 (CD138), which is subsequently internalized and degraded^[51]. Furthermore, T lymphocytes in MM also overexpress TNF-related apoptosis inducing

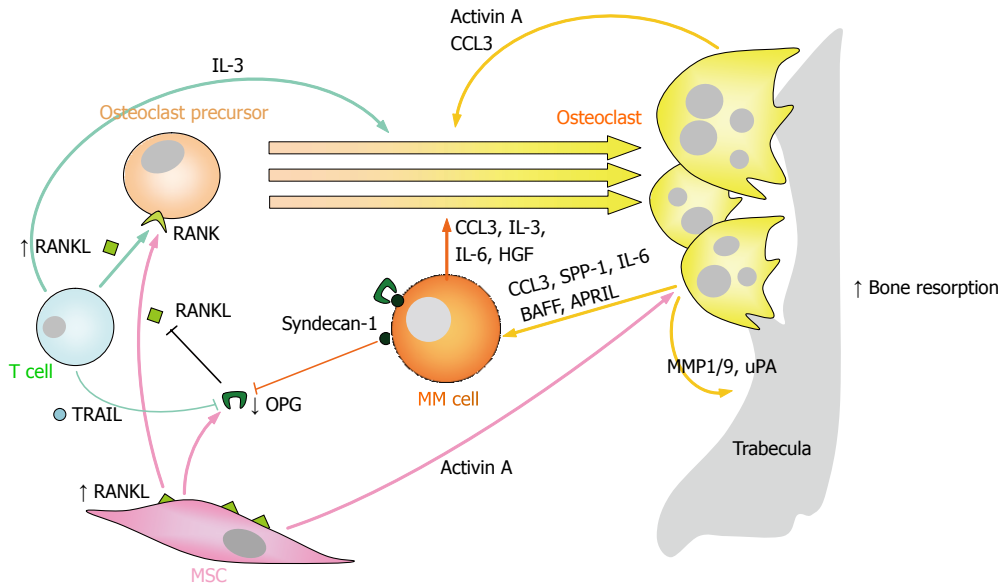


Figure 1 Enhanced osteoclast formation and resorption partially mediates the development of myeloma bone disease. Numerous “OC-activating factors” produced by multiple myeloma cells and other cells in the bone marrow microenvironment (including RANKL, CCL3, activin A, IL-3, HGF and IL-6) readily promote OC differentiation from OC precursors and/or stimulation of OC resorptive activity. In MM, the RANKL/OPG ratio is clearly favored towards RANKL, both because of increased expression of RANKL on MSCs and T lymphocytes, and because of reduced expression of OPG by MSCs and inactivation of OPG through binding to TRAIL or syndecan-1 on the surfaces of myeloma cells. On the other hand, OCs produce several factors (e.g., IL-6, CCL3, BAFF and APRIL) which promote the growth and survival of multiple myeloma cells. RANKL: Receptor activator of NF κ B ligand; NF κ B: Nuclear factor- κ B; CCL3/MIP1 α : Macrophage inflammatory protein 1- α ; IL-3/6: Interleukin 3/6; HGF: Hepatocyte growth factor; OPG: Osteoprotegerin; RANK: Receptor activator of NF κ B; TRAIL: Tumor necrosis factor-related apoptosis inducing ligand; BAFF: B-cell-activating factor; APRIL: A proliferation-inducing ligand; SPP-1: Osteopontin; MMP1/9: Matrix metalloproteinase 1/9; uPA: Urokinase plasminogen activator; MSCs: Mesenchymal stromal cells; OC: Osteoclast; MM: Multiple myeloma.

ligand (TRAIL), which binds and neutralizes OPG, further reducing its OC inhibitory activity^[52]. Whereas in physiological conditions the RANKL/OPG ratio tightly regulates OC function for adequate bone remodeling, in MM it is clearly favored towards RANKL, promoting osteoclastogenesis and bone destruction^[53,54]. Thus, the RANKL/OPG axis constitutes an important target for the treatment of MBD.

CCL3: The CCL3 (MIP-1 α) chemokine is mainly produced by both myeloma cells and OCs, and functions as a major osteoclastogenic and OC survival factor, both directly and indirectly by enhancing the osteoclastogenic activity of RANKL and IL-6^[55,56]. Interestingly, CCL3 has been found to have pleiotropic roles in MM, also inducing growth, survival and chemotaxis for malignant plasma cells^[57] and, as will be commented later, inhibition of OB differentiation^[58,59].

Activin A: Activin A is a transforming growth factor (TGF) β family member identified as a key component of MBD, having a dual role in stimulating OC formation and activity^[34,60] and as an inhibitor of OB differentiation^[61,62]. MSCs and OCs are the main sources of activin A and interacting myeloma cells further upregulate its expression in MSCs^[61].

Other factors promoting OC formation and activity: IL-3 is majorly produced by activated T lymphocytes and by myeloma cells, and may stimulate OC formation and resorption directly or by further augmenting that

of RANKL and CCL3^[63,64]. Other chemokines such as IL-7, tumor necrosis factor α (TNF α) and IL-1 β indirectly stimulate osteolytic processes, inducing RANKL expression on BM stromal cells (TNF α and IL-1 β ^[65]) and by circulating T cells (IL-7)^[66]. Several other OAFs are secreted by myeloma cells and/or stromal cells [e.g., hepatocyte growth factor (HGF)^[67,68], IL-6^[69], IL-8^[70], and vascular endothelial growth factor (VEGF)^[71]], or by dendritic cells, Th17 lymphocytes, osteocytes and megakaryocytes in the BM milieu [(e.g., IL-17, IL-11) reviewed in^[37,72]], which further increase the gradient of osteoclastogenic factors in focal lesions and contribute to OC production and activity.

At the same time, OCs readily promote MM cell survival and growth by physical cell-cell contact and by the release of several soluble factors [including IL-6, CCL3, osteopontin, B-cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL)], and thus creating a vicious circle between bone lesions and tumour expansion (reviewed in^[30,37]).

On the other hand, myeloma-OC interactions may directly contribute to bone matrix degradation *via* secreted metalloproteinases 1/9 and urokinase-type plasminogen activator from OCs^[73]. Besides, some myeloma cells may acquire resorbing capabilities and degrade bone^[74,75], and dendritic cells in the BM may transdifferentiate to bone-resorbing OCs after myeloma interaction^[76], further contributing to enhanced resorption.

Suppression of osteoblastogenesis and OB function

Myeloma-induced suppression of osteoblastogenesis

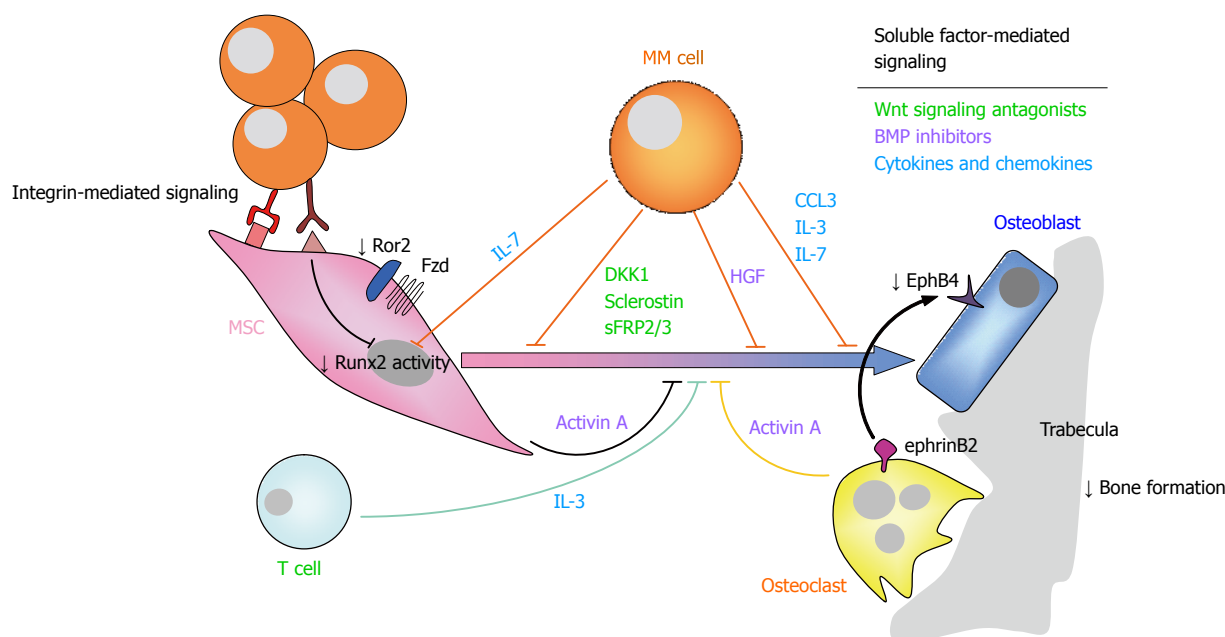


Figure 2 Suppression of osteoblastogenesis and osteoblast function in multiple myeloma is also involved in the pathophysiology of myeloma bone disease. Myeloma-induced OB suppression is partially mediated by direct cell to cell contact interactions with MSCs, leading to reduced activity of the Runx2/Cbfa1 transcription factor, and to inhibition of non-canonical Wnt5a signaling due to decreased expression of Ror2 in pre-OBs. In addition, soluble factors produced by myeloma cells and cells in the bone marrow microenvironment, such as Wnt signaling antagonists (e.g., DKK1, sclerostin, sFRP-2/3), BMP inhibitors (activin A, TGF β , HGF), cytokines and chemokines (such as IL-7, TNF α , IL-3, CCL3) and apoptotic factors also contributed to inhibition of osteogenic differentiation and function. Finally, reduced ephrinB2-EphB4 signaling (from OCs to OBs) because of diminished EphB4 expression in MSCs, further contributes to impaired OB differentiation. Ror2: Receptor tyrosine kinase-like orphan receptor 2; DKK1: Dickkopf-1; sFRP-2/3: Secreted frizzled related protein-2/3; TGF β : Transforming growth factor β ; HGF: Hepatocyte growth factor; IL-7/3: Interleukin 7/3; ephrinB2: ephrin-B2 ligand; EphB4: Eph receptor B4; MSCs: Mesenchymal stromal cells; OB: Osteoblast; OC: Osteoclast; CCL3/MIP1 α : Macrophage inflammatory protein 1- α ; TNF α : Tumor necrosis factor α ; BMP: Bone morphogenetic protein; MM: Multiple myeloma.

and OB activity is exerted both by functional inhibition of existing OBs as well as by impaired differentiation of MSCs into mature OBs. This is in accord with the findings of a significant reduction in the number of active OBs in BM biopsies^[77] and extremely low serum markers of osteoblastogenesis (such as osteocalcin and OPG) in patients with active osteolytic lesions as compared to myeloma patients not having bone lesions^[45].

In recent years, many of the molecular mediators underlying suppression of OB differentiation and function in MM have been identified, involving both direct cellular interactions and soluble factors (Figure 2).

Soluble factors contributing to OB suppression include inhibitors of the two major signaling pathways governing osteoblastogenesis [*i.e.*, Wnt and bone morphogenetic protein (BMP) signaling pathways], several cytokines and chemokines, as well as MM-induced apoptotic factors for OBs.

Wnt signaling antagonists (Dickkopf-1, sclerostin, secreted Frizzled-related proteins-2 and 3): MM cells secrete Dickkopf-1 (DKK1)^[78] and sclerostin^[79], both inhibiting Wnt canonical signaling and thus OB differentiation because of direct binding to the low-density lipoprotein receptor-related protein (LRP)5/6 co-receptor in osteoprogenitor cells^[80]. Sclerostin is also produced by osteocytes, mediating osteocyte-OC communication necessary for bone homeostasis^[81]. Interestingly, both DKK1

and sclerostin further increase the RANKL/OPG ratio on MSCs and osteoprogenitor cells by upregulating the expression of RANKL and reducing that of OPG, thus indirectly enhancing OC differentiation and activity^[79,82]. A direct stimulatory effect of sclerostin on OC formation has also been reported^[83]. Other Wnt antagonists produced by primary cells and MM cell lines are the secreted Frizzled-related proteins-2 and 3 (sFRP2 and sFRP3), which bind directly to secreted Wnt ligands and at least sFRP3 has been associated to the extent of MBD at diagnosis^[84-86].

BMP inhibitors (activin A, TGF β , hepatocyte growth factor): The BMP is another major molecular pathway involved in osteogenesis, in which members of the TGF β superfamily of cytokines (BMPs, activin A, TGF β) bind to heterodimeric receptors to activate Smad proteins, which may directly regulate the expression of osteoblastogenic genes as transcription factors (*e.g.*, DLX5-distal-less 5) or indirectly *via* Runx2/Cbfa1^[87]. Although some ligands (*e.g.*, BMP2) directly stimulate osteogenesis through this pathway, others (such as activin A and TGF β) have opposite effects. Activin A is produced by OCs and by MSCs after interaction with myeloma cells, and in addition to its commented pro-OC effect, it inhibits OB differentiation *via* Smad2-dependent DLX5 downregulation^[61]. Similarly, TGF β also downregulates DLX5^[88], and inhibits OB differentiation. The HGF is produced by MM cells and is found at high concentrations in the BM of my-

eloma patients^[89]. It has been shown to promote proliferation of human MSCs keeping cells in an undifferentiated state, and to inhibit BMP-induced Smad translocation, thus inhibiting OB formation.

Other cytokines and chemokines: Other cytokines and chemokines may additionally contribute to suppression of OB activity (*e.g.*, IL-7, TNF α , IL-3 and CCL3). IL-7 is produced by malignant plasma cells and partially mediates Runx2/Cbfa1 decreased activity in MSCs interacting with myeloma cells, and reduces the expression of OB markers^[77,90]. The pro-inflammatory cytokine TNF α inhibits the expression of both Runx2/Cbfa1 and Osterix transcription factors^[91,92] and increases the expression of sclerostin in OBs^[93]. Interestingly, IL-7 and TNF α effects on osteoprogenitor cells in MM were found to be partly mediated by increased levels of the Gfi1 transcriptional repressor of Runx2/Cbfa1^[94]. IL-3 is majorly secreted by T lymphocytes (but also by myeloma cells) and besides its commented activity on OC formation and activation^[63], it indirectly inhibits basal and BMP2-induced OB differentiation by stimulating CD45⁺ monocyte-macrophages^[95,96]. In addition to the commented activity of CCL3 related to its pro-OC activity and in support of MM growth, CCL3 has been shown to inhibit OB differentiation and function through CCR1^[58].

MM-induced apoptosis on OBs: MM-induced apoptosis on OBs and osteocytes may also account for OB suppression in MM. OBs from myeloma patients with extensive osteolytic lesions have been shown to overexpress the Fas Cell Surface Death Receptor, death receptors DR4/5 and receptors to TRAIL, and to promptly undergo apoptosis when co-cultured with myeloma cells^[97,98]. Similarly, pre-osteocytes of patients with active bone disease in co-culture with myeloma cells showed increased apoptosis and upregulated expression of IL-11, thus increasing their pro-osteoclastogenic properties^[99].

In addition to soluble factors, direct contact interactions of myeloma and pre-OBs further contribute to OB suppression in MBD leading to reduced activity of Runt-related transcription factor 2/core-binding factor Runt domain α subunit 1 (Runx2/Cbfa1), which is a critical transcriptional regulator of OB differentiation^[77]. Blocking of very late antigen 4 (VLA4)-vascular cell adhesion molecule 1 (VCAM1) interactions with a neutralizing anti-VLA4 antibody reduced the inhibitory effect on Runx2/Cbfa1 activity, thus making these adhesion molecules partially responsible for the inhibition of OB differentiation and function^[77]. Further, long-term inhibition of Runx2/Cbfa1 and Osterix in pre-OBs seems to be mediated by overexpression of the transcriptional repressor 4EBP1^[100].

Only recently, Giuliani *et al.*^[101] identified another mechanism of myeloma-induced OB impairment through contact interactions. Although canonical Wnt signaling is known to play a critical role in osteoblastogenesis^[87], the non-canonical Wnt5a ligand has been

shown to mediate the osteogenic differentiation of BM human MSCs through activation of co-receptor receptor tyrosine kinase-like orphan receptor 2 (Ror2)^[102,103]. Myeloma cells were found to inhibit the expression of Ror2 when in co-culture interaction with pre-OB cells, therefore inhibiting non-canonical Wnt5a signaling and the osteogenic differentiation of MSCs^[101].

Finally, suppressed osteoblastogenesis in MM is further mediated by dysregulation of cell surface molecules involved in OB-OC communication (*i.e.*, ephrinB2-EphB4). Bidirectional signaling between the cell-surface molecules ephrin ligands and Eph receptors controls numerous processes including OB-OC communication^[104]. Specifically, MSCs and OBs express both ephrinB2 and EphB4, whereas OC precursors mainly express ephrinB2. The ephrinB2-EphB4 signaling (from OCs to OBs) stimulates OB differentiation and leads to new bone formation; on the other hand, EphB4-ephrinB2 signaling (from OBs to OCs) blocks OC differentiation^[105]. MSCs from myeloma patients have reduced expression of both ephrinB2 and EphB4 due to interacting myeloma cells, which results in reduced osteogenic differentiation as compared to dMSCs, and in stimulation of osteoclastogenesis^[105].

A secondary consequence of suppression of OB differentiation is that it renders an excess of MSCs/immature OBs in the BM which would enhance OC activation due to higher expression of RANKL, activin A and reduced secretion of OPG, as compared to mature OBs^[106,107]. These MSCs/immature OBs pool would also further support myeloma progression and survival by providing higher levels of cytokines and growth factors than mature OBs.

COMPARISON BETWEEN MSCs FROM MM PATIENTS AND HEALTHY DONORS. CONTRIBUTION OF pMSCs TO MYELOMA BONE DISEASE

A number of studies have compared MSCs derived from the BM of newly diagnosed myeloma patients (pMSCs) and those from healthy donors (dMSCs) (reviewed in^[108]), in an attempt to gain insight into their role in the pathophysiology of MM and MBD. Although MSCs from both origins similar adipogenic and chondrogenic potential, pMSCs functionally and genetically differ from their healthy counterparts. A summary of main similarities and differences found between BM-derived pMSCs and dMSCs is shown in Table 2.

Relative to the contribution of MSCs to the pathogenesis of MBD, and despite some opposite results within groups, several features of pMSCs readily reflect their reduced osteogenic potential as compared to their healthy counterparts (*e.g.*, reduced expression of bone formation markers and critical transcription factors in OB differentiation-Runx2/Cbfa1, Osterix and TAZ)^[109]; reduced expression and activity of early OB marker alkaline phosphatase (ALP)^[110]; reduced matrix mineralization under

Table 2 Main similarities and differences between bone marrow mesenchymal stromal cells from myeloma patients and mesenchymal stromal cells from healthy donors

Study	Assay	Description
Similarities		
Adipogenic differentiation	Oil O Red staining	Both pMSCs and dMSCs showed accumulation of lipid-rich vacuoles ^[109,194]
Chondrogenic differentiation	Toluidine Blue staining	Chondrogenic differentiation potential was similar between pMSCs and dMSCs ^[109]
Differences		
Chromosomal alterations	CGH arrays, FISH	pMSCs did not carry the genomic abnormalities detectable in their correspondent myeloma cells ^[111,194,195] . Only pMSCs showed several non-recurrent chromosomal gains and losses (> 1 Mb size) and “hot-spot” regions with discrete (< 1 Mb) genomic alterations ^[195]
Gene expression profiling	Gene expression microarray	Among 145 differentially expressed genes between pMSCs and dMSCs, 46% accounted for tumor-microenvironment cross-talk. Functional assignment revealed their implication in tumor-support (<i>e.g.</i> , GDF15), angiogenesis (<i>e.g.</i> , ANGPTL4, PAI1, SCG2), and contribution to bone disease (<i>e.g.</i> , NPR3, WISP1, EDG2) ^[111] . Even a distinct transcriptional pattern was found associated to the occurrence of bone lesions in pMSCs ^[113]
Immunophenotype	Flow cytometry	Although few significant differences in cell surface marker expression were found between dMSCs and pMSCs, the latter expressed reduced VCAM1 and fibronectin ^[196] , and higher ICAM1 ^[197] compared to dMSCs
Bone formation markers	qPCR, WB	Expression of bone formation markers (<i>i.e.</i> , osteocalcin and osteopontin), master transcription factors of osteogenic differentiation (<i>i.e.</i> , Runx2/Cbfa1 and Osterix) and TAZ (a Runx2/Cbfa1 transcriptional co-activator) was lower in pMSCs than in dMSCs ^[109]
Expression and secretion of growth factors/cytokines/chemokines	RT-PCR, ELISA	Compared to dMSCs, pMSCs showed increased expression of IL-1 β ^[111] , IL-3 ^[112] , IL-6 ^[111,112,194,198] , IL-10 ^[199] , BAFF ^[199] , GDF15 ^[111,198] , TNF α ^[112] , TGF β 1 ^[112,198] , DKK1 ^[111,121,198] , RANKL ^[112] , AREG ^[111] , and decreased expression of TGF β 2, TGF β 3 and FasL ^[112]
Senescence profile	β -gal staining, propidium iodide DNA staining, qPCR	pMSCs showed an early senescence state compared to dMSCs, as assessed by increased expression of senescence-associated β -galactosidase, increased cell size and accumulation of cells in S phase ^[198]
Immunoability	Co-cultures of MSCs and lymphocytes or PBMCs	pMSCs exhibited reduced efficiency to suppress T-cell proliferation compared to that of dMSCs ^[112,194,198]
Angiogenic potential	qPCR, ELISA, tube formation assay	Angiogenic factors (bFGF, HGF and VEGF) were elevated in the CM of pMSCs compared to dMSCs. Besides, CM from pMSCs significantly promoted proliferation, chemotaxis and capillary formation of HUVECs compared to dMSCs ^[200]
Controversial points		
Proliferation rate	Cell density, CFU-F	Whereas some studies did not find differences in CFU-F number and cell density between dMSCs and pMSCs ^[111] , others found a deficient proliferative potential in pMSCs which could be partly explained by the reduced expression of receptors for several growth factors ^[121]
ALP expression and activity	BCIP-NBT staining and pNPP hydrolysis	ALP expression/activity did not differ between MSCs from both origins ^[111] , whereas other authors found it was significantly reduced in pMSCs compared to dMSCs, with lowest levels in pMSCs from patients with bone lesions ^[110]
Matrix mineralization	Alizarin Red and Von Kossa staining	Some groups have reported a significative reduction of matrix mineralization by pMSCs relative to dMSCs ^[110,111,198] , although others have not observed those differences ^[121,194]
Hematopoietic stem cell support	Long-term co-cultures	Some authors reported that the ability to support the growth of hematopoietic stem cells did not differ between dMSCs and pMSCs ^[111,194] , whilst others found that pMSCs better supported CD34 ⁺ progenitor expansion ^[198]

pMSCs: Mesenchymal stromal cells from myeloma patients; dMSCs: Mesenchymal stromal cells from healthy donors; CGH: Comparative genomic hybridization; FISH: Fluorescence *in situ* hybridization; qPCR: Quantitative PCR; WB: Western blot; RT-PCR: Reverse transcription-PCR; PBMC: Peripheral blood mononuclear cells; CM: Conditioned media; HUVECs: Human umbilical vein endothelial cells; CFU-F: Colony forming unit-fibroblast assay; BCIP-NBT: Bromo-chloro-indolyl-phosphate and nitro blue tetrazolium staining; pNPP: p-nitrophenyl phosphate; ALP: Alkaline phosphatase; IL-3: Interleukin-3; TNF α : Tumor necrosis factor α ; TGF β 1: Transforming growth factor β 1; DKK1: Dickkopf-1; RANKL: Receptor activator of NF κ B ligand; NF κ B: Nuclear factor- κ B.

osteogenic conditions^[110,111]; increased expression of OB inhibitory factors (DKK1, IL-3, IL-1 β , TGF β)^[111,112], and a discernible gene expression signature for pMSCs with or without osteolytic lesions^[113]. These characteristics on pMSCs are likely the consequence of myeloma cell interactions and exposure to multiple soluble OB inhibitory factors and microenvironment conditions (*e.g.*, hypoxia)^[114] as occurring in the BM milieu of myeloma

patients. Most of these studies have been conducted in MSCs after *in vitro* expansion and, in the case of pMSCs, after long-term absence of interaction with myeloma cells; thus, the presented differences between dMSCs and pMSCs may have been retained *in vitro* likely by epigenetic mechanisms.

Importantly, pMSCs not only contribute to MBD because of their reduced osteogenic potential, but also

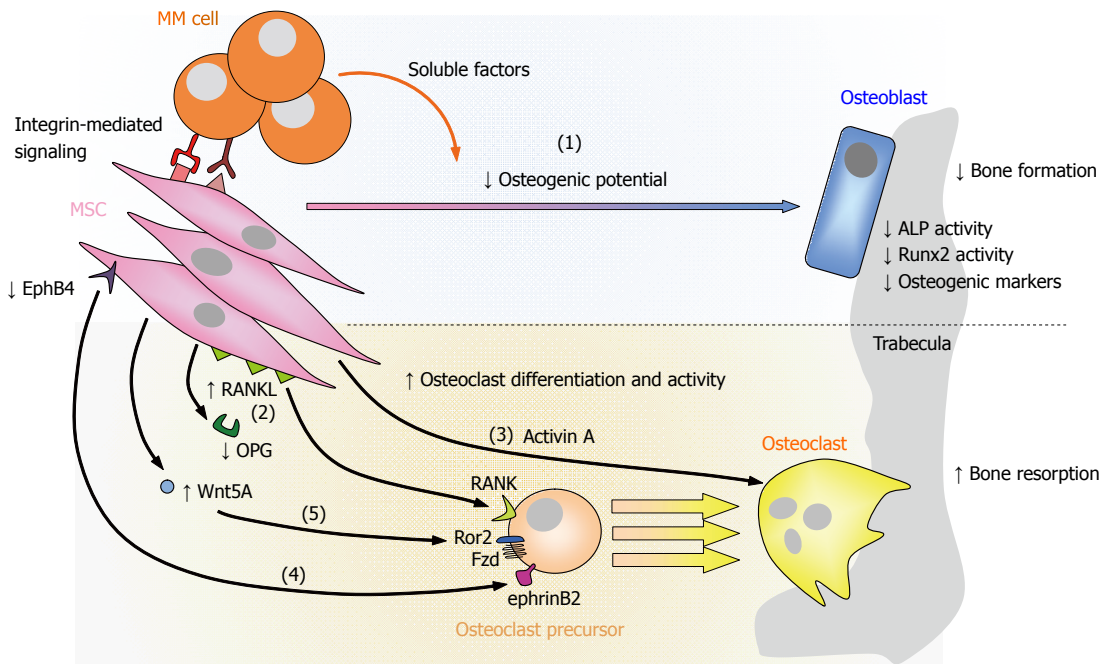


Figure 3 Contribution of mesenchymal stromal cells to myeloma bone disease. In MM, MSCs contribute to the development of osteolytic lesions not only because of their reduced osteogenic potential [(1)], but also because they promote OC differentiation and hyperactivation at various levels: pMSCs upregulate the expression of RANKL and reduce that of OPG [(2)]; pMSCs augment the secretion of activin A [(3)]; diminished EphB4-ephrinB2 signaling from pMSCs/OBs to OCs allows osteoclastogenesis [(4)]; increased Wnt5a secretion by pMSCs interacting with myeloma cells enhances RANK expression in OC precursors through Ror2, ultimately increasing their sensibility to RANKL [(5)]. RANKL: Receptor activator of NF κ B ligand; NF κ B: Nuclear factor- κ B; OPG: Osteoprotegerin; RANK: Receptor activator of NF κ B; EphrinB2: Ephrin-B2 ligand; EphB4: Eph receptor B4; Ror2: Receptor tyrosine kinase-like orphan receptor 2; MSCs: Mesenchymal stromal cells; MM: Multiple myeloma; OC: Osteoclast; OB: Osteoblast; ALP: Alkaline phosphatase; MM: Multiple myeloma.

because they ultimately lead to the differentiation and/or activation of OCs at various levels (Figure 3): (1) increased RANKL/OPG ratio: interacting myeloma cells upregulate the expression of RANKL in MSCs, whereas the expression of OPG is reduced, thus favouring osteoclastogenesis and OC activation through RANKL-RANK signaling; (2) augmented secretion of activin A: interaction with myeloma cells leads to increased secretion of activin A in MSCs *via* adhesion-mediated c-Jun N-terminal kinase (JNK) activation^[61]. Besides inhibiting OB differentiation, increased activin A levels would stimulate OC formation and activity^[60]; (3) diminished expression of ephrinB2 and EphB4 in pMSCs: myeloma cells reduce the expression of both ephrinB2 and EphB4 in pMSCs^[105] thereby dysregulating the ephrinB2/EphB4 signaling between OCs and OBs. Besides an impaired OB differentiation (due to reduced ephrinB2-EphB4 signaling from OCs to OBs), diminished EphB4-ephrinB2 signaling (from OBs to OCs) would no longer prevent OC formation, allowing increased osteoclastogenesis; and (4) increased Wnt5a production by MSCs: Wnt5a has been identified as a myeloma growth factor being overexpressed by myeloma plasma cells and pMSCs (as compared to their healthy counterparts)^[115]. Interestingly, we have found the upregulated expression of Wnt5a in MSCs after interaction with myeloma cells (our unpublished results), which would further contribute to its enhanced production in the BM. Recently, a link between Wnt5a and increased osteoclastogenesis has been found by identification of signaling between Wnt5a (secreted

by OB-lineage cells) and the membrane Ror2 receptor (expressed on OC precursors), leading to upregulated RANK expression in the latter and increased sensitivity to RANKL^[116]. Accordingly, myeloma interacting-MSCs would heighten the production of Wnt5a which in turn would increase OC formation and activity.

Thus, both a reduced osteogenic capacity and a hyperstimulation of OCs^[68] at various levels constitute the two major contributions of MSCs to the development of MBD. Other contributions of MSCs, such as modification of ECM components (in relation with retention of OC-activating and OB-inhibiting factors or growth factors) have not been addressed here, but would likely participate in the pathophysiology of the disease.

PATHOPHYSIOLOGY OF IMPAIRED OSTEOGENIC DIFFERENTIATION OF pMSCs: THERAPEUTIC OPPORTUNITIES BASED ON MSC TARGETING

In this section, we will review the major signaling pathways involved in OB differentiation and OB function [e.g., Wnt, Notch, BMP and CCL3 signaling, ephrin-Eph axis, unfolded protein response (UPR)]; at the same time, we also discuss potential therapies targeting members of these pathways in order to restore OB differentiation and activity in patients with MBD.

Bisphosphonates (BPs) are the current mainstay for

Table 3 Therapeutic targets, bone anabolic drugs and preclinical/clinical studies in the context of myeloma bone disease or other bone diseases

Drug	Mechanism of action	Signaling pathway	Cell target	Preclinical studies	Phase of clinical trials
BHQ880	Neutralizing anti-DKK1 antibody	Wnt	MSC, MMPC	[122,123,125]	II ^[126,127]
Romosozumab (AMG785)	Neutralizing anti-sclerostin antibody		MSC	[79,83]	II (postmenopausal osteoporosis) ^[129]
LiCl	GSK3 β inhibitor		MSC, MMPC	[131]	NA
DAPT	γ -secretase inhibitor	Notch	MSC, MMPC	[110,142]	NA
GSI15			OC, MMPC	[139]	
Bortezomib and second generation PIs	Proteasome inhibitor	UPR	MSC, OC, MMPC	[179,180,201]	Bortezomib and carfilzomib: Approved Oprozomib: I / II Ixazomib: III
RAP-011 (mouse)	Decoy receptor neutralizing	BMP	MSC, OC, MMPC	[61,62]	II ^[153,154]
Sotatercept/ACE-011 (human)	activin A				
SB431542	TGF β inhibitor		MSC	[150]	NA
Ki26894			MSC		
MLN3897	CCR1 antagonists	CCL3	MSC, OC, MMPC	[58,160]	NA
CCX721 (mouse)			OC, MMPC	[157]	II (rheumatoid arthritis) ^[161]
CCX354-C (human)					

MSC: Mesenchymal stromal cell; MMPC: Multiple myeloma plasma cell; OC: Osteoclast; NA: Not applicable; DKK1: Dickkopf-1; GSK3 β : Glycogen synthase kinase 3 β ; DAPT: N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; TGF β : Transforming growth factor β ; UPR: Unfolded protein response; BMP: Bone morphogenetic protein; CCR1: Chemokine (C-C Motif) receptor 1; CCL3/MIP1 α : Macrophage inflammatory protein 1- α .

the treatment of bone complications in MM patients, generally administered as supportive therapy in addition to anti-myeloma agents. BPs are pyrophosphate analogs with great affinity for mineralized matrix surfaces, causing inhibition of OC function and OC apoptosis^[117]. Second generation nitrogen-containing BPs now in use, such as pamidronate and zoledronic acid, have been shown to be superior to previous BPs and to more effectively reduce the incidence of SREs^[33]. However, adverse side-effects after long BP treatment (*i.e.*, osteonecrosis of the jaw, kidney failure and accumulation of bone microfractures^[34,118]) and eventual progress of bone disease under treatment^[33], has prompted preclinical and clinical studies for the use of alternate bone anabolic agents which may achieve a more efficacious and improved management of MBD (Table 3).

Wnt signaling

Role in MBD: Wnts are a family of 19 secreted glycoproteins that trigger several pathways involved in cell fate determination, proliferation, migration and polarity, both in embryogenesis and regeneration of adult tissues. Specifically, Wnt signaling in MSCs is critical for OB differentiation and hence, for bone metabolism^[119]. Literature categorizes this pathway in canonical or non-canonical, depending on the requirement of β -catenin or not, respectively. In the Wnt canonical pathway and in the absence of Wnt stimulation, cytoskeletal β -catenin is phosphorylated by a multi-protein destruction complex and undergoes ubiquitin-mediated degradation in the proteasome. Upon binding of canonical Wnt ligands to a Frizzled (Fzd) receptor and a LRP co-receptor, the destruction complex is inhibited allowing β -catenin to translocate into the nucleus where it interacts with T-cell factor/lymphoid enhancer factors to activate transcription

of target genes involved in osteoblastogenesis. On the other hand, the two better characterized Wnt non-canonical pathways are the planar cell polarity and the Wnt/Ca²⁺ pathways, mainly implicated in cell polarity and cell migration mediated by cytoskeletal-actin rearrangements^[119].

Several secreted factors may negatively regulate canonical and non-canonical Wnt signaling: DKK1-4 and sclerostin directly bind to the LRP5/6 co-receptor limiting its availability to Wnt ligands; on the other hand, sFRP1-5 or Wnt inhibitory factor 1 (Wif1), directly bind to Wnt ligands, preventing their functional association with Fzd receptors. Since Wnt signaling plays such a critical role in the osteogenic differentiation of MSCs, alterations in this pathway may lead to skeletal disorders as observed in MBD. In fact, newly diagnosed MM patients showed elevated DKK1^[78], sclerostin^[120] and sFRP3^[84] levels compared to that of healthy donors both in BM and peripheral blood plasma, correlating with the presence of bone lesions.

Although malignant plasma cells are the main source of these Wnt antagonists in the BM^[78,79,84-86], pMSCs secreted higher DKK1 levels than their healthy counterparts^[111,121]. Similarly, sclerostin was found to be produced by OBs derived from pMSCs co-cultured with a MM cell line, further contributing to suppression of OB differentiation and function in MBD^[83]. Even though non-canonical Wnt signaling is not usually associated to osteogenic functions, Wnt5a ligand seems to be at least partially responsible for the osteogenic differentiation of MSCs in the BM^[102,103]. As we commented before, this pathway was inhibited in MSCs from myeloma patients, due to downregulation of Ror2 co-receptor expression^[101].

Therapeutic approaches: Given that Wnt inhibition (by DKK1, sclerostin and sFRPs) has been involved in the

development of osteolytic lesions, modulation of Wnt signaling by different approaches constitutes a potential clinical strategy in MBD.

BHQ880 is a humanized monoclonal antibody against DKK1, which has been shown to reverse the hampering effects of this Wnt inhibitor on OB formation. Treatment with anti-DKK1 or BHQ880 therapy prevented OB suppression and reduced the development of osteolytic lesions in *in vivo* studies with mouse models of murine/human MM^[122-125]. Furthermore, BHQ880 showed an anti-myeloma effect, overcoming the growth advantage conferred by MSCs to MM cells in co-culture through downregulation of cell adhesion and IL-6 production by MSCs^[123], which was also corroborated in *in vivo* models^[122,123,125]. A phase I / II study of BHQ880 in relapsed or refractory MM patients with or without BPs besides standard chemotherapy (NCT00741377), assessed the bone anabolic efficacy of this DKK1 inhibitor through an increase in bone mineral density and regulation of bone metabolism markers^[126]. Ongoing phase II studies of BHQ880 in untreated patients with high risk smoldering myeloma (NCT01302886), have reported increased vertebral strength but no anti-MM activity^[127].

Inhibition of sclerostin by monoclonal antibodies has been explored in different bone disorders, leading to increased bone formation, bone mass and bone strength in preclinical models in mouse, rats and monkeys (reviewed in)^[128]. The development of romosozumab (AMG785), a humanized monoclonal antibody to sclerostin, has allowed its translation to clinical trials. In phase I studies, romosozumab was administered to healthy men and postmenopausal women resulting in a dose-related increase in bone formation markers, a decrease in bone resorption markers, and significantly increased bone mineral density at the lumbar spine and total hip^[129]. A phase II trial is currently ongoing to compare the efficacy of romosozumab with alendronate and teriparatide in the treatment of postmenopausal women with low bone mineral density (NCT00896532). In the MM setting, *in vitro* assays with neutralizing anti-sclerostin antibodies restored OB function as assessed by increased expression of bone formation markers and transcription factors Fra-1, Fra-2 and JunD, modulation of the unbalanced OPG-RANKL ratio and accumulation of β -catenin^[79,83].

Wnt3a administration was also shown to enhance Wnt signaling on OB progenitors, and promoted bone formation and attenuated MM growth in a myeloma SCID-hu mouse model^[130]. Inhibition of glycogen synthase kinase β 3 (GSK3 β), a serine-threonine kinase involved in the phosphorylation of β -catenin for proteasome degradation has also been explored. GSK3 β inhibitors such as lithium chloride^[131] ameliorated the development of MBD and inhibited tumor growth in a disseminated 5TGM1 mouse model of MM, despite some concerns about the possibility that this strategy may stimulate myeloma growth^[132].

Notch signaling

Role in MBD: Evolutionarily conserved Notch signaling

plays an important role during embryonic and postnatal life by regulating cell fate determination, proliferation, differentiation and apoptosis in a spatio-temporal manner^[133]. Notch is a family of four (Notch1-4) transmembrane receptors activated by single-pass membrane ligands (Jagged1-2 and Delta like-1/3/4). Upon Notch-ligand interactions, the γ -secretase complex cleaves the Notch intracellular domain, which then translocates to the nucleus to regulate the transcription of target genes, including Hairy enhancer of split (Hes) and Hes-related to YRPW motif^[133]. Notch signaling plays a key role in skeletal development and remodeling maintaining MSCs in an undifferentiated stage by suppressing OB differentiation (directly repressing Runx2/Cbfa1 activity^[134] or inhibiting Wnt/ β -catenin pathway^[135]). However, once Notch signaling is activated in MSCs, it stimulates early osteoblastic proliferation^[134] leading to the maintenance of an immature OB pool. Considering the well-established role of Notch in osteogenic differentiation, dysregulation of this pathway is associated with human diseases affecting the skeleton. In this sense, alterations in Notch signaling have been reported in pMSCs^[110], which maintain high gene expression levels of some Notch signaling molecules (*e.g.*, Notch1 receptor and the transcription factors Hes1 and Hes5) as compared to dMSCs, which suggests an inhibitory role of these molecules in OB differentiation.

On the other hand, it has also been reported that activation of Notch signaling may regulate osteoclastogenesis depending on the ligands and receptor isoforms involved. Notch1 and Notch3 are able to suppress OC differentiation and activity *via* ligand-mediated receptor activation^[136], whereas Notch2 is upregulated during RANKL-induced osteoclastogenesis and enhances OC formation through increased NFATc1 expression^[137].

Therapeutic approaches: To date, Notch signaling blockade has focused on inhibition of the γ -secretase complex, the intramembrane-cleaving protease with a growing list of protein substrates, including Notch receptors and the amyloid precursor protein involved in Alzheimer's disease^[138]. Treatment with N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a γ -secretase inhibitor (GSI), restored the osteogenic ability of MSCs both *in vitro* (by increasing the gene expression of bone formation-related markers, ALP activity and matrix mineralization) and *in vivo* (as assessed by increased OB cell number at the endocortical surface in naive mice)^[110]. However, GSI treatment failed to stimulate OB formation in a MM model, probably due to the lack of activity over MM cells^[110]. Looking at the OC compartment, treatment with the Notch inhibitor GSI15 blocked MM cell-induced activation of OCs, reinforcing evidences for the use of GSIs as a therapeutic option in MBD^[139]. Other preclinical studies performed with GSIs (GSI-XII, MRK003, DAPT) in the myeloma setting prevented MM cell migration, proliferation, clonogenic ability, resistance to apoptosis, angiogenesis as well as tumor growth *in vitro* and in a SCID-human model of MM^[140-144]. Moreover, it has been found that combined

treatment of GSIs and established anti-MM drugs (such as bortezomib^[145], melphalan, doxorubicin^[144]), or other agents such as ABT-737^[143] or Akt1/2 inhibitors^[141], results in a synergistic cytotoxic effect on myeloma cells. In this sense, combining Notch inhibitors with anti-MM drugs holds promise as a valuable therapeutic approach for the treatment of both MM and MBD.

BMP signaling (activin A and TGFβ)

Role in MBD: Activin A is a TGFβ superfamily member mainly secreted by BM-derived MSCs from myeloma patients and OCs^[61]. MM cell lines and primary MM plasma cells secrete very low or undetectable levels of activin A, but co-culture with MSCs induces the secretion of activin A in the latter *via* JNK pathway activation^[61]. Activin A binds to the serine/threonine kinase activin A receptor, type II A (ActR II A), which recruits and phosphorylates the receptor type I B (ActR I B), leading to phosphorylation of cytoplasmic Smad2/3 proteins. This complex associates with Smad4, which translocates into the nucleus and controls gene expression^[146]. Activin A has several roles in the development of osteolytic lesions: it enhances OC formation and activity (inducing nuclear translocation of NFκB and RANK expression in OC precursors)^[60], inhibits OB differentiation (*via* Smad2-mediated DLX5 downregulation)^[61] and alters the extracellular matrix maturation phase^[147]. Accordingly, high levels of circulating activin A correlate with extensive bone disease and inferior survival^[148].

TGFβ is abundantly deposited in the bone matrix and the enhanced bone resorption in MM bone lesions causes a marked increase in the release and activation of this factor^[149,150]. Although TGFβ enhances the recruitment and proliferation of OBs progenitors, it potently suppresses later phases of OB differentiation, maturation and matrix mineralization^[149,150].

Therapeutic approaches: Sotatercept (ACE-011) or RAP-011 are chimeric proteins derived from the fusion of the extracellular domain of ActR II A and the Fc domain of human IgG1 or murine IgG2a, respectively. These proteins sequester ligands of ActR II A (activin A among others), interfering with Smad signaling and restoring the uncoupled bone remodeling.

Treatment of MSCs with RAP-011 increased OB differentiation, even in the presence of MM cells, by rescuing DLX5 expression^[61]. The bone anabolic effect of RAP-011 could be translated to the *in vivo* setting on a SCID-hu model of MBD. RAP-011 treatment prevented bone destruction and reduced MM tumor burden^[61], providing the basis for clinical testing in myeloma patients suffering from severe bone disease. Similar results were obtained in healthy and ovariectomized mice^[151], in murine models of osteolytic disease induced by MM cells and breast cancer cells^[62], and in non-human primates^[152].

The human counterpart of RAP-011, sotatercept, has been evaluated in phase II studies in MM patients with osteolytic lesions receiving a regimen of melphalan,

prednisone and thalidomide (NCT00747123); after sotatercept treatment, patients showed an increase in bone formation markers (bone-specific ALP), improvement in osteolytic lesions, reduction of bone pain and myeloma burden^[153]. Other studies of sotatercept in combination with lenalidomide and dexamethasone in patients with relapsed and/or refractory myeloma are currently recruiting participants (NCT01562405). In accordance with these studies, a phase I trial of sotatercept in postmenopausal women has evidenced a bone anabolic and anti-resorptive effect, as observed by sustained increase in bone formation markers (bone-specific ALP) and decrease in bone resorption markers (CTX and TRACP-5b)^[154].

Relative to TGFβ, pharmacological inhibition of the TGFβ type I receptor kinase (TβRI), SB431542 and Ki26894, potently enhanced OB differentiation *in vitro*, releasing MSCs from their differentiation arrest and facilitating the formation of terminally differentiated OBs^[150]. *In vivo* administration of these agents showed anabolic and anti-catabolic effects on bone, in parallel with suppression of MM cell growth^[150,155]. Therefore, TGFβ appears to be an important therapeutic target in MBD.

CCL3 signaling

Role in MBD: CCL3 (MIP1α) is a chemokine mainly secreted by OCs and MM cells, which binds to G-protein-coupled receptors CCR1 and CCR5. Both chemokine receptors are expressed in MM cells, MSCs/OBs and OCs^[58,156], being CCR1 the major receptor on OC precursors and mature OCs^[157]. The CCL3 pathway is not only involved in the survival, growth and migration of MM cells^[156], but CCL3 also readily contributes to the imbalance between bone formation and bone resorption by enhancing OC formation^[158] and hampering OB function^[58]. BM plasma CCL3 levels were found to be elevated in MM patients, correlating directly with the extent of MBD and inversely with survival^[159] and osteocalcin expression^[58].

Therapeutic approaches: Preclinical *in vitro* and *in vivo* studies have been performed either targeting CCL3 (anti-sense construct to human CCL3^[56] and neutralizing antibody against CCL3^[57]), or the CCR1 receptor (small-molecule CCR1 antagonists MLN3897^[160] and CCX721^[157]). These treatments reduced myeloma tumor burden and prevented osteolysis, thus providing a strong rationale for the clinical evaluation of these compounds in the treatment of MBD.

Therapeutic strategies towards the inhibition of the CCL3 pathway have mainly focused on their effect on the OC compartment^[56,57,157,160], although there is also pre-clinical evidence of the anabolic effect of the CCR1 inhibitor MLN3897 in osteogenic differentiation^[58]. In the latter study, *in vitro* CCR1 inhibition suppressed CCL3-induced ERK activation and restored both Osterix and osteocalcin expression in OBs differentiated from a human stromal cell line; in the SCID-hu murine model of MM, treatment with MLN3897 reduced tumor burden,

decreased OC number and increased both the trabecular bone area and the percentage of osteocalcin-positive area in the trabeculae^[58]. These studies set the stage for development of clinical trials to assess the effects of CCR1 inhibitors in MM. CCX354, the human structural analog of CCX721, is currently in phase II studies for rheumatoid arthritis, exhibiting clinical activity with a good safety and tolerability profile^[161].

Eph/ephrin signaling

Role in MBD: Another example of a bidirectional signaling pathway capable of regulating both osteoblastic and osteoclastic lineages is the one mediated by Eph receptors and ephrins (Eph receptor-interacting ligands). There are two classes of ephrins: the B class (ephrin B1 to B3), which are ligands for EphB tyrosine kinase receptors (B1 to B6), and the A class (ephrin A1 to A5), which are ligands for glycosylphosphatidylinositol-anchored EphA receptors (A1 to A10)^[162]. Eph-ephrin complexes signal bidirectionally to orchestrate several cellular processes including immune regulation, neuronal development and cancer metastasis. The Eph/ephrin system is expressed by BM microenvironment cells (including OBs and OCs), and growing evidence point out the pivotal role of this pathway in the control of normal and pathological bone remodeling^[163]. Specifically, the ephrinB2/EphB4 axis has been involved in bone homeostasis: reverse signaling through ephrinB2 ligand (expressed by OCs and MSCs/OBs) limits OC activity, whereas forward signaling through EphB4 receptor (expressed by MSCs and OBs) enhances OB differentiation^[104]. Dysregulation of Eph/ephrin function may also contribute to other bone pathological conditions such as osteoarthritis, rheumatoid arthritis or osteosarcoma^[163].

In the MBD context, Pennisi *et al.*^[105] have found reduced levels of ephrinB2 and EphB4 in MSCs from MM patients as compared to their healthy counterparts, and also in OBs/OCs of myelomatous bones compared to non-myelomatous bones. In co-culture experiments, MM cell lines markedly downregulated EphB4 receptor and ephrinB2 ligand in human MSCs, thus confirming a MM cell-induced imbalance of ephrinB2/EphB4 signaling in the MSC-OB lineage^[105].

In addition to the EphB4/ephrinB2 axis, OB-OC, OB-OB and OC-OC interactions through other ephrins and Eph receptors do in fact occur and participate in bone homeostasis. For example, it has been reported that OC-derived ephrinA2/EphA2 interaction enhanced OC differentiation *via* reverse signaling, whereas ephrinA2 inhibited osteoblastogenesis through OB-derived EphA2 receptor *via* forward signaling, contributing to the transition phase of bone remodeling from bone formation to bone resorption^[164]. Future studies about the expression/function of A class ephrins/Eph in MSCs from MM patients may thus unravel new governing mechanisms of impaired OB differentiation in MBD.

Therapeutic approaches: The dual role of EphB4/

ephrinB2 signaling in the OB/OC compartment is especially attractive as a therapeutic approach in MBD, since its activation is able to promote both OB differentiation and function and attenuate OC formation and bone resorption. Pennisi *et al.*^[105] performed experiments with two chimeric proteins (ephrinB2-Fc and EphB4-Fc) in an attempt to induce forward and reverse signaling in MSCs and OC progenitors respectively, and to observe their effects on OB/OC differentiation. Treatment of MSCs with ephrinB2-Fc induced forward signaling (as assessed by phosphorylation of the EphB4 receptor), and increased osteocalcin expression and matrix mineralization of OBs under osteogenic conditions^[105]. On the other hand, EphB4-Fc treatment had an inhibitory effect in OC progenitors (as checked by phosphorylation of ephrinB2 and downregulated expression of NFATc1 and reduced numbers of TRAP⁺ OCs), but no effect in MSCs. In the same line of reasoning, both ephrinB2-Fc and EphB4-Fc treatments in the SCID-hu model of MM, increased bone formation and OB number, but only EphB4-Fc reduced the number of OCs^[105] (since no expression of EphB4 was found in the OC lineage). These results supported the notion that activation of either forward or reverse EphB4/ephrinB2 signaling affects bone remodeling, resulting in increased bone formation. Moreover, the anti-myeloma effect of ephrinB2-Fc and EphB4-Fc treatments was evaluated in myelomatous bones, as assessed by the area of myeloma infiltration and the human Ig monoclonal component; however, only EphB4-Fc-treated SCID-hu mice showed a reduction in tumor burden. Since no effect was found for EphB4-Fc on MM cells *in vitro*, the anti-myeloma activity of this molecule was probably due to its modulatory effects on the BM environment (inhibition of osteoclastogenesis and neo-vascularization and stimulation of OB activity)^[105]. In this sense, upregulation of the endogenous expression of EphB4 in pMSCs or osteoprogenitor cells of myeloma patients (*e.g.*, by Wnt3a administration-since EphB4 receptor is a Wnt signaling target-or directly by EphB4-Fc treatment) could restore coupling of bone homeostasis and simultaneously reduce MM tumor burden in MM patients with bone affection.

Unfolded protein response pathway

Role in MBD: The endoplasmic reticulum (ER) is a membranous compartment present in eukaryotic cells which controls the synthesis, folding and trafficking of proteins to be secreted, as well as calcium storage and synthesis of membranes^[165]. Increased load of unfolded or misfolded proteins within the ER triggers a sophisticated mechanism known as the UPR, in an attempt to re-fold those proteins and to allow cellular adaptation to the imbalance in the protein folding homeostasis, referred as ER stress. Briefly, when unfolded proteins accumulate in the lumen of the ER, three coordinated pathways are activated by the transmembrane ER stress-sensor proteins, namely: PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme

1 (IRE1). The activation of these ER sensor proteins leads to the induction of a battery of transcription factors [orchestrated by ATF4, ATF6 and X box-binding proteins (XBP1s)] to promote the transcription of ER chaperone proteins and folding enzymes to increase the protein folding capacity of the ER, as well as proteins controlling the ER-associated degradation machinery, a mechanism by which misfolded proteins are retro-translocated into the cytosol for degradation by the proteasome. Alternatively, prolonged or severe exposure to ER stress may result in the cell undergoing apoptosis^[165-167].

Although ER stress often arises in pathological situations, specialized secretory cells such as hepatocytes, insulin-producing β cells of the pancreas, plasma cells and connective tissue cells (fibroblasts, chondrocytes and OBs) are particularly sensitive to ER stress induction in their normal development and function^[167]. Therefore, ER stress is essential during osteoblastogenesis through the three arms of the UPR: IRE1-XBP1s (promoting Osterix transcription)^[168], PERK-ATF4 (increasing osteocalcin and bone sialoprotein expression)^[169] and ATF6 (enhancing osteocalcin expression)^[170].

On the other hand, a recent study showed that MSCs from MM patients displayed elevated mRNA and protein levels of endogenous XBP1s (an active transcription factor involved in the clearance of unfolded/misfolded proteins) compared with dMSCs, suggesting that the IRE1-XBP1s pathway is activated in pMSCs^[171]. Experiments with overexpression of XBP1s in MSCs led to an increase in IL-6 and RANKL secretion and VCAM1 expression, which translated into an enhanced *in vitro* ability of MSCs to support MM cell growth and OC formation^[171]. Future studies exploring the expression and role of the other components of the UPR in MSCs would be of particular value for disrupting the protective effects of the MM microenvironment on tumor cell growth and bone destruction.

Therapeutic approaches: Plasma cells seem to be exquisitely sensitive to their core protein handling machinery due to the large amounts of immunoglobulins that these cells produce and secrete. The ubiquitine-proteasome pathway, linked to the UPR response to discard misfolded proteins, has become a potential drug target for the treatment of several tumors including MM^[172]. Bortezomib was the first-in-class proteasome inhibitor (PI) introduced in the clinical practice with a significant benefit in terms of anti-myeloma response rate and overall survival in both front-line and relapsed/refractory settings^[23]. Moreover, bortezomib not only reduces myeloma tumor burden, but directly restrains the progression of MBD, clinically evidenced by changes in bone turnover markers and radiologic data favouring bone healing^[173,174]. The beneficial impact of bortezomib on bone metabolism is not merely secondary to its anti-myeloma activity, but rather this agent directly targets the OC and MSCs/OB populations, both hampering osteoclastogenesis and OC resorption and promoting osteoblastogenesis and

OB function^[175-177].

A next-generation of PIs, including peptide boronic acid analogs (delanzomib and ixazomib), peptide epoxyketones (carfilzomib and oprozomib) and a β -lactone compound (marizomib) have been developed to address the shortcomings of bortezomib treatment with the aim of retaining or improving bortezomib efficacy^[178]. Our group has investigated the potential bone anabolic and anti-resorptive effects of three of these second-generation PIs (*i.e.*, carfilzomib, its orally bioavailable analog oprozomib and ixazomib) in preclinical models of MM^[179,180]. *In vitro* studies evidenced that the three PIs were able to promote osteoblastogenesis and OB function (as assessed by augmented expression of bone formation markers, increased ALP activity and enhanced bone matrix mineralization), and to inhibit OC formation and resorption (through disruption of RANKL-induced NF κ B signaling together with reduced expression of integrin α V β 3 and F-actin ring disruption)^[179,180]. These effects were subsequently corroborated *in vivo*, since the three PIs provided a marked benefit in associated bone disease, sustained by bone anabolic and anti-resorptive activities^[179,180].

Moreover, the UPR was identified as a crucial pathway affected by PI-treatment of MSCs and osteoprogenitors resulting in enhanced osteoblastogenesis. Treatment of a BM-derived mesenchymal stromal cell line with PIs led to increased protein levels of the ER stress sensor IRE1 α . IRE1 α knockdown by siRNAs significantly diminished PI-enhanced mineralized bone formation, thus underscoring the crucial role of IRE1 α in the promotion of OB activity by these agents^[180]. In the same line, Nakamura *et al.*^[181] recently reported a critical role for other ER stress mediator, ATF4, in bortezomib-mediated osteoblastogenesis, and suggested the optimization of a dose regimen for PI-treatment in order to obtain a maximal bone anabolic response (lower doses) avoiding the induction of pro-apoptotic pathways in the MSC-OB lineage (higher doses). It is thought that the adaptive threshold for myeloma plasma cells and OBs is quite different, since UPR induced by PIs (at the same range of doses) results in a cytotoxic effect in MM cells^[172] whereas promotes OB differentiation on mesenchymal precursors^[179].

Other therapeutical approaches on OB differentiation and function

Inhibition of tyrosine kinases: Several studies showed that the tyrosine kinase inhibitor imatinib mesylate directly promoted OB differentiation and stimulated osteogenic gene expression and mineralization, majorly by inhibiting PDGFR function on osteoprogenitors^[182,183]. This partially explained the increased trabecular bone volume and bone mineral density of long-term imatinib treated patients^[182]. As expected, subsequent studies with dasatinib, a second generation tyrosine kinase inhibitor with more potency and broader target profile, also evidenced enhanced OB differentiation from mesenchymal

precursors and promotion of OB activity both *in vitro* and *in vivo*^[184-187]. Preclinical anti-myeloma and anti-angiogenic efficacy of dasatinib was also reported, but attained at higher concentrations than those required for the bone anabolic effect of this drug, and which were cytotoxic for mesenchymal osteoprogenitors and OBs^[52,187]. Therefore, the latter suggests that if dasatinib is to be used for the treatment of MBD, it should be administered in combination with another anti-myeloma agent.

MSC cytotherapy for MBD: MSCs have been considered as excellent candidates for cytotherapy studies due to their immunoprivileged nature, their ability to migrate to damaged and tumor tissues, together with their capacity to differentiate to several mesenchymal lineages^[188]. Some concerns have been raised, however, for the use of MSCs in the treatment of MBD since interacting BM MSCs have been shown to support the proliferation, survival, migration and chemotherapeutic resistance of MM cells^[27,30,31,189]. When genetically-modified human MSCs overexpressing OPG were administered to a model of medullary myeloma with associated bone disease, they reduced OC activation and restored bone volume^[190]. Moreover, human placenta or BM derived MSCs were intrabone or systemically administered in the severe combined immunodeficiency (SCID)-rab model, and found to promote bone formation, prevent MM-induced bone disease and tumor growth^[191,192].

Specific delivery of RNAi-based anabolic therapy: The use of siRNA-based bone anabolic therapies in the clinic has been hampered by lack of specific targeting to bone-formation surfaces. The (AspSerSer)₆ has been found to be a targeting moiety for bone formation sites *in vivo*, due to its great affinity to lowly crystallized hydroxyapatite and amorphous calcium phosphonate. Systemic administration of (AspSerSer)₆-labeled liposomes containing osteogenic siRNAs has been shown to be an effective therapeutic approach in a model of osteoporosis^[193] and its use may also be explored in MM to promote OB function.

CONCLUSION

In conclusion, MSCs from myeloma patients are important contributors to the development of osteolytic lesions because of their reduced osteogenic potential and because they also promote OC differentiation and/or activity at various levels (increased RANKL/OPG ratio, augmented activin A secretion, uncoupled ephrinB2/EphB4 axis and because of increased Wnt5a production). We have reviewed current therapeutic approaches targeting components of signaling pathways involved in the osteogenic differentiation and maintenance of OB activity. It is likely that due to the multifactorial character of MBD, combinations of both anti-resorptive and bone-anabolic agents may be required for an effective restoration of bone homeostasis and for an additional anti-myeloma

benefit.

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Mesenchymal stem cells as a potent cell source for articular cartilage regeneration

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Abstract

Since articular cartilage possesses only a weak capacity for repair, its regeneration potential is considered one of the most important challenges for orthopedic surgeons. The treatment options, such as marrow stimulation techniques, fail to induce a repair tissue with the same functional and mechanical properties of native hyaline cartilage. Osteochondral transplantation is considered an effective treatment option but is associated with some disadvantages, including donor-site morbidity, tissue supply limitation, unsuitable mechanical properties and thickness of the obtained tissue. Although autologous chondrocyte implantation results in reasonable repair, it requires a two-step surgical procedure. Moreover, chondrocytes expanded in culture gradually undergo dedifferentiation, so lose morphological features and specialized functions. In the search for alternative cells, scientists have found mesenchymal stem cells (MSCs) to be an appropriate cellular material for articular cartilage repair. These cells were originally isolated from bone marrow samples and further investigations have revealed the presence of the cells in many other tissues. Furthermore, chondrogenic differentiation is an inherent property of MSCs noticed

at the time of the cell discovery. MSCs are known to exhibit homing potential to the damaged site at which they differentiate into the tissue cells or secrete a wide spectrum of bioactive factors with regenerative properties. Moreover, these cells possess a considerable immunomodulatory potential that make them the general donor for therapeutic applications. All of these topics will be discussed in this review.

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Key words: Mesenchymal stem cells; Regeneration; Articular cartilage; Cell therapy

Core tip: Articular cartilage possesses only a weak capacity for repair; therefore, regeneration of its defects is considered one of the most important challenges for orthopedic surgeons. On the other hand, mesenchymal stem cells (MSCs) are specified as appropriate cell candidates for regenerating incurable defects of articular cartilage due to the following characteristics: inherent chondrogenic property, easy availability, cell homing potential and immunomodulatory function. In the past, several attempts were made to exploit MSC capacity to cure articular cartilage defects developed in osteoarthritis, rheumatoid arthritis or following trauma. All of these topics are discussed in this review.

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INTRODUCTION

Articular cartilage covers the ends of bones in diarthrodial joints. This highly specialized tissue reduces joint

friction and protects the bone ends from the shear forces associated with a high mechanical load. Furthermore, it works as a lubricant and a shock absorber. Histologically, articular cartilage is hyaline cartilage tissue with no blood, lymphatic or nerve supply.

An articular cartilage defect is an area of damaged or missing cartilage that is often caused by acute trauma. These defects usually are well defined and surrounded by normal articular cartilage. Cartilage defects may also occur following osteoarthritis (OA), osteonecrosis, osteochondritis dissecans and other pathologies^[1]. Defects caused by OA are often ill-defined, large and surrounded by osteoarthritic tissue of variable quality. If cartilage defects are restricted to the articular cartilage, they are termed chondral or partial thickness defects and if the defects penetrate into subchondral bone, they are called osteochondral or full thickness defects.

It has long been known that articular cartilage has only a weak capacity for self-repair^[2], which is partially due to its avascularity. With the lack of blood supply, a set of complex biochemical events that take place in order to repair the damage fails to occur. Wound healing in hyaline cartilage is further prevented due to the cartilage dense extracellular matrix impairing the migration capacity of chondrocytes^[3-5].

In general, while no repair process occurs in chondral defects, in osteochondral defects, a repair process is initiated by undifferentiated mesenchymal stem cells (MSCs) from the bone marrow tissue of subchondral bone^[6,7]. Repair of full thickness cartilage defects depends mainly on the patient age, defect size and location^[8]. Small full thickness defects are repaired by formation of hyaline cartilage, whereas large osteochondral defects are only repaired by formation of scar tissue (fibrous tissue) or fibrocartilage.

For a long period of time, the current regenerative treatment option for joint cartilage defects was identified as marrow stimulation techniques, including microfracture, Pridie drilling and abrasion arthroplasty, all of which involve punching or drilling holes through the subchondral plate^[9]. The main disadvantage of such techniques is the formation of repair tissue that is similar to fibrocartilage rather than hyaline cartilage. Fibrocartilage is a poorly organized tissue containing significant amounts of collagen type I. It exhibits inferior mechanical and biochemical characteristics compared to normal hyaline articular cartilage. The matrix of fibrocartilage breaks down with time and loading, leading to development of secondary OA in injured cartilage^[10].

Autologous osteochondral mosaicoplasty, known also as the osteoarticular transfer system, is the other therapeutic option for cartilage repair. Unfortunately, its clinical application is a technically challenging procedure. Osteochondral tissue is usually obtained from a non-weight bearing area of the patient's own articular cartilage cells. These methods have some disadvantages, including donor site morbidity, tissue supply limitation, unsuitable mechanical properties and thickness of the obtained tissue^[11,12]. The use of allogeneous tissue could be considered

an alternative option but it is associated with high cost, risk of immunological rejection and transmission of pathogens^[13].

There are two types of cell-based treatments for cartilage defects, the autologous chondrocyte implantation (ACI) and stem cell-based cell therapy^[14]. ACI technique involves a two-step surgical procedure as follows: (1) collecting tissue and (2) transplantation. According to the literature, the effectiveness of ACI is still controversial. While some scientists have reported that this technique is more likely to be applicable for small articular cartilage defects, others believe that even after ACI, some defects have continued to persist in the articular cartilage. It is noted that obtaining sufficient chondrocytes from biopsies is challenging; therefore, *in vitro* expansion of chondrocytes is inevitable. It has been reported that expanded chondrocytes in culture gradually undergo dedifferentiation, so lose morphological features and specialized functions^[15]. Limitations associated with chondrocyte-based treatment have motivated investigators to search for alternative reliable cellular materials. In this context, embryonic stem cells (ESCs), inducible pluripotent stem cells (iPSCs) and MSCs have gained considerable attention.

ESCs are pluripotent cells derived from a blastocyst inner cell mass. These cells have the characteristics of self-renewal as long as they are exposed to a feeder cell layer or leukemia inhibitory factor (LIF). Differentiation is initiated upon removal of the feeder cell layer or LIF, resulting in the formation of three dimensional cell aggregates known as embryoid bodies (EBs). These EBs can be regionally differentiated into derivatives of three germ layers: the mesoderm, ectoderm and endoderm^[16]. Thus, ESCs can be a potential stem cell source to fabricate cartilage-like tissue constructs in the field of tissue engineering; however, immunological incompatibility, the possibility of teratoma formation in transplantations, as well as certain ethical concerns make scientists hesitant to use them as cellular materials for tissue regeneration^[17]. To consider these concerns, scientists have established ESC-like stem cells, known as iPSCs, from somatic cells by plasmid or adenovirus-based transduction. Actually, iPSCs are patient-specific ESCs without ethical concerns and immunogenicity^[18,19].

Among the potential cell sources for cartilage regeneration, MSCs are considered an appropriate candidate owing to several specific characteristics. These properties will be reviewed and followed by the examples of investigations using MSC-based treatment for articular cartilage defects.

MSCS

MSCs, as non-hematopoietic cells, are originally derived from bone marrow tissue. Historically, Cohnheim was the first scientist who suggested the presence of MSCs in bone marrow tissue following some wound healing experimental studies in rabbits. By intravenous injection of non-soluble aniline stain, this German pathologist found some stained cells at the site of the wound experi-

mentally created in the animal's distal limb. He concluded that the stained fibroblastic cells would be derived from bone marrow and transferred to the wound site *via* the circulatory system^[20,21]. Many years after this suggestion through a series of bone marrow transplantation experiments, scientists found that marrow cells are able to produce cartilage and bone-like tissue *in vivo*^[22,23] but they were unable to determine the cells responsible for this property. Friedenstein *et al.*^[24] were the first to isolate and describe a fibroblastic population as the cellular equivalent of chondrogenic and osteogenic features of marrow tissue. They referred to these cells as colony forming unit fibroblasts. Thus far, the fibroblast-like cells have been referred to as marrow stromal cells, marrow progenitor cells, marrow stromal fibroblasts and MSCs. MSC is the most frequently used nomination, particularly in recently published investigations.

As with any stem cell type, MSCs possess two important properties, long-term self-renewal ability and the capacity to differentiate along multiple cell lineages, such as bone, cartilage and adipose cells. There is controversy regarding the profile of surface marker expression on MSCs. According to the suggestion of the International Society for Cellular Therapy, CD70, CD90 and CD105 have been used as positive markers, while CD34 has been used as a negative marker^[25]. In this context, some scientists believed that the three positive markers are co-expressed in various cells so they are unable to identify MSCs *in vivo*, whereas expression of the negative marker, CD34, has been shown on native adipose-derived MSCs^[26]. Furthermore, Stro-1 is the other frequently used marker of MSCs^[27,28]. This surface epitope has been shown to be an endothelial antigen but whether it can identify MSCs *in vivo* remains unknown^[29].

Investigations have shown that MSCs occur in low quantity in bone marrow aspirate. In spite of their limited numbers, these cells are easily expandable through standard culture techniques. The propagation of MSCs is strongly dependent on the bovine serum content of culture media. The cells assume a spindly-shaped morphology upon cultivation. MSC primary culture has been reported to be heterogeneous, containing multiple colonies with various differentiation capacities. Pittenger *et al.*^[30] showed that nearly one third of these colonies have osteogenic, adipogenic and chondrogenic differentiation potentials, while the other two thirds exhibit either bipotent or unipotent capacity to differentiate into osteogenic/chondrogenic and adipogenic lineages, respectively. In addition to differentiating into bone, cartilage and adipose cells, MSCs have been reported to possess differentiation capacity along non-mesenchymal cell lineages, such as neurons, keratinocytes, liver, intestine and kidney epithelial cells^[31,32]. This property is referred to as MSC plasticity or transdifferentiation.

INHERENT CHONDROGENIC POTENTIAL OF MSCS

The chondrogenic differentiation property is among the

first differentiation capacities of MSCs reported at the time when Friedenstein *et al.*^[33] isolated and described the cells. These investigators plated marrow cells in plastic dishes and removed non-adherent cells four hours after culture initiation. The adherent cells remained quiescent for two to four days and then underwent proliferation. The culture tended to uniformly consist of fibroblastic cells after several rounds of subcultures. The most important feature of the cells reported is the capacity of producing small deposits of bone and cartilage-like tissue.

To promote/maintain cartilage differentiation/phenotype in culture, one critical requirement is to provide a 3D cellular condensation in which cells could experience a microenvironment of low oxygen tension. Research has demonstrated that MSCs hardly differentiate into cartilage cell lineage in a 2D culture system. The current technique for chondrogenic differentiation of MSCs is the micro-mass culture system which Johnstone used for chondrocyte culture in 1998. These authors reported that chondrocytes from a growth plate cultured in the micromass system could maintain chondrocytic phenotype without undergoing dedifferentiation. In micromass culture, the cells are placed in a tube and centrifuged into a condensed aggregate. A chondrogenic medium providing appropriate inducers for cell differentiation is then added to the resulting pellet. Transforming growth factor (TGF)- β 3 is the most crucial inducer included in chondrogenic medium^[34-38]. This growth factor probably acts by inducing the expression of Sry-related high-mobility-group box-9^[39], which in turn regulates the expression of aggrecan and collagen type II, type IX and type XI during chondrocyte differentiation^[40]. Furthermore, research has indicated that addition of bone morphogenetic proteins enhances chondrogenesis under the specific conditions employed by Steinert *et al.*^[41]. Insulin-like growth factor-1 has also been shown to have a synergistic effect with TGF- β 1 in promoting chondrogenesis^[42]. Furthermore, fibroblast growth factor-2 (FGF-2) may possess a chondrogenic function. It has been demonstrated that in human marrow MSC culture, FGF-2 in combination with dexamethasone enhances production of collagen type II, glycosaminoglycan (GAG) and aggrecan. Platelet-rich plasma has also been reported to possess chondrogenic effects owing to the presence of FGF-2 and TGF- β 2^[43-46].

DIFFERENT SOURCES OF MSCS

Since the MSC population exists in many tissues in body, they could be considered readily available cells for application in regenerative medicine. Besides bone marrow, multiple tissues have been reported to contain MSCs. These include adipose tissue^[47], trabecular bone^[48], periosteum^[49], synovial membrane^[50], skeletal muscle^[51], as well as teeth^[52], among which bone marrow and adipose tissue are widely used sources. Furthermore, some researchers have paid special attention to synovial membrane as a potent source of stem cells with good chondrogenic potential.

Unlike bone marrow MSCs, adipose MSCs can be

isolated in large quantities with minimal morbidity and discomfort^[53,54]. Moreover, the frequency of MSCs in the whole bone marrow of skeletally mature adults ranges from 1 in 50000 to 1 in 100000 cells, corresponding to a yield of a few hundred MSCs/milliliter of marrow. Fraser *et al.*^[54] reported that the frequency of MSCs in adipose tissue is in the order of 1 in 100 cells, about 500-fold more than that found in bone marrow^[55]. In view of these practical advantages, MSC from adipose tissue could be considered an alternative option for bone marrow MSCs in cell-based cartilage regeneration strategies.

MSCs derived from synovial membranes have been shown to possess multilineage potential. These cells can be stimulated to undergo chondrogenesis *in vitro* with appropriate inducers. The study by Shirasawa *et al.*^[56] showed that human synovial-derived cells have greater chondrogenic potential than bone marrow MSCs, adipose MSCs, as well as periosteal- or muscle-derived cells from the same patients. Furthermore, a follow-up study by the same authors indicated that synovial-derived MSCs produce consistently larger cartilage than bone marrow MSCs from the same patients^[57].

HOMING PROPERTY OF MSCS

MSCs are known to have a homing potential to the damaged site which could possibly help to repair in two ways: (1) differentiation to tissue cells and restoration of lost morphology and function; and (2) secretion of a wide range of bioactive factors and creation of a repair environment with anti-apoptotic effects, immunoregulatory function and the stimulation of endothelial progenitor cell proliferation^[58].

The precise mechanisms of the MSC homing process have not been thoroughly understood. In this regards, it has been proposed that chemokines and their receptors on the surface of MSCs are the key players^[59] which enable MSCs to migrate towards chemokine gradients secreted by injured tissues^[60] or tumors^[61]. MSCs express multiple chemokine receptors, allowing their migration in response to the chemokine-attractive gradients created by the inflamed injured site. Some chemokine receptors expressed by MSCs include CCR1, CCR7, CCR9, CXCR3, CXCR4, CXCR5 and CX3CR1^[62]. To consider the relationship between gradient of chemokine concentration and cell migration, it can be concluded that MSCs must be transplanted to an adjacent area of injured site following the establishment of the gradient of the chemokine concentration.

IMMUNOMODULATORY FUNCTION OF MSCS

Some scientists consider MSCs a valuable cellular material for applications in a variety of autoimmune and allo-immune diseases since these cells possess a considerable immunomodulatory potential. In this context, research has indicated that MSCs can suppress proliferation and

activity of CD4⁺ and CD8⁺ T lymphocytes, as well as T memory cells^[63,64]. This is directed mainly by targeting the inhibition of cyclin D2, which leads the T cells into cell cycle arrest regulating anergy^[65]. Furthermore, for this effect, there is no need for major histocompatibility complex (MHC) identity between MSC and the target immune effector. Similarly, it has been observed that B lymphocyte neither proliferates nor differentiates into immunoglobulin-producing cells in the presence of MSCs^[66]. Moreover, MSCs have been shown to inhibit the proliferation and cytotoxicity of interleukin (IL)-2 or IL-15-stimulated natural killer cells *in vitro*^[67]. MSCs could also inhibit the maturation of monocytes into dendritic cells (DCs) *in vitro*. Mature DCs incubated with MSCs display a decreased cell-surface expression of MHC class II molecules, CD11c, CD83 and co-stimulatory molecules, resulting in impaired antigen-presenting cell function. In addition, MSCs have been shown to inhibit pro-inflammatory potential of DCs by inhibiting their production of tumor-necrosis factor (TNF)^[66-69].

A range of mechanisms have been proposed to explain MSC immunomodulatory capacity. For example, it has been reported that MSCs exert their immunomodulatory effects through secretion of soluble inflammatory mediators, including IL-6, IFN- γ , TNF- α , IL-1 α and IL-1 β ^[70]. These effects are created through enzymatic action such as the expression of inducible nitric oxide synthase (iNOS) and indoleamine 2,3-dioxygenase (IDO), and through production of human leukocyte antigen class I molecule HLA-G and prostaglandin E2 (PGE2)^[70,71]. Moreover, it has been indicated that MSCs can mediate immunosuppression (modulation of T cell proliferation, gene expression and cell migration) by releasing galectin-1, an intracellular and cell surface protein, in a soluble form^[72]. Furthermore, research has suggested a relationship between MSC immunosuppressive function and the expression of Toll-like receptors (TLR). MSCs have been shown to express a range of functional TLRs, specifically TLR-2 through TLR-8, leading to the production of IL-6 and IL-8 which subsequently affects T-cell function. In support of this idea, some authors have demonstrated that the inhibition of these receptors *in vitro* is conversely associated with a reduction in immunosuppressive activity of MSCs^[72,73].

Regarding the mechanism of MSC-mediated immunosuppression, it must be emphasized that some mechanisms are constitutively involved (*i.e.*, production of PGE2), whereas others are induced when MSCs are exposed to an inflammatory environment (*i.e.*, IDO is expressed when MSCs are stimulated with IFN γ). In addition, according to the evidence, cooperation of several molecules (rather than a single molecule) is responsible for the MSC immunomodulatory function^[66]. Finally, there are differences among species regarding the mechanism of immunosuppression. For example, in human MSCs, IDO-mediated suppression is one of the most prominent mechanisms. This enzyme depletes the cellular microenvironment of the essential amino acid trypto-

phan required for T-cell proliferation. In contrast, in murine MSC, immunosuppression is mediated by iNOS^[74].

MSCS AND CARTILAGE GENE THERAPY

Gene therapy approaches could be considered a promising strategy for efficient promotion of regeneration in cartilage defects. In this context, MSCs could readily be transduced by viral vectors. Also, specific liposomal formulations have been reported as a safe gene delivery system into MSCs with some efficiency^[75]. MSC-based gene therapy offers some advantages for articular cartilage repair. Using this approach, therapeutic proteins could be designed to overexpress in MSCs transplanted into articular cartilage defect. This in turn could enhance the structural features of the repair tissue formed at the defect site. Furthermore, MSC-based gene therapy is an applicable approach to deliver genes with complementary mechanisms of action (*i.e.*, chondrogenic and proliferative factors) into a cartilage defect.

In many studies, MSC-mediated gene delivery has been applied for cartilage repair using a variety of chondrogenic growth factors. For example, it has been indicated that overexpression of IGF-1 in concert with TGF- β 1 or BMP2 *in vitro* in MSCs could induce greater chondrogenic tissue than either growth factor alone^[42,76]. According to this research, overexpression of IGF-1 alone could not induce chondrogenic differentiation of MSCs in culture. In contrast to this finding, Gelse *et al*^[77] indicated the differentiation-promoting effect of IGF-1. In this *in vivo* study, MSCs from rib perichondrium of rat were subjected to adenoviral transduction with adenoviral vectors encoding BMP-2 and adenoviral vectors encoding IGF-1. The cells were then mixed with fibrin glue matrix and delivered to cartilage partial thickness lesions of the patellar groove. Both treatments with BMP-2 and with IGF-1 have been shown to improve repair tissue compared with the naïve and Ad.LacZ controls after eight weeks. However, the majority of BMP-2 treated joints showed signs of ectopic bone formation and osteophytes, which were not present in the knees of the IGF-1 treated defects^[77]. In addition to IGF-1 and BMP-2, some other growth factors, including BMP-4^[78] and growth differentiation factor 5^[79], were also employed in MSC-based gene delivery to cartilage defects which resulted in an enhanced cartilage repair.

POTENTIAL PITFALLS OF USE OF MSCS

In spite of the above mentioned potential, there are some pitfalls associated with MSC application for articular cartilage regeneration. Some research has indicated the expression of cartilage hypertrophy markers such as collagen type X, matrix metalloproteinase-13, alkaline phosphatase, parathyroid hormone-related protein receptor and vascular endothelial growth factor after inducing MSCs to undergo chondrogenesis. Since hypertrophy could finally lead to ossification of cartilage tissue, scientists are concerned about the clinical application of MSCs for regenerating articular cartilage defects^[80-83].

Furthermore, it has been reported that the thickness of the regenerated cartilage by MSC transplanted into cartilage defects was too thin to resemble mature cartilage^[84]. However, there have been promising attempts to overcome these issues, such as that co-culture of MSCs with mature chondrocytes has been reported to result in decreased expression of hypertrophy markers^[85]. Further investigation has revealed that such an anti-hypertrophic effect is created by the parathyroid hormone-related peptide secreted by mature chondrocytes^[86]. Moreover, a recent study indicated that the immature cartilage treated with FGF-2 and TGF- β 1 displays increased nano-compressive stiffness, decreased surface adhesion, decreased water content, increased collagen content and smoother surfaces, indicating characteristics of mature cartilage^[87].

REGENERATION OF ARTICULAR CARTILAGE WITH MSC TRANSPLANTATION

During the past years, valuable attempts have been made to evaluate MSC potential in regeneration of articular cartilage defects. Examples of such efforts in animal models and human are described.

MSCS FOR CARTILAGE REGENERATION IN ANIMAL MODELS

In order to study the regenerative potential of MSCs in cartilage defects *in vivo*, rabbit has frequently been used as an animal model. In some studies, MSCs have been applied alone without any biomaterial. Im *et al*^[88] isolated MSCs from rabbit marrow and transplanted them into a full thickness osteochondral defect which was artificially made on the same rabbit's patellar groove. Evaluation of repair 14 wk post transplantation indicated that the histological score of experimental group was higher than the corresponding value of the control group (untreated); therefore, they concluded that repair of cartilage defects can be enhanced by the implantation of cultured MSCs.

Most investigators preferred transplantation of cells combined with scaffold. Wakitani *et al*^[89] used this strategy to create regeneration of full thickness articular cartilage following experimentally created defects in rabbit knee joint. Cells were combined with collagen I gel and surgically transplanted into the medial femoral condyle defect. Two weeks post-surgery, evaluations indicated that MSCs differentiated into chondrocytes contribute to regenerate damaged tissue and within 24 wk the defect was completely repaired. Interestingly, a mechanical test indicated good mechanical strength of repair tissue. Recently, Berninger *et al*^[90] attempted to promote regeneration of osteochondral defects in rabbit knee joint by implantation of allogeneic MSC in fibrin clots.

Also, Grigolo *et al*^[91] used MSCs with scaffold to promote regeneration in an osteoarthritic defect induced in rabbit knee by cutting the cruciate ligaments. Upon es-

tablishment of an OA model eight weeks post induction, marrow-derived MSCs combined with hyaluronan were transplanted into the osteoarthritic knee. Six months post-transplantation, a statistically significant difference in the quality of the regenerated tissue was found in the implants with scaffolds carrying MSCs compared to the scaffold alone.

In addition to rabbit, goat has been also used as an animal model for investigation of the regenerative potential of MSCs for cartilage defects. Guo *et al.*^[92] loaded goat marrow-derived MSC in tricalcium phosphate scaffolds and transplanted them into a cartilage defects of 4 mm × 8 mm dimensions created in femur articular surface at the animal knee joint. About 24 mo after transplantation, evaluation indicated that the defect was filled by a hyaline-like cartilage. According to their findings, the graft tended to integrate with the subchondral bone. About 12-24 mo post-surgery, the GAG content of the repair tissue increased significantly.

Non-surgical administration of MSCs for articular cartilage repair has also been investigated. Using this strategy, Murphy *et al.*^[93] reported transplantation of marrow derived MSCs which were suspended in hyaluronan through injection into the cavity of an osteoarthritic knee in a caprine model created by cutting cruciate ligaments and the meniscus. According to their findings, injected cells tended to regenerate meniscus and thereby delayed the formation of OA in the animal knee joint. Recently, an injection approach was evaluated in a sheep model of OA by Al Faqeh *et al.*^[94]. These authors reported marrow MSCs transplanted either as undifferentiated cells or chondrogenically induced cells could retard the progression of OA. According to their findings, the induced cells indicated better results, especially in meniscus regeneration.

MSCS-MEDIATED CARTILAGE REGENERATION IN HUMANS

Cartilage defects following trauma

Articular cartilage of the knee joint is often injured after a fall in athletes, which is considered a challenging surgery for orthopedists. In this context, some authors have tried to apply the regenerative potential of MSCs. For example, Kuroda *et al.*^[95] attempted to reconstruct a 20 mm × 30 mm full thickness cartilage defect (International Cartilage Repair Society Classification grade IV) in the weight-bearing area of the medial femoral condyle of the right knee in a 31-year-old male judo athlete. They transplanted MSC/collagen gel into the cartilage defects and observed the formation of hyaline cartilage in the histological sections. The patient returned to a normal life seven months post-implantation. Similarly, Wakitani *et al.*^[96] transplanted autologous MSC combined with collagen gel into two patients with patellar full thickness articular cartilage defects and observed significant improvements in patient pain and walking ability six months post-transplantation.

In another clinical study, Wakitani *et al.*^[97] tried to treat three patients, including a 31-year-old female, a 44-year-

old male and a 45-year-old male, with full thickness articular cartilage defects in their patellofemoral joints. An undifferentiated MSC/collagen sheet was transplanted into the defects and evaluated for a six month follow-up period, at the end of which the clinical symptoms were significantly improved. The improvements were maintained over a period of 17-27 mo. One year post-transplantation, histological examination of the repair tissue from one patient revealed that the defect was repaired by fibrocartilaginous tissue. Magnetic resonance imaging of the second patient revealed a complete coverage of the defect but was unable to determine the nature of the material covering the defect^[97]. The formation of repair tissue with fibrocartilage nature in this study would be due to the inappropriate microenvironment (*i.e.*, collagen type I solution) that was used for transplantation of MSCs. Hyaline cartilage naturally contains plenty of collagen type II and hyaluronic acid (HA) macromolecules. In this study, the addition of matrix substance in the form of HA could provide chemical signals for right matrix production by the cells. The effect of HA-synthetic hydrogel matrix has been recently emphasized in the MSC cartilage differentiation process^[98].

OA

OA is a group of progressive joint disorders in which biomechanical characteristics of cartilage changes and so results in patient disability^[99]. This disease progressively involves articular cartilage, subchondral bone, ligaments and synovial membrane. Some attempts have been made to treat osteoarthritic joints using MSCs. In this context, a report about the treatment of 24 patients with knee OA revealing MSC transplantation by Wakitani *et al.*^[100] is remarkable. In this clinical trial, adherent cells from bone marrow aspirates were embedded in collagen gel and transplanted into articular cartilage defects in the medial femoral condyle of 12 patients, while the other 12 subjects served as cell-free controls. Outcomes indicated that although clinical improvement was not significantly different, the treatment group showed a better arthroscopic and histological grading score.

In the above-mentioned study, MSCs were introduced through an invasive approach (surgery) into the defective area. Some authors have attempted to introduce the cells by injection. Using this approach, Centeno *et al.*^[101] applied culture expanded autologous MSCs and transplanted the cells through an intra-articular injection into the knee of a 46-year-old OA patient. They reported that 90% of the patient's pain was reduced two years post-injection. Furthermore, Davatchi *et al.*^[102] used this strategy to introduce the cells into knee joints of four OA patients and reported the strategy as an encouraging method. Using this strategy, Emadedin *et al.*^[103] injected autologous MSCs in six female volunteer patients with knee OA and observed more satisfactory outcomes.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflam-

matory disorder that may affect many tissues and organs. It principally attacks synovial joints. This systemic autoimmune disease is associated with progressive reduction of extracellular matrix and joint destruction. Pro-inflammatory cytokines including TNF- α and IL-6 are believed to be responsible for the creation of RA symptoms^[104,105]. Current therapy is based mainly on suppressing the symptoms using analgesia and anti-inflammatory drugs, including steroids. Although such therapy is effective in relieving pain and inflammation, it is not able to regenerate damaged cartilage. Furthermore, it has been reported that cartilage-regenerating methods, including cell-based treatment strategies using autologous chondrocytes, is not considered an efficient method for RA patients due to prevention of cartilage formation by the presence of the inflammatory condition in the joint or destruction of the newly-formed cartilage.

In contrast to chondrocyte-based cell therapy, it has been suggested that injection of an allogeneic MSC results in a considerable reduction in inflammation and a formation of new cartilage in RA due to their immunosuppressive and anti-inflammatory features^[106]. In support of this concept, injection of MSCs in the mouse animal model with collagen-induced arthritis has been reported to prevent severe arthritis and to lower the serum level of inflammatory cytokines^[107].

CONCLUSION

MSCs are specified as appropriate cell candidates for regenerating incurable defects of articular cartilage due to the following characteristics: inherent chondrogenic property, easy availability, cell homing potential and immunomodulatory function. In the past, several attempts were made to exploit MSC capacity to cure articular cartilage defects developed in OA, rheumatoid arthritis or following trauma. Taken together, the outcomes of these trials show promising results. Furthermore, many clinical trials have been registered at www.clinicaltrial.gov regarding application of MSCs for regenerating articular cartilage. With a worldwide extensive effort, MSCs will be routinely applicable in articular cartilage defects in the near future. Special attention must be given to improve the quality of repair tissue formed following MSC transplantation into the cartilage defect. First, efficient protocols must be developed to prevent hypertrophy of chondrocytes produced by MSC differentiation. Second, a practical solution must be explored regarding production of mature cartilage by MSC differentiation. Third, optimal biomaterial mimicking the matrix of hyaline cartilage must be developed in order to provide appropriate chemical signals for right matrix production by MSCs following transplantation. Fourth, in most clinical trials, MSCs are applied in the undifferentiated state. This approach exhibits a major potential drawback. MSCs represent a heterogeneous population containing multiple colonies with various differentiation capacities; therefore, to improve MSC regenerative outcome in cartilage defects, the cell population must be enriched

for chondrogenic cells. Otherwise, pre-differentiation of MSCs will be essential in clinical applications in order to ensure appropriate lineage commitment and to avoid undesired heterotopic tissue formation. Finally, a gene therapy approach offers the potential of addressing most of these issues (*i.e.*, chondrocyte hypertrophy, production of immature cartilage and pre-differentiation of MSCs) but this approach requires further improvement for MSC engraftment. More importantly, in this context, a safe highly efficient gene delivery system into MSCs with sustained duration of transgene expression and the optimal therapeutic gene(s) for cartilage repair must be identified. Moreover, determination of an optimized combination of genetically modified MSCs with scaffolds is of utmost importance for producing a high quality repair tissue *in vivo*.

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Umbilical cord fibroblasts: Could they be considered as mesenchymal stem cells?

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Core tip: Umbilical cord matrix derived fibroblasts [umbilical cord-mesenchymal stem cells (UC-MSC)] have gained importance in the last years. We have studied these cells and noticed some differences when compared with bone marrow-MSC in term of differentiation and phenotype. Other studies pointed out similar differences. Recently, some studies have doubted of the MSC nature of these cells. Starting from our own results and those from literature, we summarized in this minireview different studies that isolated and characterised UC-MSC. In the discussion we confronted the contradictory data about the differentiation and immunophenotype of UC-MSC and highlight what remains to be done to answer the question: are cord matrix isolated fibroblasts stem cells or not?

Abstract

In cell therapy protocols, many tissues were proposed as a source of mesenchymal stem cells (MSC) isolation. So far, bone marrow (BM) has been presented as the main source of MSC despite the invasive isolation procedure related to this source. During the last years, the umbilical cord (UC) matrix was cited in different studies as a reliable source from which long term *ex vivo* proliferating fibroblasts were isolated but with contradictory data about their immunophenotype, gene expression profile, and differentiation potential. Hence, an interesting question emerged: Are cells isolated from cord matrix (UC-MSC) different from other MSCs? In this review, we will summarize different studies that isolated and characterized UC-MSC. Considering BM-MSC as gold standard, we will discuss if UC-MSC fulfill different criteria that define MSC, and what remain to be done in this issue.

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Key words: Mesenchymal stem cells; Cord matrix; Bone marrow; Differentiation; Immunophenotype

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INTRODUCTION

Initially, mesenchymal stem cells (MSC) were defined as a rare population of multipotent progenitors with a defined immunophenotype, having the capacity for self-renewal and differentiation into various lineages of mesenchymal tissues^[1]. Based on this definition, and in order to find an alternative to the invasive bone marrow (BM) isolation procedure, fibroblasts from various adult tissues such as adipose tissue, muscle, heart and liver^[2,3], or fetal tissues like umbilical cord (UC) blood^[4], or umbilical cord matrix^[5,6] were analyzed for stemness. Scientists focused their interest on the available tissues, easy to isolate and without serious ethical considerations. According to these criteria, many protocols which show MSC isolation from

the umbilical cord matrix were proposed. Most of the studies were based on the published minimal criteria defining multipotent mesenchymal stromal cells. First, the cells must be plastic adherent, with an important proliferation potential. Second, these proliferating fibroblasts must express CD105, CD73 and CD90, and lack the expression of CD45, CD34 and CD14. Third, they should be able to differentiate *in vitro* under specific conditions, at least in osteocytes, adipocytes and chondrocytes^[1]. Fibroblasts isolated from UC (UC-MSC) presented the same aspect as MSC from BM (BM-MSC), but with a significantly higher proliferative potential^[6]. However, with the apparition of more MSC exclusive immunophenotype, some differences between UC-MSC and BM-MSC were highlighted. Furthermore, different studies presented contradictory results about the capacity of UC-MSC to differentiate into defined cell lineage. Here, we underlined the most striking immunophenotype and gene expression differences between UC-MSC and BM-MSC. We also discussed the contradictory data concerning the differentiation potential of UC-MSC, in an attempt to clarify whether these cells have different stemness potential in comparison with standard BM-MSC.

COMPARATIVE IMMUNOPHENOTYPE AND GENE EXPRESSION OF UC-MSC AND BM-MSC

Until recently, immunophenotyping of mesenchymal stem cells was essentially concentrated on the determination of the expression of CD90, CD73, CD105, CD13, CD44 and the absence of CD14 and CD34^[1]. However, it is now admitted that these markers are not exclusive for MSCs. Indeed, foreskin fibroblasts also show this phenotype without being ranked as MSC^[7]. Using more specific markers, UC-MSCs were distinguished from MSC of other tissues. We demonstrated that UC-MSCs were totally negative for SSEA-4 and LNGFR antigens, whereas BM-MSC presented an important fraction of positive cells for these markers^[6]. SSEA-4 is an early embryonic glycolipid antigen, commonly used as a marker for undifferentiated pluripotent human embryonic stem cells. On the other hand, LNGFR (CD271) was found to be involved in the development, survival and differentiation of neural cells. These two markers have been proposed to identify the adult mesenchymal stem cell population^[8,9]. Other differences based on the expression of CD56 and CD146 were described between UC-MSC and BM-MSC. Indeed, immunophenotyping analysis has distinguished UC-MSC (CD56⁺, CD146⁺⁺) from BM-MSC (CD56⁻, CD146⁺⁺⁺)^[7].

Proteomic is an excellent tool to study and compare expressed protein profile of MSCs. 2D gel analysis revealed that BM-MSCs highly express proteins involved in cell migration (CTSB, CTSD and PHB), which correlates with their important migration potential^[10]. These migration-enhancing proteins were minimally expressed in UC-MSC, which expressed migration inhibitory proteins

(PAI-1 and MnSOD).

Other studies reported further differences in UC-MSC in contrast to BM-MSC and cord blood MSCs. Indeed, UC-MSC exhibited a different expression profile for *HOX*-gene; a transcription factor implicated in embryologic development^[7].

As far as we know, no study has identified MSC with a single marker in humans. In mice, Méndez-Ferrer *et al.*^[11], identified a population of Nestin expressing population (Nestin⁺ MSC). In a Nestin-GFP transgenic mouse, they identified the entire mesenchymal stem cell activity of bone marrow CD45⁻ cells within the Nestin⁺ population. These results suppose that Nestin could be a potential single marker able to define murine MSCs. It would be interesting to check for Nestin expression in human BM-MSC and UC-MSC.

We also compared the cytokine expression profile in BM-MSC and UC-MSC. BM-MSC expressed leptin that was enhanced in the presence of glucocorticoids, whereas UC-MSCs were not able to express this adipokine^[12]. We found a high constitutive pSmad2 expression in UC-MSC, while it was low and modulated in BM-MSC. Indeed, the investigation of leptin expression mechanism showed pSmad-2 as an inhibitory factor^[12].

Taking into account the above listed studies, it appears that UC-MSC constitute a cell population that can be distinguished from BM-MSC in term of immunophenotype and the expression of some genes implicated in development, differentiation and migration.

The main immunophenotype differences between UC-MSC and BM-MSC are summarized in Table 1.

UC-MSCS DIFFERENTIATION POTENTIAL

Despite *in vivo* transplantation assays are the most suitable to assess the MSC differentiation potential, *in vitro* differentiation assays were performed in most of the studies. Conventional staining for adipogenic, osteogenic and chondrogenic differentiation (Oil red O, Alkaline phosphatase, Von Kossa, *etc.*) were used to establish UC-MSC differentiation potential. Compared to cells derived from other tissues, UC-MSCs were reported to fail in osteogenic differentiation^[7,13]. We have also published a lack of *in vitro* osteogenic differentiation potential of UC-MSC, demonstrated by the absence of alkaline phosphatase staining, and runx-2 expression, even when cells were cultured in the presence of osteogenic mixture for more than 4 wk^[12]. We demonstrated that this osteogenic inability of UC-MSC was due to their incapacity to express leptin^[12]. In fact, leptin was admitted to be implicated in osteogenic differentiation^[14].

Furthermore, BSP, a marker for osteoblastic differentiation was shown to be highly expressed in cell-lines with high osteogenic capacity, while non-osteogenic cell line did not. Human UC-MSC did not express BSP, which can account for their inability to differentiate into osteoblasts^[7].

UC-MSCs were also shown to differentiate into adipocytes in a very limited manner^[13,15]. Adipogenic potential

Table 1 Immunophenotype and differentiation potential comparison between umbilical cord matrix derived fibroblasts and bone-marrow-mesenchymal stem cells

		Immunophenotype	Gene expression	Differentiation potential
BM-MSC	Positive for:	CD90, CD73, CD105, CD13, CD44 ^[1] SSEA-4, LNGFR ^[6] , CD146 ^{+++ [7]}	<i>CTSB</i> , <i>CTSD</i> , <i>PHB</i> ^[10] , <i>BSP</i> ^[7] , <i>Leptin</i> ^[12] <i>HOX-gene</i> ^[7] , <i>DLK-1</i> ^[16] , <i>pSmad2</i> (low and modulated) ^[12]	No report for osteo-, adipo or chondrogenic differentiation failure
	Negative for:	CD14, CD34 ^[1] , CD56 ^[7]		
UC-MSC	Positive for:	CD90, CD73, CD105, CD13, CD44 ^[1] CD56, CD146 ^{++ [7]}	<i>PAI-1</i> , <i>MnSOD</i> ^[10] , <i>HOX-gene</i> (different expression pattern) ^[7] <i>pSmad2</i> (high and constitutive) ^[12]	Reports for adipo- ^[13,15] and osteogenic ^[7,12] differentiation failure
	Negative for:	CD14, CD34 ^[1] , SSEA-4, LNGFR ^[6]	<i>CTSB</i> , <i>CTSD</i> , <i>PHB</i> ^[10] , <i>BSP</i> ^[7] , <i>Leptin</i> ^[12] , <i>DLK-1</i> (or weakly) ^[16]	

UC-MSC: Umbilical cord-mesenchymal stem cells; BM-MSC: Bone marrow-mesenchymal stem cell.

was inversely correlated with DLK-1 expression in mesenchymal stem cells isolated from cord blood-MSC (CB-MSC). UC-MSCs do not or weakly express DLK-1; which can explain their failure to differentiate into adipocytes^[16].

Bosch *et al*^[7], went further by wondering if UC-MSC are true “mesenchymal stromal stem cells”. In fact, UC-MSC isolated by this group failed to differentiate into adipo-, osteo- and also into chondrocytes. Indeed, UC-MSC did not express Sox9 factor after 21 d incubation in an *in vitro* pellet culture system.

The above-summarized studies clearly presented results that demonstrated failure of UC-MSC to differentiate into osteo-, adipo-, and chondrocytes, at least in these conditions. The majority of these studies also provided explanations for the described UC-MSC differentiation inability. However, one should not forget that many other studies have concluded to successful UC-MSC differentiation.

DISCUSSION

It seems confusing to see the above contradictory data about the immunophenotype and the differentiation potential of UC-MSC! So what could explain these contradictions? It could be the contamination of umbilical fibroblasts by endothelial cells of the vein and arteries embedded in jelly connective tissue. These cells could proliferate over UC-MSCs and give contradictory results, especially in terms of differentiation. To address this question, we have looked to UC-MSC isolation procedure used by different laboratories and found that even in the studies that concluded to different immunophenotype and differentiation potential between UC-MSC and BM-MSC, the removal of veins and arteries before cord matrix processing was carefully performed^[7,12].

Umbilical cord blood was also proposed as a source of MSC (CB-MSC). It could also be possible that cord matrix handling method may have caused the transfer of circulating MSC from the cord blood compartment in the endothelial/subendothelial layer of the umbilical cord matrix. So the studies that concluded by identical characteristics of BM-MSC and UC-MSC may have in reality characterized umbilical CB-MSC. In fact, despite CB-MSCs are difficult to isolate, studies are unanimous about resemblance of these cells with BM-MSC in term of phenotype and differentiation potential. In addition, the use of different media and culture conditions may also

have an effect on the heterogeneity of UC-MSC population isolated in different laboratories; contributing to the selection and expansion of specific cell populations, which may have not the same behavior under specific culture conditions. In this context, a strategy based on counterflow centrifugal elutriation was used to identify different subpopulations in cultured UC-MSC^[17]. The authors revealed that UC-MSC cultures were composed of different sized populations. The smallest cells exhibited the highest proliferative capacity, with a reduced amount of aging cells, compared with larger diameter cells. This study is a clear proof of UC-MSC heterogeneity. It is obvious that the MSC-like characteristics fit with the small sized subpopulation, however this might be proven. Performing comparative analysis on sorted small and large diameter UC-MSCs for differentiation potential could give key information that may explain the above listed contradictions.

Another technique used to detect heterogeneity among MSCs is the single cell transcriptional profiling, where more than 48 genes could be analyzed. This study was performed on adipose-derived stromal cells (ASC). Based on transcriptional profiles, ASCs were grouped into different clusters. Statistical analysis was used to find out correlations between the expression of specific markers and an increased differentiation gene expression. One striking finding was that low expressing endoglin (CD105) ASC subpopulation showed an increased osteogenic differentiation potential^[18]. Such technique would be of great interest to study UC-MSCs heterogeneity.

It clearly appears that UC-MSC is a heterogeneous population, composed of distinct subpopulations, with distinct characteristics that may account for the contradictory results presented by different laboratories. However, a question remains unanswered: Is it necessary for all mesenchymal cells to have the same differentiation characteristics as BM-MSC to be defined as stem cells, or is there different MSCs with different development stages that make them different in their behavior under specific conditions?

Finally, unless a clear explanation of the contradictions in the literature about the immunophenotype and the differentiation potential of UC-MSC is proposed, cord matrix could not be considered as a reliable source for MSC for the moment. Furthermore, the above-cited studies raise the necessity to update the criteria that de-

fine multipotent stromal cells.

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New advances in the mesenchymal stem cells therapy against skin flaps necrosis

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pears to hold substantial promise in the treatment against skin flaps necrosis. This review involved four out of the top 10 innovations of the 20th century and four out of the 10 most important, current innovations. We hope that these contents could help you to pick up the new advances in the MSCs therapy against skin flaps necrosis.

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Abstract

Mesenchymal stem cells (MSCs), multipotential cells that reside within the bone marrow, can be induced to differentiate into various cells, such as osteoblasts, adipocytes, chondrocytes, vascular endothelial progenitor cells, and other cell types. MSCs are being widely studied as potential cell therapy agents due to their angiogenic properties, which have been well established by *in vitro* and *in vivo* researches. Within this context, MSCs therapy appears to hold substantial promise, particularly in the treatment of conditions involving skin grafts, pedicle flaps, as well as free flaps described in literatures. The purpose of this review is to report the new advances and mechanisms underlying MSCs therapy against skin flaps necrosis.

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Key words: Mesenchymal stem cells; Skin flaps; Endothelial progenitor cells

Core tip: Mesenchymal stem cells (MSCs) therapy ap-

INTRODUCTION

According to the report by Hultman *et al*^[1] from the American Council of Academic Plastic Surgeons and the Southeastern Society of Plastic and Reconstructive Surgeons, four out of the top 10 innovations of the 20th century were myocutaneous flaps, microsurgery, skin grafts, and transplantation, and four out of the 10 most important, current innovations are hand/face transplantation, fat grafting, stem cells, and perforator flaps. So these separately important contributions may lead to such a promising prospect by the combination of two or three or even more of them.

Mesenchymal stem cells (MSCs) mainly include widely applied bone marrow MSCs (BMSCs), adipose tissue-derived SCs (ADSCs), and Human umbilical cord matrix stem cells (HUCMSCs). Circulating BMSCs homed to perivascular sites in critically ischemic tissue, exhibited paracrine function and augmented microhemodynamics. These effects were mediated through arteriogenesis and angiogenesis, which contributed to vascular regeneration^[2]. MSCs are relatively easy to isolate and expand in culture and therefore have potency as a therapeutic tool in ischemic disease and in transplantation. The current

dilemma is that MSCs may not have a long lifespan after administration^[3,4]. A rapid disappearance of MSCs raises the question of how MSCs therapy might work. It is possible that partial administered MSCs escape from death and migrate to sites of injury and inflammation and that MSCs are able to rapidly pass on their effect to other cells that subsequently mediate tissue repair or immunomodulation. The dramatically decreased quantity of MSCs will absolutely affect their angiogenic and immunomodulatory function. Fortunately some authors have suggested that the combination of MSCs and gene therapy might generate a synergistic effect on stem cells therapy against skin flaps necrosis^[5,6].

This review is willing to elicit the stem cells treatment of conditions involving skin grafts, pedicle flaps, as well as free flaps and stem cells immunomodulation in skin flaps therapy based on the published data.

MSCS THERAPY AGAINST FREE FLAPS NECROSIS

For majority of surgeons, one of the most amazing medical miracles is the total or partial human face transplantation. Dubernard *et al*^[7] reported the encouraging outcomes 18 mo after the first human partial face transplantation which was performed on November 27, 2005. Then human face transplantation was successively reported.

Some free flaps combined with or without MSCs therapy already have clinical applications, but, for some cases they are lack of appropriate research models, and, for the remains the mechanisms are still disputed. A novel murine free flap model of acute hindlimb ischemia-reperfusion combined with Laser-Doppler Flowmetry, quantitative immunohistochemistry and immunofluorescence detection is maybe a suitable and reproducible experimental procedure of translational research that allows *in vivo* investigation of diverse molecular and cellular mechanisms^[8]. Some authors considered the patient body as an ideal bioreactor to induce vascularisation in large volumes of grafted tissues. But, for volumes limited by the lack of vascularisation, engineering a bone free flap for maxillofacial reconstruction still exists technical restrictions^[9].

It's good news for some patients who had undergone ablative tumor surgery, radiochemotherapy and primary reconstruction to receive the secondary reconstruction of the mandible by the prefabricated bony radial forearm flaps consisting of iliac crest and radial forearm flaps. And the iliac bone graft might be replaced with scaffold seeded with stem cells for further reduction of donor site morbidity^[10]. And the fact that MSCs combine with growth factors therapy is extremely promising. MSCs transduced by stromal cell-derived factor-1 α (SDF-1 α) definitely augmented ischemic free flaps survival, which was initially reported by us previously^[5]. Although the free flaps are versatile, they are deserted sometimes. For example, ADSCs enhance the survival of fat grafted into

the face, and a microfat graft with simultaneous ADSCs injection may be utilized to treat Parry-Romberg disease without the need for microvascular free flap transfer by Koh *et al*^[11], which has demonstrated the promising prospect by the combination of two current innovations.

MSCS THERAPY AGAINST PEDICLE FLAPS NECROSIS

At the very beginning, flap anti-necrosis therapy might be performed without directly division and culture of MSCs. For example, an intramedullary muscle flap could improve the functional results of joints reconstructed with partially demineralized and lyophilized osteochondral allografts by providing both vascularity and an increased population of MSCs capable of responding to bone morphogenetic proteins^[12]. By far the major pathway of cranial defects repair induced by implantation of demineralized bone matrix is by the direct induction of resident MSCs to osteoblasts and by the direct formation of bone through upgrading osteocalcin and Collagen type I mRNA^[13].

A series of endogenous growth factors and chemokines may do great contribution to flaps survival. A positive correlation existed between MVD and the high expression of SDF-1 and Chemokine receptor type 4 (CXCR4) following hyperbaric oxygen treatment in promoting neovascularization, which might be explained by the upregulation of SDF-1 and CXCR4 expression in the skin flaps of rats^[14]. Human umbilical cord mesenchymal stem cells (HUCMSCs) could improve the survival of ischemic skin flaps by promoting vascularization, which might be attributed to the increased expression of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF)^[15]. Hypoxia preconditioned BMSCs or ADSCs transplantation improved ultra-long random skin flaps survival *via* promoting angiogenesis by upgrading VEGF^[16,17]. ADSCs could enhance the survival of random-patterned skin flaps in streptozotocin-induced diabetic mice *via* elevated expression of hypoxia-inducible factor-1 α ^[18], be capable of promoting flap prefabrication by VEGF-A^[19], and prevent ischemia-reperfusion injury, mainly by regulating the growth factors, such as VEGF, bFGF and transforming growth factor-beta (TGF- β)^[20]. However, the angiogenic effect by ADSCs is still controversial. Although the mean survival area ratios in the ADSCs treatment group and in the BMSCs treatment group had no significant difference, higher levels of bFGF and VEGF were found in the BMSCs transplantation group^[21]. In addition, ADSCs also had a significantly angiogenic response^[22], better immune compatibility and potential for enhancing the blood supply. These together suggest that both ADSCs and BMSCs have proangiogenic effect, but their promoting angiogenesis mechanisms may be quite different.

A large sum of exogenous stem cells and growth factors are utilized to promote flaps survival. An optimal delivery route should have been screened through a lot

of researches. There are mainly five methods to deliver MSCs: (1) intravenous injection; (2) subcutaneous injection; (3) intramuscular injection; (4) application with collagen sponge seeding; and (5) application with fibrin glue seeding. Hu *et al.*^[23], as many did, suggested that intravascular delivery of BMSCs increased wound healing and promoted flap survival following ischemia-reperfusion injury of cutaneous tissue flaps. Lee *et al.*^[24] suggested that the collagen sponge method delivered ASCs most effectively within the flaps and increased flap vascularity. VEGF and MSCs had synergetic effect when they were used together^[6], as we suggested the synergetic effect by SDF-1 α and MSCs co-application^[5], which could help rebuilding the blood circulation of the ischemic region in random flaps.

Recent advances about the flap anti-necrosis therapy should not neglect the prefabricated flaps and/or tissue engineering flaps. The prefabricated groin flaps with skin substitutes provided a useful vehicle for the implantation of MSCs to serve as an autologous microvascular bioscaffold^[25]. Poly(L-lactic-co-glycolic acid) or poly(ϵ -caprolactone) scaffolds seeded with co-cultured chondrocytes and BMSCs, were wrapped in a pedicle muscle flaps^[26].

MSCS THERAPY AGAINST SKIN GRAFT NECROSIS

Autologous transplantation of BMSCs was a promising therapeutic strategy for prevention of skin-graft contraction^[27]. A typical combined graft consisting of a free full-thickness skin graft and cultured autologous fibroblast-like BMSCs was effectively implanted and healed on the facial soft tissue defect^[28]. Some scholars suggested that the autologous ADSCs transplantation increased full-thickness skin graft survival and showed promise for use in skin graft surgery. This might be both due to in situ differentiation of ADSCs into endothelial cells and increased secretion by ADSCs of growth factors, such as VEGF and TGF- β 3 that enhanced angiogenesis^[29].

The most vital role of skin graft may not only be to repair a defect, but also to study the immunomodulatory mechanisms. Human MSCs have immunomodulatory properties. They inhibited lymphocyte (especially, T-cell) proliferation to mitogens and alloantigens *in vitro* and prolonged skin graft survival *in vivo*^[30,31]. MSCs increased interleukin (IL)-2 and soluble IL-2 receptor in MSCs and lymphocyte co-cultures and antibodies against IL-10 further suppressed proliferation, that is to say, MSCs induced suppression was a complex mechanism affecting IL-2 and IL-10 signaling and might function differently, depending on T-cell stimuli^[30]. Alloreactivity was marked by pronounced CD45+ T-cell infiltration consisting of CD4+ and CD8+ T cells and increased skin graft IFN- γ expression which was significantly inhibited by both BMSCs and ADSCs^[32].

Human MSCs and their stromal cell antigen 1 Stro-1 positive [Stro-1(+)] subgroup possess immunosuppres-

sive properties. Stro-1(+) MSCs induced greater prolongation of skin graft in mice than unsorted MSCs^[33]. Transplantation of allogeneic bone marrow-derived flk-1+Sca-1- MSCs led to stable mixed hematopoietic chimerism, permanent donor-specific immunotolerance in allogeneic host and long-term allogeneic skin graft acceptance^[34]. The co-infusion of MSCs with unmodified donor bone marrow limited the toxicity of allogeneic bone marrow transplantation, treated graft vs host disease (GVHD), enhanced mixed chimerism and improved vascularized skin graft survival^[35]. The high level of TNF- α also demonstrated a possible immunogenic role for donor (allogeneic) MSCs against skin allograft rejection^[36]. Lee *et al.*^[37] suggested that ADSCs and their secretome had the potential to induce immunologic tolerance in full-thickness skin allotransplantation model. Moreover, the immunosuppressive properties of ADSCs were mediated by the ADSCs secretome. However, these chimerism induced tolerance theories were still disputed, for example, as Carrier *et al.*^[38] suggested, microchimerism did not lead to the induction of a high degree tolerance after utero transplantation but instead lead to the development of alloreactivity to donor cells.

Furthermore, infusion of MSCs exosomes enhanced the survival of allogeneic skin graft in mice and increased Tregs to help MSCs to show their immunosuppressive characters^[39]. In addition, infusion of ADSCs dramatically increased skin allograft survival by inhibiting the Th-17 pathogenic immune response and enhancing the protective Treg immune response^[40]. However, this viewpoint might be controversial. Co-administration of allogeneic hematopoietic stem cells and third-party myeloid progenitor (MP) transplantation simultaneously with placement of a MP-matched skin graft demonstrated that the organ donor matched Treg was not essential for tolerance but MP did^[41].

Donor specific immune tolerance could be effectively induced by intra-bone marrow-bone marrow transplantation combined with BMSCs treatment without any additional cytoreductive recipient treatment, which provided a promising allograft transplantation strategy whenever the donor bone marrow was available^[42]. Moreover, third-party BMSCs transplantation could prolong skin graft survival time by inhibiting T lymphocyte activation and proliferation^[43]. Third-party MSCs were able to suppress allo-specific antibody production *in vitro*, and they might rescue patients with life-threatening GVHD *in vivo*^[44]. Likewise, the donor haematopoietic stem cells had the capacity to reduce the risk of GVHD^[45]. A split-thickness skin graft from the donor was accepted, however, a third-party graft was rapidly rejected without the help of the third-party MSCs^[46]. And all dogs received donor bone marrow at the time of vascularized composite allograft (VCA) transplantation were tolerant to their donor skin graft and promptly rejected the third-party skin grafts. These data demonstrated that donor-specific tolerance to all components of the VCA could be established through simultaneous allogeneic hematopoietic third-party stem

cells transplantation^[47].

For some extreme situations, such as diabetic and radiation-induced tissue defects, stem cells therapy shows their unique advantages. Autologous ADSCs transplantation could enhance skin graft survival in diabetic rats through differentiation, vasculogenesis, and secretion of growth factors, such as VEGF and TGF- β 3. This might represent a novel therapeutic approach in skin graft surgery for diabetic wounds^[48]. MSCs combined with plastic surgery or skin graft therapy may be a promising therapeutic approach for improving radiation-induced skin and muscle damages^[49].

Recently, tissue engineered skin graft develops rapidly when taking micro-environment into consideration. Chitosan-modified poly(3-hydroxybutyrate-co-3-hydroxyvalerate) scaffold loaded with HUCMSCs or unrestricted somatic stem cells could significantly contribute to full-thickness skin defects repair and be potentially used in the tissue engineering^[50,51]. Laser microporous porcine acellular dermal matrix, which provided a “cell niche-like” micro-environment for the migration and differentiation of the BMSCs population, could induce exogenous differentiation of BMSCs *in vivo* and achieve the reconstruction of skin appendages, when combining with the split-thickness skin graft^[52].

CONCLUSION

Thanks to all published data, we have to acknowledge that we yet know little about how MSCs therapy against skin flaps necrosis works. Further studies aimed at exploring angiogenic signaling pathway after administration and optimal treatment approach will shine light on effective MSCs therapy against skin flaps necrosis.

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Secretion of immunoregulatory cytokines by mesenchymal stem cells

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ed from different tissue sources, although some papers report some quantitative but not qualitative differences in cytokine secretion. The present review focuses on the basic cytokines secreted by MSCs as described in the literature by which the MSCs exert immunomodulatory effects. It should be pointed out that MSCs themselves are objects of cytokine regulation. Hypothetical mechanisms by which the MSCs exert their immunoregulatory effects are also discussed in this review. These mechanisms may either influence the target immune cells directly or indirectly by affecting the activities of predominantly dendritic cells. Chemokines are also discussed as participants in this process by recruiting cells of the immune systems and thus making them targets of immunosuppression. This review aims to present and discuss the published data and the personal experience of the authors regarding cytokines secreted by MSCs and their effects on the cells of the immune system.

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Key words: Mesenchymal stem cells; Immunomodulation; Cytokines; Chemokines; Dendritic cells

Abstract

According to the minimal criteria of the International Society of Cellular Therapy, mesenchymal stem cells (MSCs) are a population of undifferentiated cells defined by their ability to adhere to plastic surfaces when cultured under standard conditions, express a certain panel of phenotypic markers and can differentiate into osteogenic, chondrogenic and adipogenic lineages when cultured in specific inducing media. In parallel with their major role as undifferentiated cell reserves, MSCs have immunomodulatory functions which are exerted by direct cell-to-cell contacts, secretion of cytokines and/or by a combination of both mechanisms. There are no convincing data about a principal difference in the profile of cytokines secreted by MSCs isolat-

Core tip: Autoimmune diseases affect approximately 5% of the human population, leading to serious disability and effective methods to treat these diseases are still not perfect. Mesenchymal stem cells (MSCs) are assumed to be promising agents, both for regenerative medicine and cell therapy for autoimmune disorders. Under the influence of some factors, mesenchymal stem cells secrete cytokines which induce suppression of the immune response. Studies on the secreted cytokines and the precise mechanisms involved in these suppressive mechanisms would create possibilities for efficient application of MSCs as a therapeutic means for treatment of autoimmune diseases.

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INTRODUCTION

Maintenance of immunological self-tolerance and immune homeostasis in the organism is under the control of a complex and sophisticated process of immunoregulation and its dysfunction could be a critical factor in the development of autoreactive and potentially life-threatening conditions. Profound understanding of the precise mechanisms underlying this immunoregulatory process could lay the ground to develop a more suitable and efficient therapy for autoimmune diseases. Regulation of the immune response by mesenchymal stem cells (MSCs) is mediated by a number of cell subtypes and secreted factors and recently new cell-based therapeutic approaches have emerged as successful strategies for treatment of various inflammatory and autoimmune conditions. In the last decades, mesenchymal stem cells, one type of adult stem cells, have gained considerable interest as extremely promising cell therapeutic agents^[1,2] due to their unique combination of immunomodulatory properties and self-renewal and multilineage differentiation capacity^[3,4]. MSCs have been shown to exert profound anti-inflammatory and immunomodulatory effects on almost all the cells of the innate and adaptive immune systems *via* a variety of mechanisms, notably cytokine and chemokine secretion^[5].

Mesenchymal stem cells are a population of undifferentiated multipotent adult stem cells that naturally reside within the human body and are generally defined as plastic-adherent, fibroblast-like cells possessing extensive self-renewal properties and potential to differentiate *in vitro* and *in vivo* into a variety of mesenchymal lineage cells^[4,6]. MSCs were initially described in the bone marrow by Friedenstein *et al.*^[7,8] as a small subpopulation of colony-forming unit fibroblasts which could be distinguished from the rest of the bone marrow cells on the basis of their plastic adherence, spindle-shaped appearance and rapid expansion^[7].

After their initial discovery in bone marrow (BM-MSCs), MSCs were isolated and characterized from a wide variety of other adult and fetal tissues, including adipose tissue (AT-MSCs), umbilical cord, dental pulp, skin, tendon, skeleton, muscle, spleen, brain, liver, periosteum, placenta, synovial and amniotic fluids^[9,10]. MSCs from different sources may display some differences in the expression of surface markers. However, in general, the phenotypes of these cells are very similar and in the absence of an individual specific marker, MSCs are commonly defined by a panel of cell surface markers that include CD73, CD90 (Thy-1), CD105 (endoglin) and MHC class I, as well as the adhesion molecules CD44, CD29, CD54 (ICAM-1; intercellular adhesion molecule 1), CD106 (VCAM-1;

vascular cell adhesion molecule) and CD166^[11]. MSCs do not express hematopoietic markers such as CD34, CD45, CD14 and CD11 or co-stimulatory molecules like CD80, CD86 and CD40^[11].

According to the minimal criteria of the International Society of Cellular Therapy (ISCT, 2006), the required functional and phenotypic features for defining MSCs include: (1) plastic adherence of the isolated cells under standard culture conditions; (2) positive expression of CD105, CD90 and CD73 markers in at least 95% of a cell population and lack of expression of CD34, CD45, CD11b, CD14, CD19 or CD79a and HLA-DR markers in greater than 95% of the culture, as measured by flow cytometry; and (3) trilineage differentiation potential into osteoblasts, adipocytes and chondroblasts in *in vitro* culture with specific stimuli^[12].

Besides this, trilineage multipotency experimental data have demonstrated that MSCs can also differentiate into other mesodermal lineages, such as skeletal myocytes^[13,14], cardiomyocytes^[15], tenocytes^[16,17] and endothelial cells^[18,19]. Moreover, it has been reported that under appropriate conditions, MSCs have the capacity to differentiate into types of cells of endodermal and ectodermal lineages, including hepatocytes^[20,21], neuronal cells with neuron-like functions^[22-24], insulin-producing cells^[25,26], photoreceptor cells^[27], renal tubular epithelial cells^[28], epidermal and sebaceous duct cells^[29]. In addition to their comprehensive differentiation potential, MSCs have the ability to migrate and engraft at sites of inflammation and injury in response to cytokines, chemokines and growth factors^[30,31]. At a wound site, they can exert local reparative effects through transdifferentiation into tissue-specific cell types or *via* the paracrine secretion of soluble factors with anti-inflammatory and wound healing activities^[32-34].

Another aspect that makes MSCs of particular clinical interest is the finding that they exert a wide range of immunomodulatory activities affecting both cell-mediated and humoral immune response. A search in the PubMed data base reveals 149 papers, while the ScienceDirect data base contains 495 papers in peer-reviewed journals describing animal models developed to study various aspects of the immunomodulatory effects of MSCs in the period of 2001-2014. The promising results obtained prompt clinical trials in humans using MSCs as a biological agent for immunomodulation. According to the web site of Clinical Trials.gov (a service of the United States National Institutes of Health), more than 418 clinical trials are currently under way to assess the clinical effects of mesenchymal stem cells isolated from various sources, with the greater part of the trials studying the immunomodulatory effect of autologous or allogeneic MSCs in autoimmune diseases such as ulcerative colitis, multiple sclerosis, primary Sjogren's syndrome, systemic sclerosis, Crohn's disease *etc.* Similarly, numerous trials are devoted to the effect of MSCs on modulating the reactions after allogeneic transplantation, such as chronic graft-versus-host disease (GVHD), poor graft function, *etc.*

Table 1 Cytokines secreted by mesenchymal stem cells and the corresponding target cells

Cytokines secreted by MSCs	Target cells
IL-10	Mph, Neu, DCs, Th1, Tregs, Tr1, tumor cells
IL-6	Neu, Mo, DCs, B, Th2, Tregs, Th17, CD8+FoxP3+
TGFβ	Mph, NK, DCs, B, T, Tregs
Chemokines	Neu, Mo, NK, Eo, Baso, DCs, Ly
CCL-2/MCP-1	Mph, EC, PL, Th2, Th17
CCL-5/RANTES	Neu, Mo, DCs, Th1, Tregs, CD8+FoxP3+
IDO	Mo, DCs, B, T, Tregs
VEGF	DCs, EC, Th1, Th17, Tregs
ICAM	T, MSCs
PGE2	Mph, Mo, NK, DCs, T, Tr1

MSCs: Mesenchymal stem cells; TGFβ: Transforming growth factor β; CCL: CC chemokine ligand; MCP-1: Monocyte chemoattractant protein 1; RANTES: Regulated on activation, normal T cell expressed and secreted; IDO: Indoleamine-2,3-dioxygenase; VEGF: Vascular endothelial growth factor; ICAM: Intercellular adhesion molecule; PGE2: Prostaglandin E2; Mph: Macrophages; Neu: Neutrophils; DCs: Dendritic cells; Th: T helpers; Tregs: T regulatory cells; Tr1: T regulatory 1; Mo: Monocytes; B: B cells; NK: Natural killers; T: T cells; Eo: Eosinophils; Baso: Basophils; Ly: Lymphocytes; EC: Endothelial cells; PL: Plasma cells.

In general, the data from these studies have shown that MSCs exert immunomodulatory effects by both cell-to-cell contacts and by secreting biologically active substances, growth factors, cytokines and chemokines.

MSCs have been shown to inhibit T-cell activation and proliferation triggered by mitogenic or antigenic stimulation with allogeneic cells (mixed lymphocyte cultures) or nominal antigens^[35,36]. MSCs can also influence T-cell responses indirectly through suppression of CD34⁺ progenitor cell and monocyte-derived dendritic cell differentiation, as well as through inhibition of their antigen-presenting functions^[37-40]. A number of studies have demonstrated that MSCs have the capacity to inhibit B-cell proliferation, differentiation and immunoglobulin production *in vitro*^[41,42] as well as to down-regulate the proliferation, cytokine production and cytotoxicity of NK cells^[43,44]. Their ability to promote the generation and to maintain the activity of different subtypes of regulatory T cells (Tr1, CD4+FoxP3⁺, CD8+FoxP3⁺) is well documented, especially CD4+FoxP3⁺, also known as Tregs^[45-48]. In addition, MSCs are considered as not being inherently immunogenic as they express low-intermediate levels of HLA class I antigens and either do not express or express negligibly low levels of HLA class II antigens and co-stimulatory molecules, such as CD80, CD86 and CD40^[49,50]. Therefore, they should be able to escape not only from the recognition by alloreactive T cells^[49,51], but also the cell-specific lysis by cytotoxic T lymphocytes (CTLs)^[52] and freshly isolated alloreactive NK cells^[53]. Some of these *in vitro* properties have already been successfully clinically exploited for the treatment of disorders such as acute graft-versus-host disease^[54,55], multiple sclerosis^[56] and systemic lupus erythematosus^[57].

Although the precise mechanisms underlying MSCs immunomodulation are still not completely understood, a number of soluble factors involved in the process have already been identified.

The present review discusses some MSC secreted cytokines which are involved in regulation of the immune

response. For the purposes of this review, the term “immunoregulation” is used in a very strict sense as an influence on immunocompetent cells. It should be pointed out that the immunomodulatory effects of MSCs are jointly executed by both secretory factors and direct cell-to-cell contacts. In that case, cytokines most commonly do not directly affect the target cells but interact with other biologically active factors to achieve the effect of immunosuppression. There are some papers describing fine differences in MSC secreted cytokine profiles with immunoregulatory effects but in this review the generally accepted cytokines most often cited in the literature are discussed. The mechanisms of immunomodulation by direct cellular contacts will not be discussed in this review.

MSCs isolated from different tissues are different in some fine specifics as mentioned above. However, no data have been published describing significant differences in the profiles of secreted cytokines by different types of MSCs. Most authors report either a lack of differences or find some quantitative differences in the levels of cytokines secreted by AT-MSCs or BM-MSCs^[58-60]. Our experimental data also show some quantitative differences in the cytokine secretion^[37]. Similar findings are reported when embryonic, fetal and adult MSCs have been compared^[61].

MSCs secrete cytokines either “spontaneously” or after induction by other cytokines, the most important being IFNγ, TNFα and IL-1β^[62-64], and it should be underlined that MSCs are not always immunosuppressive. It is assumed that their effects are determined by the local conditions of the microenvironment and sometimes the pro-inflammatory IFNγ, TNFα and IL-1β cytokines may induce secretion of anti-inflammatory immunosuppressive factors. Engagement of certain Toll-like receptors (TLR) expressed by MSCs can determine their pro or anti-inflammatory effects^[65-67]. The definition of cytokines as pro or anti-inflammatory is quite far from their real effects because it seems that there is not a single cytokine which is not engaged in both types of reactions. Nev-

ertheless, that definition is quite convenient and will be used further in the present review. The most important immunoregulatory cytokines described in the literature are presented in Table 1.

INTERLEUKIN 10

Interleukin 10 (IL-10) is pleiotropic cytokine identified in the 1980s and characterized by its anti-inflammatory effect related to the induction of immune tolerance^[68-71]. It has been established that IL-10 suppresses the functions of macrophages and neutrophils^[70,72], inhibits the Th1 immune response^[70,73-76], influences NF- κ B synthesis^[77] and causes expression of anti-inflammatory molecules, such as protease inhibitors^[78] and IL-1 and TNF α antagonists^[79].

The major function of IL-10 in induction of immune tolerance is its effect on the antigen presenting cells and particularly on the dendritic cells (DCs). IL-10 suppresses the secretion of pro-inflammatory cytokines (TNF α , IL-1, IL-6, IL-8, IL-12) by DCs and the expression of MHC II molecules, as well as co-stimulatory complex B7 on their surface^[75-77]. In parallel to that, IL-10 is capable of inducing anergy of T lymphocytes by directly inhibiting the phosphorylation of CD28. In that way, one of the basic immunosuppressive mechanisms is executed by IL-10 by inducing a tolerogenic type of dendritic cells with reduced HLA- II and B7 expression and by suppression of CD28 (the partner of B7) expression on the surface of the T lymphocytes. This “two sided” suppression of the second signal which is unconditionally needed for activation of the T lymphocytes induces a deep anergy in this cell population^[37,69-71,76].

Further on, IL-10 is directly engaged in the induction of immune tolerance by two types of T regulatory lymphocytes: Tregs and Tr1^[76]. IL-10 is one of the cytokines related to the generation of Tregs^[73] which secretes IL-10 by itself and this process has been described both for “natural” FoxP3+ Tregs and for FoxP3+ Tregs generated after response to a specific antigen^[70,73,80].

A specific feature of IL-10 and some other cytokines is that the producing cells are both the source and target of the cytokine effect and this predominantly affects the dendritic and T regulatory cells. A good example is that tolerogenic DCs secrete IL-10 and thus induce the generation of regulatory T helpers (FoxP3 and Tr1) which secrete IL-10 inducing tolerogenic phenotype of DCs^[70,71,76]. Likewise, many other cytokines IL-10 can also act in an autocrine loop.

The effect of IL-10 is mediated *via* its binding to its specific receptor (IL-10R) and subsequent interaction between JAK1 and STAT-3^[73,77], a mechanism which is common for many other cytokines. Besides the antigen-presenting cells and particularly tolerogenic DCs, Tregs and Tr1, other immune cells secrete IL-10 and these include T and B lymphocytes, NK cells, neutrophils and macrophages^[76,80]. The role of IL-10 secreted by Th2 helpers is well known^[76,80] but some recently published

data show that this cytokine in a somewhat paradoxical manner is secreted by both Th1 and Th17 cells. Quite often these “double secreting” cells (IL-10 simultaneously with IFN γ or IL-17) use IL-10 to suppress their own pro-inflammatory effect, both directly and/or with the help of tolerogenic antigen-presenting cells^[71,74].

IL-10 is considered to be a classical cytokine inducing immune tolerance but there are data which show that, similarly to most cytokines, IL-10 acts in more than one way. Its pro-immune effect has been described in tumorigenesis^[70,72] and IL-10 is detected in a tumor environment and shown to have an anti-tumor effect. It is assumed that this effect is due to inhibition of the tumor angiogenesis and enhancement of the nitric oxide secretion. IL-10 is also connected to the inhibition of the expression of MHC by the tumor cells which makes them an easier target for the NK cells. Some pro-inflammatory effects of IL-10 have been demonstrated which seem to lead to enhanced apoptosis of Tregs and stimulation of the antigen up-take by the antigen-presenting cells^[70].

IL-10 is the cytokine most commonly discussed in relation to the immunoregulatory effects of MSCs. Nevertheless, the published data demonstrating the secretion of IL-10 by MSCs are quite contradictory. Almost half of the papers discussed in the present review report positive secretion of IL-10 by MSCs^[62,66,81-84], while the other half and our own experimental results reject such a possibility^[60,64,69,78,85-88]. It is quite logical to support the concept proposed by some authors claiming that MSCs secrete IL-10 under specific conditions with the inflammatory environment and presence of cytokines (IFN γ , IL-1b and TNF α) which activate certain Toll-like receptors on MSCs^[63,65,67]. Although there is no definite opinion about the conditions under which MSCs secrete IL-10, their role is indisputable as a factor which causes indirect stimulation of IL-10 secretion by other cells. It has been shown that MSCs secrete factors which up-regulate the secretion of IL-10 by peripheral blood mononuclear cells (PBMCs)^[59], as well as by tolerogenic macrophages^[89] and tolerogenic DCs^[37,69,90]. It is also assumed although not undoubtedly proven that MSCs induce generation of Tregs^[59,62,90] and our results show that when cultured in MSC conditioned medium, the fraction of CD4+FoxP3+ lymphocytes is increased and this effect is directly induced by MSCs without any involvement of DCs^[69].

IL-6

IL-6 was identified in 1986 as a factor stimulating B lymphocytes^[91]. It is now known that it is a pleiotropic cytokine with a key role in a multitude of processes such as regulation of the immune response, hematopoiesis, inflammation, cell survival, apoptosis, cell proliferation and oncogenesis^[91,92]. The action of IL-6 is mediated by its binding with a membrane IL-6 receptor (mIL-6R) and gp130 as gp130 interacts with the JAK-STAT system^[91]. A small fraction of cells show expression of mIL-6R but almost all cell types express gp130. Cells express-

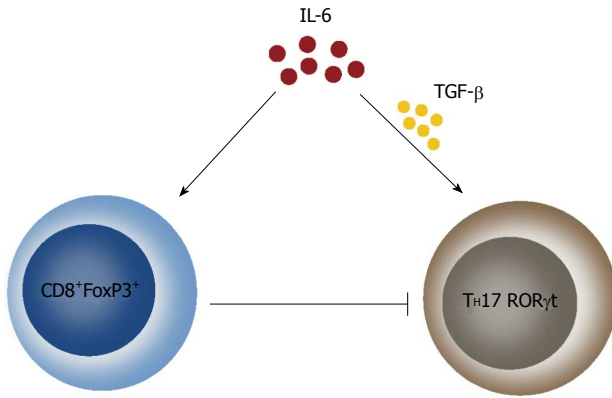


Figure 1 Effect of Interleukin 6 on Th17 formation. IL-6 exerts a dual effect on the generation of Th17 cells. On one side, IL-6 in concert with TGF β facilitates the generation of this cell population and on the other side, IL-6 inhibits the Th17 cells by inducing the generation of CD8 $^{+}$ FoxP3 $^{+}$ T lymphocytes. TGF β : Transforming growth factor β .

ing only gp130 can bind the complex IL-6/soluble IL-6R (sIL-6R), a process known as trans-signaling, which makes a lot of cell populations susceptible to the effects of IL-6^[93,94]. Some authors believe that the effect of IL-6 mediated by trans-signaling (IL-6/sIL-6R) is related to a pro-inflammatory effect, while the “classical” pathway (IL-6/mIL-6R) of activation is connected to the anti-inflammatory action of the cytokine^[94]. Such an assumption sounds quite logical, keeping in mind the dual nature of IL-6 because of its pro-inflammatory and/or anti-inflammatory effects^[75,82]. IL-6 is routinely described as a classical pro-inflammatory cytokine based on well proven effects of this cytokine. In concert with IL-1 and TNF α , this cytokine induces secretion of acute phase proteins, causes neutrophil recruitment, expression of cell adhesive molecules and a switch from neutrophil to macrophage induced inflammation^[72,75,94]. IL-6 stimulates T cell proliferation^[64,72] and together with IL-4 participates in the generation of the Th2 immune response^[94]. IL-6 has a significant role in the triggering of humoral immune response by stimulating the B cell differentiation and secretion of antibodies^[95]. Some recent data demonstrate that IL-6 together with TGF β is engaged in regulation of the balance between the pro-inflammatory Th17 and immunosuppressive response mediated by Tregs as both cytokines acting together induce expression of ROR γ t which is the major transcription factor defining the Th17 cells^[94,95].

Recently, simultaneously to the proven pro-inflammatory function of IL-6, quite convincing data have piled up about its function as an anti-inflammatory cytokine. It has been established that IL-6 suppresses the secretion of many pro-inflammatory cytokines, such as IL-1, TNF α , GM-CSF, IFN γ , but on the other hand, it induces the synthesis of glucocorticoids, IL-10, IL-1 receptor antagonist and soluble receptor for TNF α ^[72,75,79,96]. It has been demonstrated that IL-6 exerts its anti-inflammatory effect both locally and systemically because mice deficient to IL-6 gene have increased production of pro-inflammato-

ry TNF α , GM-CSF and MIP-2^[72]. Moreover, some new results show that IL-6 is a key factor in the formation and functions of the CD8 $^{+}$ FoxP3 $^{+}$ cell population, which is related to suppression of the Th17 immune response^[97]. Altogether, these data show that IL-6 has a “two sided” engagement in the modulation of the immune response by regulatory and Th17 cells. On one hand, IL-6 together with TGF β induces formation of Th17 cells and on the other hand, IL-6 directly inhibits this response by its effect on CD8 $^{+}$ FoxP3 $^{+}$ (Figure 1).

Contrary to IL-10, there is no doubt that IL-6 is secreted by MSCs and almost all authors agree with this statement^[69,78,82,84,86,87]. Its secretion by MSCs has been demonstrated both in mice and in humans^[62,66] and is detected either after induction with TNF α , IL-1b and IFN γ or spontaneously^[37,61-63,72,95]. When MSCs were tested for 120 cytokines at mRNA and protein levels, it was established that IL-6 has the highest expression and the conclusion was made that IL-6 was the basic cytokine responsible for the immunoregulatory effects of MSCs^[60]. After the secretion of IL-6 by MSCs, a suppression of the apoptosis of neutrophils is observed^[64,68,81] and this effect could be very important for the connection between defects of apoptosis and triggering of autoimmune reactions. However, it is still not truly clarified whether IL-6 causes generation of classical Treg cells (CD4 $^{+}$ FoxP3 $^{+}$), although there is no doubt about its direct effect on the generation of CD8 $^{+}$ FoxP3 $^{+}$ cells. The fact that MSCs secrete active factors, increasing the numbers of Tregs, has been proven in a number of experiments^[59,62,69] but there are no sure data that this effect is mediated *via* IL-6. It should be stated that such a mechanism is quite probable, keeping in mind the effect of IL-6 on the generation of CD8 $^{+}$ FoxP3 $^{+}$.

MSCs can be both a source and a target of the effects of IL-6. It has been established that under the influence of IL-6, MSCs can transform malignant cells and have tumorigenic properties and this effect is mediated through the mechanism of trans-signaling^[98]. These facts raise questions about the interactions between MSCs and the tumor microenvironment which is most commonly very rich in IL-6.

INTERACTIONS BETWEEN IL-6 AND IL-10

IL-6 stimulates the secretion of IL-10 by different types of cells and this effect has been proved without any doubt but the reverse interaction has not been demonstrated so far^[96]. The effect of IL-6 on monocytes and dendritic cells is of particular importance for the complex process of immunoregulation. Some publications describe a pathway in which MSCs secrete IL-6 which directly or *via* induction of autocrine secretion of IL-10 influences the monocyte activity inhibiting their differentiation as dendritic cells^[37,81]. Both IL-6 and the autocrine reacting IL-10 also suppress the capacity of DCs to present antigens and thus a population of immature tolerogenic dendritic cells is formed which secrete

IL-10^[37,38,59,64,69,99,100]. Its effect stimulates the generation of T regulatory cells secreting IL-10 by themselves and potentiating further formation of tolerogenic DCs^[62,85]. However, it should be noted that IL-6 and IL-10 are not the only cytokines involved in these complex interactions, for example, prostaglandin E2 (PGE2) which is another immunosuppressive factor secreted by MSCs interacting with IL-6 in suppression of the DCs differentiation^[68].

TRANSFORMING GROWTH FACTOR BETA

One of the most prominent immunomodulatory cytokines produced and constitutively secreted by MSCs is transforming growth factor beta (TGFβ). As a pleiotropic cytokine, TGFβ regulates multiple fundamental cellular functions, including proliferation, differentiation, migration, adhesion and apoptosis, that affect numerous biological processes such as development, wound healing, carcinogenesis, angiogenesis and immune responses^[101]. TGFβ is a member of a superfamily of dimeric polypeptide growth factors that consists of about 40 members in vertebrates, also including bone morphogenetic proteins (BMPs), activins, inhibins, growth differentiation factors (GDFs) and glial cell line-derived neurotrophic factor (GDNF)^[102]. In mammals, three homologous TGFβ isoforms have been identified (TGFβ1, TGFβ2 and TGFβ3) that are controlled by specific genes^[103]. Each isoform may exert a distinct role which depends on the target cell type, its state of differentiation and growth conditions^[103].

TGFβ is now established as a principal mediator of immune regulation which plays an essential role in orchestrating the initiation and resolution of inflammatory responses, as well as in induction and maintenance of immune tolerance by influencing leukocyte proliferation, differentiation, activation and survival^[104,105]. The diversity of modulatory activities that TGFβ exerts on the immune cell functions is quite extensive and includes effects such as inhibition of effector T-cell proliferation and function, generation of regulatory T cells from naïve T lymphocytes, attenuation of cytokine production and cytolytic activity of NK cells, suppression of B cells, dendritic cells and macrophages^[105].

As TGFβ is constitutively produced by MSCs and most of its effects on immune cells mentioned above have also been demonstrated to be intrinsic features to MSCs, it is reasonable to assume the putative involvement of TGFβ as a mediator of their broad immunoregulatory properties.

It has been reported that MSCs isolated from human bone marrow were able to suppress CD4+ and CD8+ T-cell proliferation induced by cellular or nonspecific mitogenic stimuli and that this effect could be reversed by the addition of monoclonal anti-TGFβ1 neutralizing antibodies^[55]. Later, it was shown that human bone marrow-derived MSCs, activated by blood CD14+ monocytes, secreted TGFβ1 which is responsible for inhibition of T-lymphocyte responses^[106]. It has also been observed

that TGFβ1 was involved in a cell contact-dependent inhibition of T-cell proliferation by MSCs^[107]. Furthermore, MSCs obtained from dental pulp were found to produce TGFβ and to suppress the proliferation of PBMCs, which could be neutralized with anti-TGFβ antibodies^[108]. In contrast, the addition of TLR-3 agonist augmented the suppressive potential of dental pulp-derived MSCs and potentiated TGFβ secretions by these cells^[108].

Numerous mechanisms have been suggested to be involved in TGFβ-mediated inhibition of T-cell proliferation, differentiation and effector functions. One pathway by which TGFβ exerts its anti-proliferative effect on T lymphocytes is through blockade of the production of the T-cell mitogenic cytokine IL-2^[109]. Functional analysis revealed that this is most likely due to impaired IL-2 gene transcription as a result of inhibition of IL-2 promoter/enhancer activity^[109]. In another study^[110], the transcription factor Smad3 was also shown to be critical for TGFβ1-mediated inhibition of IL-2 expression. Moreover, it has been demonstrated that the addition of exogenous IL-2 partially but not completely reversed the antiproliferative effects of TGFβ, indicating the suppressive activity of TGFβ on both production and intracellular signaling of IL-2^[111].

TGFβ also inhibits cell proliferation through controlling the expression of cell cycle regulators, including up-regulation of cyclin-dependent kinase inhibitors (CKIs) p15, p21 and p27 and down-regulation of cell cycle-promoting factors, such as c-myc, cyclin D2 and cyclin E^[112-115]. However, it has been reported that TGFβ is able to suppress the proliferation of T cells from mice deficient for all three CKIs mentioned above, demonstrating their dispensable role in this process^[116]. In addition, a Smad3-dependent down-regulation of CDK4 has been described, suggesting a potential mechanism underlying resistance of Smad3-/- T cells to the induction of growth arrest by TGFβ^[116].

TGFβ is a strong suppressor of T-cell differentiation and effector functions. In the presence of TGFβ, CD8+ T cells fail to acquire CTL function and CD4+ T lymphocytes do not become Th1 or Th2 cells^[117]. The inhibition of T-cell differentiation occurs even in the presence of added IL-2, while at the same time T-cell proliferation remains unaffected^[118].

One of the possible mechanisms of inhibition of T-cell differentiation by TGFβ is associated with decreased expression of IL-12 receptor β2-chain (IL-12Rβ2) and therefore with possible blockade of IL-12 signaling, which is required for Th1-cell development^[119]. However, a more recent study has demonstrated that inhibition of T-bet (T-box expressed in T cells), a transcriptional activator of Th1 development, was critical for TGFβ-induced suppression of Th1-cell differentiation and that down-regulation of IL-12Rβ2 expression appeared not to be important for the TGFβ-mediated effect but rather was an event secondary to T-bet inhibition^[120]. It has also been shown that restoration of T-bet expression through retroviral transduction of T-bet into

developing Th1 cells abrogated the inhibitory effect of TGF β ^[120] which indicated that T-bet was the most critical and primary target for the inhibition of Th1 differentiation by TGF β . In addition, TGF β can also function indirectly to suppress Th1-cell differentiation by inhibiting IFN γ production by NK cells^[121]. In this regard, it has been found that bone marrow-derived MSCs were able to suppress NK cell proliferation and IFN γ production through the secretion of TGF β 1 and prostaglandin E2^[43].

TGF β has also been found to potently down-regulate Th2-cell differentiation. A few studies^[122,123] have shown that TGF β -mediated prevention of Th2-cell development is due to suppressed expression of the transcription factor GATA-3, a key transcriptional activator of Th2-cell differentiation^[124]. Moreover, TGF β is able to induce the transcription factor Sox-4 and therefore negatively regulate GATA-3 function indirectly by two distinct mechanisms^[125]. First, Sox-4 binds directly to GATA-3, preventing its transcriptional activity, and second, Sox-4 binds to the promoter of IL-5, a Th2 cytokine, and prevents GATA-3-mediated induction of gene expression^[125].

In addition to suppressing proliferation, TGF β has also been demonstrated to inhibit CD8+ T-cell effector functions through down-regulation of the expression of several essential CTL effector molecules such as perforin^[126], Fas ligand (FasL)^[127] and IFN γ ^[128,129]. Furthermore, the release of cytolytic granules by CTLs can be selectively suppressed by Tregs in a TGF β -dependent manner^[130].

Another important immunosuppressive activity of TGF β could be its implication in the development of regulatory T cells. TGF β promotes the conversion of naive CD4+T cells to Treg cells by induction of transcription factor FoxP3^[131-133]. Several reports have indicated an essential role for both Smad2 and Smad3 transcription factors in TGF β -mediated induction and maintenance of Foxp3 expression^[134-137]. For instance, it was demonstrated that Smad2 and Smad3 double deficiency lead to complete ablation of FoxP3 upregulation by TGF β , suggesting a functional redundancy between these two transcription factors in the induction of Tregs^[137].

A recent paper has shown that both TGF β 1 and prostaglandin E2 derived from MSCs contributed to allogeneic MSCs induction of CD4+CD25+ FoxP3+ regulatory T cells that possess the ability to suppress alloantigen-driven proliferative responses in a mixed lymphocyte reaction^[46]. Later, MSC-derived TGF β 1 was reported to be largely responsible for the increase in Treg frequency based on knockdown studies, thereby protecting breast cancer cells from immune clearance^[138].

Recently, a mouse model of ragweed-induced asthma was described in which iv injected MSCs were capable of suppressing Th2-driven allergic responses *via* secretion of TGF β ^[139]. The results suggested that IL-4 and/or IL-13 were able to activate the STAT6 pathway in MSCs which resulted in an increase of their TGF β production. It seemed that TGF β secreted by MSCs could mediate its beneficial effects (*i.e.*, inhibition of eosinophil infiltration and excess mucus production in the lung, decreased levels of Th2 cytokines (IL-4, IL-5 and IL-13) in bronchial la-

vage and lowered serum levels of Th2 immunoglobulins (IgG1 and IgE), either alone or together with recruited Treg cells^[139].

CHEMOKINES

Chemokines are a family of structurally related peptides with comparatively small molecules (7,5-12,5 kDa) with chemoattractive properties^[140]. Their physiological role is participation in processes like regulation of inflammation, cell differentiation and migration of immune cells, as well as angiogenesis^[141]. Chemokines are produced and secreted by various cell types as a response to pro-inflammatory stimuli with the aim to attract and activate neutrophils, monocytes, lymphocytes and other effector cells to sites of infection^[140].

It has been established that *in vitro* cultured MSCs constitutively secrete a multitude of different members of the chemokine family, such as CCL2 (MCP-1), CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL5 (RANTES), CCL7 (MCP-3), CCL20 (MIP-3 α), CCL26 (eotaxin-3), CXCL1 (GRO α), CXCL2 (GRO β), CXCL5 (ENA-78), CXCL8 (IL-8), CXCL10 (IP-10), CXCL11 (i-TAC), CXCL12 (SDF-1) and CX3CL1 (fractalkine)^[142]. Our own data have shown that MSCs isolated from human bone marrow or adipose tissue secrete IL-8, GRO α , MCP-1, RANTES and SDF-1 and these chemokines can be demonstrated to be present in MSC conditioned medium^[59,69].

It is quite reasonable to assume that the types and the combinations of chemokines expressed by MSCs could vary depending on the specific microenvironment and contacts with surrounding cells, especially as the latter are immune cells. The target cells attracted by the cited group of chemokines are neutrophils, monocytes, eosinophils, basophils, T and B lymphocytes, DCs, NK cells, hematopoietic and endothelial progenitors^[142].

These data might suggest that the MSC secreted chemokines just have a chemoattractive effect which does not seem to be related to immunoregulation. Nevertheless, chemokines could be considered a crucial element in exerting the immunomodulatory activity of MSCs *in vivo* because it is assumed that the chemokines mediate the interactions between MSCs and other types of immunocompetent cells. By attracting immune cells in close proximity with MSCs, the secreted chemokines provide direct cell-to-cell contact as well as a possible paracrine immunoregulatory effect of other effector molecules also secreted by the MSCs. Thus, Ren *et al.*^[143] established that the chemokines CXCL9, CXCL10 and CXCL11 stimulate the migration of T cells in the proximity of MSCs and that these cells are targets of the local suppressive effect of nitrogen oxide secreted by the stem cells. Nevertheless, MSC secreted chemokines predominantly exert chemotactic activity and many data point to their direct role in the process of immunomodulation.

Monocyte chemoattractant protein-1 (CCL2/MCP-1)

CCL2 is a key chemokine regulating the recruitment and migration of cells of the monocyte-macrophage system.

It is secreted from monocytes and other types of cells, including endothelial cells, microglial cells, NK cells *etc*^[144]. CCL2 is related to multiple disorders associated with accumulation of activated monocytes, including atherosclerosis, bronchial asthma, inflammatory processes of the intestines *etc*^[144]. CCL2 plays a role of direct mediator for angiogenesis and its effect is manifested by formation of new blood vessels, as proven in animal models^[145]. Much data shows that CCL2 modulates the T cell immune response, causing a switch from Th0 to Th2 with predominant secretion of IL-4^[146,147]. The role of CCL2 in immune regulation has been proven by the fact that it induces secretion of MCP-1 (MCP-1 induced protein-1) which acts as RNase and stimulates mRNA degradation for some cytokines such as IL-6 and IL-1^[148]. MCP-1 acts as a negative regulator of CCL2 and inhibits macrophage activation^[149]. It has also been established that CCL2, CCL5 and some other chemokines induce proliferation and activation of specific CD56+ cytolytic cells designated as CHAK (CC chemokine-activated killer) which act similarly to the IL-2 activated cells (LAK)^[150].

Some recent studies report that CCL2 is one of the factors associated with the immune modulation caused by MSCs. Secretion of this chemokine by the MSCs causes enhanced FasL dependent apoptosis of T lymphocytes. The apoptotic T cells stimulate secretion of higher levels of TGF β by macrophages and the latter cytokine is associated with generation of CD4+FoxP3+ Tregs^[151]. Other authors comment about the anti-apoptotic effect of CCL2 and describe inhibition of caspase 3 in the cell line of embryonic cardiomyoblasts cultured in the presence of MSC conditioned medium^[152]. So it seems that there are data suggesting a dual function of CCL2, either pro-apoptotic or anti-apoptotic depending on the micro-environment and the general cytokine profile. It has been shown that CCL2 mediated in an autocrine manner the migration of MSCs towards the site of inflammation, ischemic damage, trauma or a developing malignant process and there the MSCs exert their immunomodulating effect^[152].

Some data have been reported demonstrating that the inhibiting effect of MSCs on the immunoglobulin production by plasma cells is the result of the effector effect of CCL2 and CCL7 chemokines secreted by the MSCs^[153]. It has been established that this effect is due to inhibition of the phosphorylation of STAT3 which causes activation of the transcription factor PAX5 and suppression of the immunoglobulin synthesis^[153]. This assumption is substantiated by the fact that neutralizing the CCL2 neutralizes the suppressive effect of MSCs on plasma cells^[153]. A possible participation of CCL2 in the inhibition of the pro-inflammatory CD4+ Th17 cells caused by MSCs has been hypothesized as an alleviation of clinical symptoms observed in EAE (experimental autoimmune encephalomyelitis)^[154]. Furthermore, it has been established that MSC conditioned medium exerts an inhibitory effect on the activation of CD4 T cells obtained from EAE mice. This effect is mediated *via* CCL2-

dependent suppression of STAT3 phosphorylation^[154]. In addition, the key role of CCL2 produced by MSCs has been supported by the fact that MSCs isolated from CCL2 knock-out mice and injected in EAE mice do not demonstrate any therapeutic effect^[154].

Regulated on activation, normal T-cell expressed and secreted (RANTES/CCL5)

RANTES/CCL5 was initially identified as a product secreted by activated T lymphocytes^[155] which mediates the chemotactic activity of some cell types, including monocytes, lymphocytes and dendritic cells. It is engaged in regulation of leucocyte migration, angiogenesis^[156,157] and some processes of wound healing^[158]. CCL5 is a mighty activator of leucocytes and neutrophils, the effect of which is similar to that of mitogenic stimuli^[159]. Besides its functions as a chemokine, CCL5 participates in the anti-viral immune response by blocking HIV replication *in vitro* and the disease progress^[160,161]. CCL5 inhibits the T cell response and maybe functions as a blocking factor (suppressor of alloantigen specific T cells) by inducing cell apoptosis by modulating Bcl-2 levels and by a caspase independent mechanism^[162]. There are data that CCL5 is involved in blocking the development of monocytes and memory Th1 cells^[163]. CCL5 secreted by NKT cells leads to formation of CD8+ FoxP3+ cells which is the probable mechanism to induce tolerance in alloreactive T cells^[164]. CCL5, similarly to CCL2, stimulates the migration of MSCs to sites of tissue damage in an autocrine manner and there are data that some tumors stimulate *de novo* secretion of CCL5 by MSCs with the aim to support metastases, the invasiveness and the mobility of tumor cells^[165,166].

Data reported by different authors show that the effects of the chemokines should not be interpreted in one way. Most probably, chemokines secreted by MSCs do not only recruit various types of immune cells in order to exert immunomodulation but on the other hand, they act in an autocrine manner leading to migration of stem cells to the sites of tissue damage and at a later stage, support the immunomodulatory properties of the MSCs.

INDOLEAMINE-2,3-DIOXYGENASE

Indoleamine-2,3-dioxygenase (IDO) is the tryptophan-catabolizing enzyme that possesses immunosuppressive and antimicrobial effects. IDO is one of the key immunoregulators secreted by MSCs, tumors and during pregnancy. IDO is expressed by a wide range of MSCs, like decidual MSCs^[167], amniotic fluid MSC^[168], multipotent adult progenitor cells (MAPCs)^[169], umbilical cord MSCs^[170], AT-MSCs^[171] *etc*. IDO expression is species specific. Murine MSCs possess very little IDO^[172], while human MSCs do just the opposite - express an abundant amount of IDO. MSCs from monkey, pig and humans utilize IDO, whereas mouse, rat, rabbit, hamster^[173] and equine^[174] MSCs do not produce IDO. This variation should be considered when mouse MSCs are used as a

model for studying immunoregulation properties since differences in expression of molecules involved in the process by murine and human MSCs are unquestionable.

Not activated MSCs normally express low levels of IDO, but on stimulation with inflammatory cytokines, mainly IFN γ , the IDO mRNA levels are found to be elevated^[175]. IDO is not an exclusive mechanism for MSCs immunomodulation in basal states but is essential for MSC suppression in the presence of IFN γ ^[176]. Glucocorticoids, budesonide or dexamethasone treatment of MSCs also lead to enhanced IDO expression and is able to regenerate IDO synthesis in over-passaged MSCs^[177]. Damage associated molecular patterns (DAMPs) are also involved in the IDO expression regulated by MSCs^[178]. IDO is expressed after stimuli generated by the crosstalk of MSCs and cells co-cultured with them^[168,179].

The cross-talk of MSCs and PBMCs causes increased IL-10 and IDO expression from MSCs that seems to be the mechanism responsible for the immunosuppressive action of the human amniotic fluid stem cells^[168]. After IFN γ priming of MSCs, the IDO expression leads to B-cell growth arrest and apoptosis^[180], in contrast to not activated MSCs that are IDO negative and support B-cell proliferation and survival. The addition of 1-methyl-DL-tryptophan (1-MT), an IDO inhibitor, also restored the proliferative capacity of both naive and pre-activated T cells^[181]. The feedback regulation between MSCs and activated T cells may limit the immunosuppressive effects of MSCs only to sites containing ongoing inflammatory responses where the activated T cells induce the up-regulation of IDO from MSCs^[179].

Direct and indirect pathways are engaged in MSC mediated immunosuppression. The catabolic activity of IDO, secreted by MSCs, can directly suppress T-cell proliferation as a result of rapid tryptophan degradation.

In addition to the direct mechanism, an indirect pathway is described and is provided through the MSC mediated differentiation of monocytes into IL-10 secreting, CD206+ immunosuppressive M2 macrophages which contribute to T-cell suppression^[182].

Induction of regulatory T cells is another indirect mechanism for immunoregulation explored by MSCs. IDO expression is responsible for induction of IL-10+IFN γ +CD4+ regulatory T type 1 [T(R)1]-like cells by MSCs^[183]. Neutralization of IDO is also a reason for Treg reduction^[184]. Feedback regulation between Tregs and MSCs exist since Tregs do not alter the secretion of IFN γ by immune cells and hence contribute to MSC activation. MSCs by themselves secrete IDO and are able to induce the production of IL-10 from Tregs^[185].

As described above, MSCs can suppress dendritic cell maturation and function, mediated by soluble factors which also include IDO. It was demonstrated that MSCs inhibit the maturation of DCs through the stimulation of IL-10 secretion and by activating the JAK1 and STAT3 signaling pathway^[186].

VASCULAR ENDOTHELIAL GROWTH FACTOR

The VEGF family are the key mediators of angiogenesis and it is largely known that this process plays a critical role in tumor progression as well as in acute and chronic inflammation. The main mechanism of action of VEGF is as endothelial cell mitogen that stimulates angiogenesis by promoting endothelial cell survival, proliferation, migration and differentiation.

Six proteins of the vascular endothelial growth factor (VEGF) family are described (VEGF-A, -B, -C, -D, -E and PlGF). VEGF-A interacts with two receptors, VEGF-R1 and -R2, which are expressed on endothelial cells and on some immune cells. In addition to its best known function in angiogenesis, VEGF has a role in immunity and inflammation. VEGF is responsible for recruitment of inflammatory cells and expression of co-stimulatory molecules on recruited and resident mononuclear cells. As a result, pro-inflammatory Th1 and Th17 cytokines are up-regulated^[187]. Vascular endothelial growth factor is a key mediator in the development of T cell priming and in the polarization to type 1 and type 17 T helper cells in the airways. Affecting functions of memory T cells in pro-inflammatory responses has also been described after VEGF stimulation^[188]. VEGF also have an indirect immunosuppressive function on lymphocyte activation and proliferation by increasing IDO secretion from dendritic cells^[189].

VEGF-A secreted by tumor cells is involved in immunosuppression *via* down regulation of the transcription factor NF- κ B and as a result, there is an inhibition of dendritic cell maturation, trafficking and antigen presentation^[190-192]. An increased VEGF plasma level in cancer patients correlated to the presence of immature DCs and immature myeloid cells in the peripheral blood^[193,194]. These findings are substantiated by results from mouse model studies showing that treatment with anti-VEGF antibody increases the numbers and enhances the functions of DCs^[195-197]. VEGF-A administration decreases splenic T cells and suppresses their function^[198]. Placental growth factor (PlGF), a VEGF-R1 ligand, also impedes DC differentiation^[190]. *In vitro* experiments have demonstrated that PlGF could block the capacity of human myeloid-derived DCs to stimulate a Th1 response^[199].

MSCs are a potent source of VEGF. It has been shown that high expression levels of VEGF were maintained during prolonged culture periods and that *in vivo* hMSCs engrafted into immunodeficient mice could survive and secrete human VEGF^[200]. MSCs from decidua were also found to secrete VEGF^[167]. Measurement of secreted VEGF-A by ELISA in serum-free medium from cultured MSCs showed a reproducible concentration of 4.1 ± 0.9 ng^[201]. Wang *et al*^[202] hypothesized that hypoxia or TNF α activates MSCs which are able to release VEGF by STAT3 and p38 MAPK dependent mechanisms. Human MSCs that released VEGF in response to TLR-2

and NOD-1 ligands were also described^[203].

INTERCELLULAR ADHESION MOLECULE

Intercellular adhesion molecule-1 (ICAM-1) is a membrane glycoprotein belonging to the immunoglobulin superfamily. Expressed on endothelial cells, leukocytes (lymphocytes and monocytes) and MSCs^[204,205], ICAM-1 (CD54) is a ligand that binds primarily the heterodimeric, leukocyte-restricted β 2-integrin receptors- α L β 2 (LFA-1), α M β 2 (MAC-1). ICAM-1 plays important functions in leukocyte transmigration through vessels, cell to cell adhesion impacting immune responsiveness during infections and disease pathogenesis. The level of membrane expression of ICAM on endothelia and MSCs is up-regulated by pro-inflammatory cytokines (IL-1, IL-6, TNF α) and IFN γ from activated T cells^[204,206,207] and does not depend on intercellular adhesion. Generally, MSCs are renowned for their immune-suppressive function which is crucially dependent on membrane expression of ICAM-1, as demonstrated in a mouse experimental model. It was unambiguously shown that blocking antibodies against ICAM-1 receptors or ICAM-1 deficiency of MSCs abrogated the suppressive effect of MSCs on activated T cells. Strengthening the adhesion of MSCs to T cells *via* ICAM-1 proportionally potentiates the function of MSCs represented by lagging of T cells proliferation^[204]. Besides the direct role of ICAM-1 in MSCs interaction with immune cells, the importance of membrane expression of ICAM-1 spans MSCs migration^[208], proliferation and differentiation capacity^[209]. Seemingly indirectly related, the essence of the processes like migration, proliferation and differentiation of MSCs is also regulated by the inflammatory environment^[66]. Therefore, specifically attracted to the sites of inflammation, like tissue damage, carcinogenesis and infection, MSCs participate in immune modulation, tissue repair and cell differentiation processes.

Apart from the membrane form of ICAM, a soluble ICAM (sICAM) also exists which is formed after shedding by proteolytic cleavage from the cell membrane^[210,211] or by coding of specific mRNA transcripts in cells^[212]. Elevated amounts of a biologically active form of sICAM is detected in serum, cerebrospinal fluid, synovial fluid, urine and sputum in pathologies with an underlying inflammatory status, like autoimmune and degenerative diseases^[213-215] and tumor pathogenesis^[216,217]. Different reports point to various cell sources of sICAM in health and pathologies, including endothelial cells^[218], peripheral blood mononuclear cells, keratinocytes, epidermoid carcinoma cell lines, melanoma cells^[219] and tumors^[216,217,220]. sICAM can be secreted spontaneously or after specific inductions^[220]. Limited data demonstrate that some but not all MSCs are a source of sICAM. Profiles of cytokine arrays revealed high expression of sICAM from human MSCs derived from umbilical cord and deciduas^[221,222] and null expression from bone marrow-derived MSCs^[221]. The exact physiological role of sICAM in health and pathology is still not completely revealed but reports

demonstrate its potential to stimulate endothelial cell differentiation in conditions with angiogenic growth in tumorigenesis^[223]. In relation to this finding, a speculation imposes that the process of massive angiogenesis which takes place during placentation might be related to the secretion of sICAM from umbilical cord-derived and decidua-derived MSCs. Hypothetically, the lack of such a requirement for bone marrow-derived MSCs suggests acquisition of varying functions of MSCs according to the tissue localization. Furthermore, the importance of sICAM secreted from human umbilical cord-derived MSCs for microglia functioning and neuronal survival is depicted in a model of Alzheimer's disease^[222].

Insufficiently explored, the paracrine function of sICAM seems to counteract the classical biological function of membrane ICAM by preventing leukocyte interactions. sICAM affects trafficking of immune cells *via* hampering attachment to endothelial cells^[224] and blocks immune response development due to deteriorated immune cell contacts. In addition, the increased sICAM during inflammation probably affects MSC migration, proliferation and differentiation and detailed exploration of their biology can help understand and modulate the regulatory properties of MSCs in different pathologies.

PROSTAGLANDIN E2

Prostaglandins (PGs) are products of cyclooxygenases (COX) synthesis from arachidonic acid. COX1 is constitutively expressed from virtually all tissues, while COX2 is induced under inflammatory conditions (by LPS, IL-1, TNF α for example)^[225]. COX2 is shown to preferentially metabolize prostaglandin E2 (PGE2)^[226] that acts as a messenger molecule through a paracrine and autocrine manner on surrounding cells.

Together with IDO, PGE2 is another major effector molecule responsible for immunoregulatory competence of MSCs^[183]. MSCs constitutively produce detectable levels of PGE2^[44,227,228]. Under inflammatory conditions of the environment, PGE2 is induced, substantially increasing secreted amounts from MSCs. LPS as well as cytokines like IFN γ , TNF α , IL-1 β are mediators directly regulating PGE2 production from MSCs^[227,229,230]. Multiple studies show that direct contact of PBMCs, monocytes and NK cells with MSCs induces PGE2 augmentation *via* the mentioned cytokines^[44,227-229]. Activated by environmental signals, PGE2 from MSCs exert regulatory influence on the activation status, proliferation, differentiation and function of immune cells from adaptive and innate immunity. Acting by a contact or paracrine manner^[229,231], PGE2 has a systemic anti-inflammatory effect of reducing TNF α , IL-6 and vascular permeability in an experimental model of sepsis^[230]. Particularly, the cellular targets of PGE2 are PBMCs, NK cells, monocytes, macrophages and the transitional processes of differentiation of monocytes into immature DCs^[228,230,232]. PGE2 indirectly affects polyclonally or allo-genically activated PBMCs by substantial suppression of

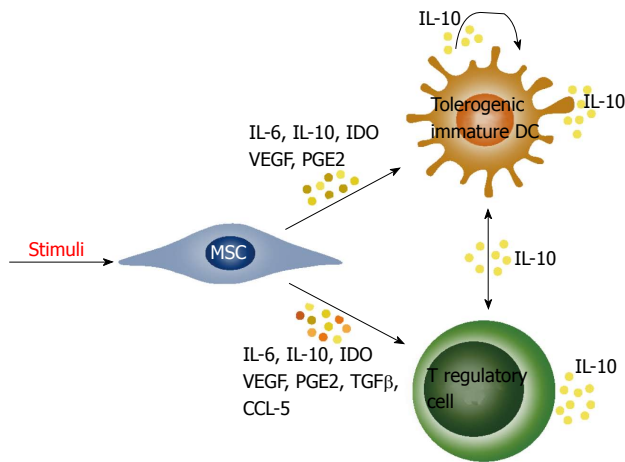


Figure 2 Mesenchymal stem cells provide an immunoregulatory effect by interactions with dendritic cells and T regulatory cells. Under the influence of cytokines secreted by MSCs and autocrine secreted interleukin-10 (IL-10), the dendritic cells acquire an immature tolerogenic phenotype characterized by a low expression of MHC II and B7 molecules, as well as a higher secretion of IL-10. The secretion of IL-10 induces generation of different subtypes of regulatory T cells which further secrete IL-10 and induce tolerogenic phenotype in dendritic cells. Cytokines secreted from MSCs also lead directly to formation of regulatory T cells. VEGF: Vascular endothelial growth factor; PGE2: Prostaglandin E2; MSCs: Mesenchymal stem cells; IDO: Indoleamine-2, 3-dioxygenase; TGFβ: Transforming growth factor β; DCs: Dendritic cells; IL: Interleukin; CCL: CC chemokine ligand.

proliferation and IFN γ secretion^[227,229,231]. Simultaneously, the effect on T cells is accompanied by a prevailing bias towards IL-4 production^[227] and induction of regulatory IL-10 secreting T cells^[183,233]. The influence of MSCs on T cells that represent the effector arm of adaptive immunity is shown to be mediated *via* the antigen presenting cells (APCs). They are subjected to the direct effect of PGE2, resulting in reduced effectiveness of reaching the stage of immature DCs from monocytes showing an affected phenotype as a low number of CD1a cells and decreased expression of co-stimulatory CD80, CD86^[228] and antigen-presenting molecules MHC II^[231]. Furthermore, when co-cultured with MSCs, the production of IL-12 from APCs (especially DCs) is low^[228,231,232], while IL-10 (from DCs and macrophages) is increased^[227,230]. In total, when differentiating in the presence of MSCs, DCs stay immature in a tolerogenic state and unable to elicit a Th1 immune response. On the other hand, MSCs do not affect the differentiation of immature into mature DCs. The latter demonstrates normal expression of CD80, CD86, CD83 receptors, normal capacity for T cell activation and even increased IL-12 secretion^[228]. Retained in an undifferentiated state, DCs when in co-culture with MSCs largely deteriorate/aggravate the cytotoxicity properties of NK cells as well. Investigations show that due to changed chemokine profile and reduced IL-12 secretion from DCs, NK cells do not properly recruit to DCs^[232]. Under these circumstances, NK cells have low activation, diminished IFN γ secretion and cytotoxicity against their targets^[232], including the reactivity against MSCs^[234]. Summarizing the influence of PGE2 on immune cells with regulatory function endows MSCs a central place in

controlling of inflammatory responses. Extremely sensitive to activation signals from the environment, MSCs seem to link the crossroads between innate and adaptive immunity. By suppressing inflammatory mediators, they participate in the activation of feed-back processes, counteracting non-self^[55,183] and autoimmune reactivity^[233,235,236], leading the immune system to a steady homeostatic state.

CONCLUSION

A general conclusion can be drawn that MSCs can realize their immunoregulatory functions even when they are an object of different stimuli. One of the mechanisms to exert these functions is secretion of cytokines which can directly influence the effector immune cells. In addition to that, when secreting cytokines MSCs are involved in complex multi-directional interactions, including predominantly dendritic cells and different subtypes of T regulatory cells (Figure 2). Detailed elucidation of these interactions might be of key importance for the effective application of mesenchymal stem cells in therapy for autoimmune diseases.

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Potential advantages of acute kidney injury management by mesenchymal stem cells

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Abstract

Mesenchymal stem cells are currently considered as a promising tool for therapeutic application in acute kidney injury (AKI) management. AKI is characterized by acute tubular injury with rapid loss of renal function. After AKI, inflammation, oxidative stress and excessive deposition of extracellular matrix are the molecular events that ultimately cause the end-stage renal disease. Despite numerous improvement of supportive therapy, the mortality and morbidity among patients remain high. Therefore, exploring novel therapeutic options to treat AKI is mandatory. Numerous evidence in animal models has demonstrated the capability of mesenchymal stem cells (MSCs) to restore kidney function after induced kidney injury. After infusion, MSCs engraft in the injured tissue and release soluble factors and microvesicles that promote cell survival and tissue repairing. Indeed, the main mechanism of action of MSCs in tissue regeneration is the paracrine/endocrine secre-

tion of bioactive molecules. MSCs can be isolated from several tissues, including bone marrow, adipose tissue, and blood cord; pre-treatment procedures to improve MSCs homing and their paracrine function have been also described. This review will focus on the application of cell therapy in AKI and it will summarize preclinical studies in animal models and clinical trials currently ongoing about the use of mesenchymal stem cells after AKI.

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Key words: Mesenchymal stem cells; Paracrine; Soluble factors; Microvesicle; Acute kidney injury; Regenerative medicine; Preclinical; Clinical trial

Core tip: Mesenchymal stem cells (MSCs) may have an important therapeutic potential in acute kidney injury management. A body of evidence has demonstrated that MSCs act through a paracrine/endocrine secretion of soluble factors and microvesicles. We summarize preclinical studies and ongoing clinical trials that evaluate the role of MSCs in restoring kidney function. We critically explain the current concerns about the use of MSCs and microvesicles that limit their applications in clinical trials. Then, we propose the future directions that could lead to extend MSCs use in humans.

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INTRODUCTION

Acute kidney injury (AKI) is a complex clinical syndrome that affects up to 20% of hospitalized patients. Ischemia/

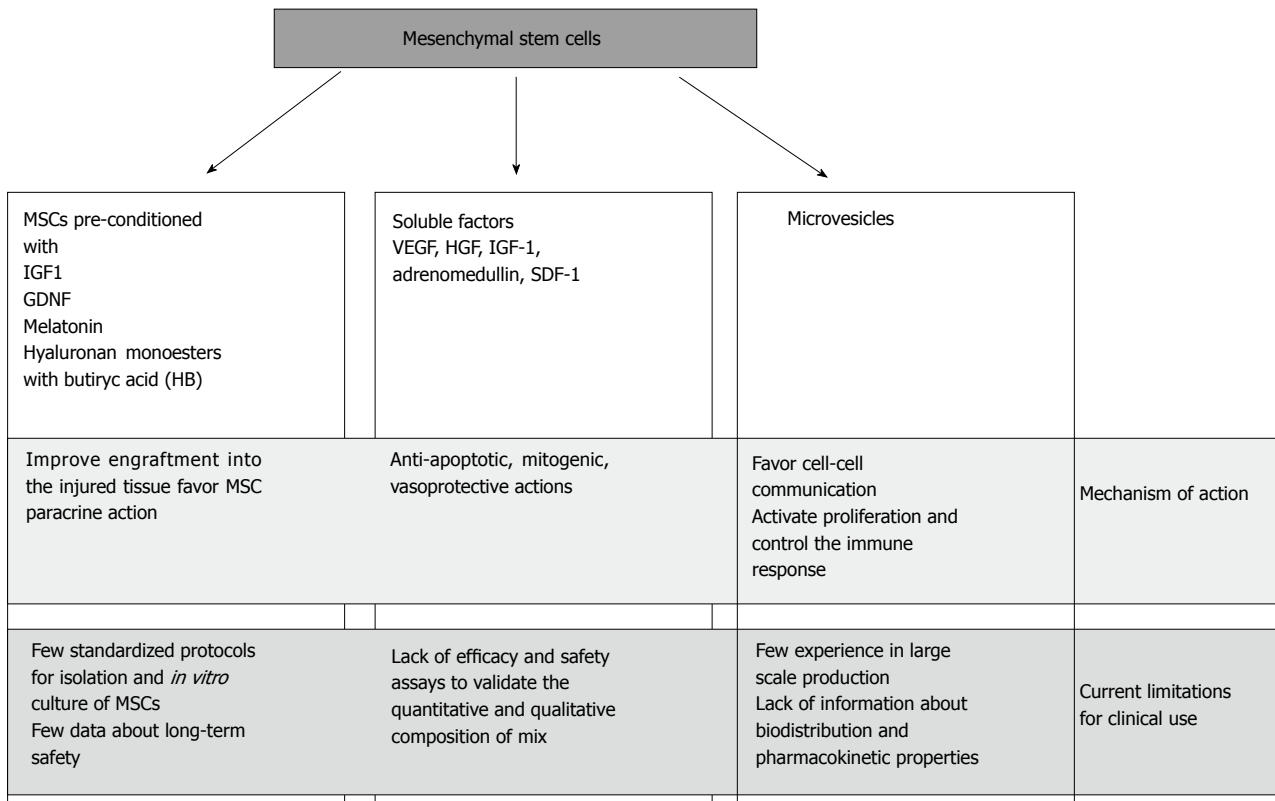


Figure 1 Therapeutic potential of mesenchymal stem cells and their derivatives. MSCs: Mesenchymal stem cells; GDNF: Glial derived-cell line neurotrophic factor; VEGF: Vascular endothelial growth factor.

reperfusion injury (IRI) is a major cause of AKI, and it is characterized by acute tubular injury and rapid renal dysfunction, generally caused by ischemic or toxic insults^[1-3]. The kidney undergoing IRI presents an extensive and complex inflammatory/oxidative stress response, that may result in fibroblast proliferation and excessive deposition of extracellular matrix and has been recognized as a major contributor to end-stage kidney disease^[4]. Although many efforts have been made to deal with this problem, such as new drugs and modern dialysis techniques, innovative interventions beyond supportive therapy are not available yet^[5]; therefore, a potent therapeutic intervention for ischemia AKI is imperative. In recent years, a promising approach to manage renal IRI is the use of mesenchymal stem cells (MSCs). Their use in treating different kind of diseases as immunological, vascular, cardiac and renal diseases has been extensively explored^[6,7]. MSCs can be isolated from various sources, such as bone marrow or adipose tissue, but other organs have their own niches of MSC-like cells, such as the kidney. Besides their broad distribution in the body and an easy isolation, the interest in MSC was originally raised by their capacity to differentiate into other cell types, suggesting that they could be a source of healthy cells to repair/replace injured tissue^[8]. There is evidence from both *in vitro* studies and animal models of AKI that MSCs can promote regenerative responses in the injured kidney, leading to tissue repair and improvement of renal function^[9-11]. These beneficial effects have been initially ascribed to the trans-differen-

tiation of MSCs into organ specific cells. However, at least in the kidney, this is a very rare event and the kidney-protective effects of MSCs have been attributed mainly to paracrine mechanisms^[12]. This review will focus on the application of cell therapy in AKI, and it will summarize the recent preclinical and clinical results about the use of MSCs in renal IRI (Figure 1).

THERAPEUTIC POTENTIAL OF MESENCHYMAL STEM CELLS

Mesenchymal stem cells are undifferentiated adult stem cells derived from mesodermal embryonic layer that can differentiate into a broad range of different mesenchymal tissues, including cartilage, bone, muscle, stroma, fat, tendon, and other connective tissues^[13]. These cells have been originally isolated from bone marrow where they regulate the self-renewal, maturation and recruitment of hematopoietic stem cells to vascular compartment^[14], thanks to their peculiar property to adhere to tissue culture plastic^[15]. MSCs are able to *in vitro* differentiate into cells of mesodermal lineages, such as adipocytes, chondrocytes and osteocytes by the exposure to appropriate conditioning media. A variety of protocols for isolation and expansion are currently used to prepare mesenchymal stem cells for preclinical and clinical use. However, the International Society for Cellular Therapy has identified some potential biomarkers useful to fully characterize MSCs, including the surface antigens CD105, CD73 and

CD90, and the lack of the hematopoietic markers CD34, CD45, CD14 or CD11, CD79 α or CD19, and HLS class II^[16].

Other sources of MSCs are the blood cord and the adipose tissue. Blood cord MSCs have characteristics and immune phenotype similar to BM MSCs, but a differentiating potential limited to osteocytes and chondrocytes while MSCs from adipose tissue have more potent anti-inflammatory and immune-modulatory properties than BM MSCs^[17]. MSCs from adipose tissue can be easily prepared after non-invasive liposuction according to the guidelines of International Federation of Adipose Therapeutics e International Society for Cellular Therapy^[18] that has appositely proposed a document to standardize international parameters to use MSC from adipose tissue in preclinical and clinical use. This attempt to standardize the use of MSC in biomedical research is pivotal and should be extended to other sources of MSCs in order to promptly define functional and qualitative criteria for these cells. Indeed, the heterogeneity of protocols of isolation and expansion has the results that investigators have used MSCs with different properties without frequently being aware of these differences^[19]. The use to record in process data during MSCs preparation and the availability of this information in the supplemental material could be useful to partially overcome the problem and optimize the comparison among different studies.

MECHANISMS OF RENOPROTECTION IN AKI MODELS: THE PARACRINE ACTIVITY OF MSC

It has widely documented that extra-renal MSCs contribute to kidney repair after injury. Interestingly, renoprotection derives from a paracrine/endocrine secretion of bioactive factors and exosomes^[20-22] and not from direct homing the injured tissue by MSCs. The infusion of MSCs in AKI animal models has demonstrated that few cells are able to engraft the damaged renal tissue and are preferentially localized into the peritubular and, less frequently, in the tubular epithelium^[23,24]. Although the cellular scarcity, the regenerative outcomes in terms of functional restoring and animal survival are evident, thus supporting the notion that MSCs act through a trans-differentiation-independent mechanism^[25]. The evidence of paracrine/endocrine secretion of bioactive factors to recover renal function has achieved in mice injected with cisplatin to generate tubular injury and apoptosis. When a conditioned medium from BM-SC culture was injected with intraperitoneal administration in these mice, tubular cell apoptosis diminished, survival increased, and renal injury improved, as well as when MSCs were directly injected^[26]. Interestingly, similar results have been obtained in an *in vitro* model of AKI with a conditioned medium produced by genetically modified MSCs. MSCs were manipulated to over-express Lnc2; the conditioned medium produced from these cells was used to treat that cisplatin

treated HEK 293 kidney cells in which it prevented apoptosis and increased the expression of growth factors, thus ameliorating and repairing injured cells^[27].

MSCs secrete a number of factors, including VEGF, HGF, IGF-1, adrenomedullin, SDF-1, that exert anti-apoptotic, mitogenic, vasoprotective, and angiogenic actions in AKI. In particular, it seems that a pivotal role in kidney regeneration is played by VEGF and IGF1. VEGF knock out mice and IGF1 silencing models show limited renal function restoring and tubular repair after injury^[28,29]. Chemotactic factors, including the SDF1-CRCX4 axis and CD44 interacting with hyaluronic acid, are important during MSC engraftment: BM-MSC isolated from CD44 KO mice lost the ability to migrate in the injured kidney and failed to improve the functional and morphological recovery of acute renal failure induced by glycerol treatment^[30].

Several molecular strategies to improve MSC homing into the injured renal tissue have been exploited in order to maximize the paracrine action of MSC in the site of injury. Pre-treatment with growth factor and cytokines, or genetic modifications seem the most promising techniques. Retroviral transduction of MSCs to overexpress the homing receptors CRCX4 or serine protease kallikrein improves renal function recovery and enhances the protective anti-inflammatory action in ischemic injured kidney^[31,32]. IGF1 preconditioning before infusion increases the expression of IGF1 and CRCX4 in BM-MSCs and improves cellular migration and renal functional restoring after AKI^[11]. The glial derived-cell line neurotrophic factor (GDNF) favors the up-regulation of CD44/HA axis and CRCX4, and the release of IL6, VEGF, SDF1 in cultured human amniotic fluid stem cells. After infusion in AKI animal models, these preconditioned cells show enhanced paracrine activity and improved renoprotection capacity^[33]. Pre-treatment with melatonin ameliorates survival, mitogenic and angiogenic properties of rat BM-MSCs, up-regulating the expression of HGF and bFGF and anti-oxidant enzymes^[34]. The hyaluronan monoesters with butyric acid (HB) show significant properties to induce metanephric differentiation, formation of capillary-like structures, and secretion of angiogenic cytokines *in vitro*. *In vivo* infusion of human mesenchymal stem cells from fetal membranes (FMhMSCs) in AKI rat models after pre-treatment with HB reduces inflammation and accelerates renal function recovery^[35]. In addition to MSCs treatment, other molecules, such as NGAL, should be used to regulate the immune response to inflammation and facilitate renal functioning^[36]. The combined intravenous administration of bone marrow MSC and muscone in rat with gentamycin induced AKI induces the expression of CXCR7 and CRCX4 on cell surface, thus promoting migration and proliferation of MSCs^[37].

All these preclinical murine models offer the proof of concept that the use of MSCs in the management of acute renal failure is rational and feasible. Before implementing clinical studies, it is important to validate the model and standardize some parameters to facilitate the

comparison among different protocols of MSCs application. Firstly, it could be important to determine the better route of MSCs administration. The direct comparison of the administration through the tail vein, carotid artery or renal artery has demonstrated that an injection of 10^5 cells in the renal artery of rat results in a greater improvement of renal function and morphology than those obtained with the other administration routes^[38]. Secondly, the choice of MSC source is another issue that has to be solved. Bone marrow is the most common source of MSC in preclinical studies, but the use of stem cells from other tissues is also reported. KaSzuno *et al*^[39] have compared the regenerative potential of human MSC derived from adipose tissue or bone marrow and cultured *in vitro* in presence of high serum or low serum. In rat AKI model, only MSCs derived from adipose tissue and cultured in low serum condition have ameliorated AKI *via* HGF-mediated paracrine effect^[39]. CD133(+) renal progenitors from the human inner medulla has been compared with bone marrow derived cells in glycerol induced tubular damage model. CD133(+) progenitor cells promoted the recovery of renal function, preventing tubular cell necrosis and stimulating resident cell proliferation and survival, similarly to mesenchymal stem cells^[40]. Therefore, the choice of MSCs or, more generally, stem cells is critical to implement the use of cell-based protocol in regenerative medicine. The feasibility of cell-based protocol is strictly dependent on the procedure to obtain and amplify stem cells. In this contest, the possibility to recover MSCs from adipose tissue after liposuction seems to be favorable because the procedure is inexpensive and non-invasive. Another point to fix is the use of autologous or allogeneic cells. Tögel *et al*^[41] have compared the outcomes in terms of renoprotection after injecting in rat AKI model autologous or allogeneic bone marrow stromal cells. Identical doses of autologous MSCs were more effective than allogeneic, but both autologous and allogeneic cells were able to reduce late renal fibrosis and loss of renal function in surviving animals^[41]. However, some factors, such as age or systemic disease, may influence and lessen the regenerative potential of autologous MSCs, therefore it is important to assess patient's suitability for autologous transplantation. Bone marrow MSCs from remnant rat with chronic renal disease showed no benefit in healing glomerular lesions and exhibited cellular modifications and other deficit *in vivo*, likely due to cellular senescence^[42].

Therefore, even if some preliminary evidence is available in terms of safety and protocol validation, further studies are required to meet the quality and safety criteria for the use of MSCs in humans.

POTENTIAL APPLICATION OF MICROVESICLES TO AKI

Recently, several groups have demonstrated the potent therapeutic activity of microvesicles (MVs), termed as exosomes and shedding vesicles^[43]. MVs are released

from stem cells and are particularly enriched in certain molecules, including adhesion molecules, membrane trafficking molecules, cytoskeleton molecules, heat-shock proteins, cytoplasmic enzymes, signal transduction proteins and, importantly, functional mRNAs and microRNAs. Their role *in vivo* may be related to cell-to-cell communication and to proteins and RNAs exchange among cells both locally and at distance^[44]. The discovery of mRNAs and miRNAs in exosomes foreshadows an important new direction for their application as delivery vehicles for therapeutics. *In vitro* and *in vivo* experiments have demonstrated that MVs released by BM MSC activate proliferation in tubular epithelial cells and restore renal function after glycerol-induced injury. This regenerative activity is related to the presence of specific mRNAs that encode proteins responsible for controlling proliferation, transcription and immune response^[45]. MVs intravenously injected in rat immediately after inducing ischemic-reperfusion injury reduced apoptosis and increased cellular proliferation of tubular cells. Inactivation of MV cargos with RNAase determined the lack of protective effects^[46]. Multiple injections of MVs in cisplatin-induced lethal model of AKI SCID mice reduced mortality and produced a normal histological phenotype with normal renal function in surviving animals^[47]. The analysis of bio-distribution and renal localization of MVs has demonstrated that MVs accumulated specifically in the kidneys of the mice with AKI compared with the healthy controls. Two different protocols have been used to dye MVs: the near infra-red dye was added to cell culture medium or MVs were stained after purification. Interestingly, the signal generated by the labeled MVs produced by cells was more specific for the injured tissue than those from directly labeled MVs^[48]. A therapeutic effect in renal ischemia-reperfusion injury has been shown also by MVs derived from human Wharton-Jelly MSCs. Indeed, a single administration of these MVs in rat immediately after inducing AKI reduced inflammation, and, as long-term outcome, improved renal function and decreased fibrosis^[49]. The mechanism of action of Wharton-Jelly MSC derived MVs has not been completely elucidated, but it has been observed that these MVs mitigated the oxidative stress and declined NOX2 expression and reactive oxygen species generation^[50]. A further source of MVs with renoprotective activity is the kidney mesenchymal stem cells (KMSC). These microparticles were isolated from the supernatants of KMSC cultured in anoxic conditions in serum-deprived media for 24 h; when injected in mice with acute renal ischemia, they significantly improved renal function, favoring endothelial cells proliferation and ameliorating peritubular microvascular rarefaction^[51].

USE OF MSC IN CLINICAL TRIALS AGAINST AKI

The interesting results obtained in preclinical studies prompt to the translation of MSC-based treatments into humans, although the clinical studies are still limited (Ta-

Table 1 Clinical studies on the application of mesenchymal stem cells in acute kidney injury

Study	Phase	Aim	Enrolled patients	Status
NCT00733876	Phase 1	To determine the safety of the administration of allogeneic MSCs at defined doses in patients with high risk of developing AKI after undergoing on-pump cardiac surgery	15	Completed ^[52]
NCT01275612	Phase 1	To test the feasibility and safety of systemic infusion of donor <i>ex-vivo</i> expanded MSCs to repair kidney and improve function in patients with solid organ cancers who develop acute renal failure after chemotherapy with cisplatin	3 (estimated enrollment 9 patients)	Ongoing and recruiting patients
NCT01602328	Phase 2	To evaluate kidney recovery after a single injection of allogeneic bone marrow derived MSCs in patients who experience kidney injury within 48 h of their cardiac surgery	156	Terminated

MSCs: Mesenchymal stem cells; AKI: Acute kidney injury.

ble 1). A phase I clinical trial (NCT00733876) has been designed to determine if the administration of allogeneic MSCs at defined doses is safe in patients who are at high risk of developing AKI after undergoing on-pump cardiac surgery. Preliminary data shown that kidney function is preserved up to 16 mo and that none of the patients required dialysis. Any therapy-related adverse events were noted in these patients^[52]. The explorative study (phase I) on three patients who have developed acute renal failure after cisplatin treatment for solid cancer has demonstrated that intravenous infusion of autologous *ex-vivo* expanded MSCs improves renal function and the procedure is safe (NCT 01275612). Another phase II trial (NCT 01602328) to assess human MSC safety and efficacy in patients that develop AKI after cardiac surgery is ongoing with 156 patients enrolled. The results from these clinical studies will clarify the potential of mesenchymal stem cells in AKI management. An overall view of the preliminary results currently available confirm the safety of the treatment, but other data are required to assess clinical benefit and long term safety. Up to date, none clinical study on microvesicles and AKI is ongoing.

CONCLUSION

The use of MSC for AKI therapy is encouraging and is generally considered as safe. The experience from the increasing use of mesenchymal stem cells before or after renal transplant will furnish important suggestions to implement other clinical protocols with MSC in acute kidney injury. However, some concerns about the use of living cells should keep in account. In progressive rat model of glomerulonephritis, intrarenal injection of MSCs initially ameliorated acute renal failure; however, long-term examination has demonstrates that approximately 20% of the glomeruli of MSC-treated rats contained single or clusters of large adipocytes with pronounced surrounding fibrosis, thus indicating an abnormal and detrimental adipogenic differentiation of MSC^[53].

MVs should be evaluated as a possible alternative of living MSCs. The delivery and internalization of MVs are receptor- mediated and targeted within specific cells and MVs may contain biological macromolecules that can be protected from degradation enzymes of plasma and

tissue. Before moving to clinical trials, some important issues should be addressed, especially in terms of safety. Large scale production of MVs should be validated and optimized before clinical use; bio-distribution and pharmacokinetic properties should be determined and also long-term safety in animal models has to be tested before implementation in humans. Finally, the use of soluble factors that are released from MSCs for renoprotection may be pursued. Efficacy and safety assays are required to validate the quantitative and qualitative composition of mix of soluble factors to achieve functional restoring after acute renal injury.

Based on promising preliminary results in animal models and in ongoing preclinical studies, mesenchymal stem cells and their derivatives represent a potential therapeutic intervention to treat AKI.

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Impact of parathyroid hormone on bone marrow-derived stem cell mobilization and migration

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Abstract

Parathyroid hormone (PTH) is well-known as the principal regulator of calcium homeostasis in the human body and controls bone metabolism *via* actions on the survival and activation of osteoblasts. The intermittent administration of PTH has been shown to stimulate bone production in mice and men and therefore PTH administration has been recently approved for the treatment of osteoporosis. Besides to its physiological role in bone remodelling PTH has been demonstrated to influence and expand the bone marrow stem cell niche where hematopoietic stem cells, capable of both self-renewal and differentiation, reside. Moreover, intermittent PTH treatment is capable to induce mobilization of progenitor cells from the bone marrow into the bloodstream. This novel function of PTH on modulating the activity of the stem cell niche in the bone marrow as well as on mobilization and regeneration of bone marrow-derived stem cells offers new therapeutic options in bone marrow and stem cell transplantation as well as in the field of ischemic disorders.

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Key words: Parathyroid hormone; Stem cells; Bone

marrow; Mobilization; Migration

Core tip: Parathyroid hormone (PTH) is the principal regulator of calcium homeostasis in the human body and controls bone metabolism. Besides to its physiological role in bone remodelling PTH has been demonstrated to influence and expand the bone marrow stem cell niche as well as to induce mobilization of progenitor cells from the bone marrow into the bloodstream. This novel function of PTH on modulating the activity of the stem cell niche in the bone marrow as well as on mobilization and regeneration of bone marrow-derived progenitor cells offers new therapeutic options in bone marrow and stem cell transplantation as well as in the field of ischemic disorders.

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INTRODUCTION

Parathyroid hormone (PTH) is a peptide hormone secreted from the parathyroid glands that mainly acts on bone and kidney cells^[1]. PTH is one of the two major hormones modulating calcium and phosphate homeostasis through its action to stimulate renal tubular calcium reabsorption and bone resorption^[2]. Human PTH is an 84-amino acid peptide, but the first two amino acids in the N-terminal region of the hormone are mandatory for activation of the PTH 1 receptor (PTH1r), a membrane surface receptor expressed in multiple tissues including bone and kidney^[3]. It has been appreciated that recombinant PTH 1-34 retains all of the biologic activity of the intact peptide (1-84)^[4]. Patients with primary or sec-

ondary hyperparathyroidism and subsequently chronic exposure to high serum PTH concentrations revealed increased bone resorption^[5]. However, in contrast to this observations after chronic exposure to high serum PTH concentrations, the intermittent administration of recombinant PTH in mice and men has been demonstrated to stimulate bone production more than resorption^[6]. These observations finally guided the approval of intermittent recombinant PTH (1-34) for the treatment of osteoporosis in postmenopausal woman and subsequently in men^[7-9]. Besides to its physiological role in bone remodeling, PTH has been shown to modulate the haematopoietic stem cell (HSC) niche in the bone marrow (BM)^[10]. This review will focus on the molecular interplay between PTH and the HSC niche and will also discuss the ability of PTH to mobilize bone marrow-derived stem cells (BMCs) to the peripheral blood, which opens new therapeutic options for PTH in the field of bone marrow and stem cell transplantation as well as a potential role of PTH in the treatment of ischemic disorders.

PTH AND THE BM STEM CELL NICHE

BM is a complex organ, consisting of many different haematopoietic and non-haematopoietic cell types, that is surrounded by a shell of vascularized and innervated bone^[11]. In the last years, there have been a lot of research and discussions about the existence and localizations of “niches”, specific local tissue microenvironments that maintain and regulate stem cells within the bone marrow^[12]. The niche hypothesis has been proposed for the first time by Schofield *et al.*^[13] in 1978 and since then tremendous progress has been made in elucidating the location and cellular components of the HSC niche. It is now appreciated that the HSC niche is perivascular, created partly by mesenchymal stromal cells and endothelial cells and often, but not always, located near trabecular bone^[11,14-19]. Calvi *et al.*^[10] were first able to demonstrate that osteoblastic cells regulate the haematopoietic stem cell niche and that PTH is a pivotal regulator of the HSC microenvironment. They used transgenic mice carrying constitutively activated PTH/PTHrP receptors (PPRs) under control of the osteoblast-specific $\alpha 1(I)$ collagen promoter and were able to detect a 2-fold increased number of Lin- Sca-1+ cKit+ (LSK) cells. PPR-stimulated osteoblastic cells produced high levels of the Notch ligand jagged 1 and supported an increase in the number of haematopoietic stem cells with evidence of Notch1 activation *in vivo*. Likewise, blocking Notch signaling with γ -secretase inhibitors inhibited the enhanced ability of these PPR activated osteoblasts to support long-term hematopoietic cultures. In a next step, they assessed whether PPR activation with PTH could have a meaningful physiological effect *in vivo*. They administered PTH to animals undergoing myeloablative bone marrow transplantation using limiting numbers of donor cells to mimic a setting of therapeutic need. Survival at 28 d in control mice that received mock injections after transplant was 27%. In sharp

contrast, animals receiving pulse dosing of PTH had improved outcomes with 100% survival. The bone marrow histology of the two groups was also substantially different, with an increase in cellularity and a decrease in fat cells in the PTH-treated group^[10]. That Jagged1 may play a critical role in mediating the PTH-dependent expansion of HSC, as well as the anabolic effect of PTH in bone was confirmed by Weber *et al.*^[20]. They showed the ability of PTH to augment Jag-1 expression on osteoblasts in an AC/PKA-dependent manner following 5 consecutive days of PTH administration. Jag-1 protein was increased on specific populations of osteoblasts including those at the endosteum and spindle-shaped cells in the bone marrow cavity^[20]. PTH stimulation also augments the expression level of N-cadherin on osteoblasts^[21,22]. N-cadherin-mediated adhesion may link to the canonical Wnt and Notch1 pathway through b-catenin signaling^[23]. Wnt and Notch signaling pathways are known to be important in hematopoietic stem cell renewal^[11,24-26].

As another important regulator of PTH-driven HSC expansion, a number of cytokines have been identified^[11]. Several studies demonstrated increased expression of cytokines like IL-6, IL-11, G-CSF and stem cell factor (SCF)^[27-31]. In this context, PTH signalling to osteoblasts resulted in an increase in the number of SCF⁺ cells^[30,32]. Likewise, exposure to PTH resulted in enhanced expression of IL-6 and IL-11 in osteoblasts^[33]. Jung *et al.*^[34] were able to demonstrate that expression of the chemokine stromal derived factor-1 (SDF-1, also termed CXCL12) by osteoblasts was increased following PTH administration. SDF-1 and its major receptor CXCR4 are pivotal in mediating both retention and mobilization of HSCs^[35] and will be discussed at a later stage in this review. Brunner *et al.*^[36] compared a treatment regimen with G-CSF and PTH in a mouse model. They found that in contrast to G-CSF, PTH treatment resulted in an enhanced cell proliferation with a constant level of lin-/Sca-1+/c-kit+ cells and CD45+/CD34+ subpopulations in bone marrow^[36]. Altogether the data on PTH and the bone marrow suggest an important role of PTH on the niche which allows the use PTH as a therapeutic tool to increase the number of BMSC. In the following chapter we will focus on the potential role of PTH to mobilize cells from the bone marrow to the bloodstream.

PTH AND STEM CELL MOBILIZATION

Under normal and pathological conditions there is continuous egress of hematopoietic stem and progenitor cells out of the bone marrow to the circulation, termed mobilization^[37]. Stem cell mobilization can be achieved experimentally in animal models or clinically by a great variety of agents, such as cytokines (*e.g.*, G-CSF, SCF, Erythropoietin)^[36,38-43] and small molecules (*e.g.*, AMD3100)^[44].

Following the intriguing data of Calvi *et al.*^[10] showing that PTH is a pivotal regulator of the HSC microenvironment and is able to increase the number of HSC in

the BM, several preclinical studies investigated the effect of PTH administration on stem cell mobilization in mice. Adams *et al.*^[45] used three mouse models that are relevant to clinical uses of HSCs to test the hypothesis that targeting the niche might improve stem cell-based therapies. They treated mice with PTH for 5 wk following a 5-d regimen of G-CSF to mobilize BMCs from the bone marrow to the peripheral blood. They demonstrated that PTH administration increased the number of HSCs mobilized into the peripheral blood for stem cell harvests, protected stem cells from repeated exposure to cytotoxic chemotherapy and expanded stem cells in transplant recipients^[45]. These results were corroborated by a study of our group where we explored the potency of PTH compared to granulocyte colony-stimulating factor (G-CSF) for mobilization of stem cells and its regenerative capacity on bone marrow. Healthy mice were either treated with PTH, G-CSF, or saline. HSCs characterized by lin-/Sca-1+/c-kit+, as well as subpopulations (CD31+, c-kit+, Sca-1+, CXCR4+) of CD45+/CD34+ and CD45+/CD34- cells were measured by flow cytometry. Immunohistology as well as fluorescein-activated cell sorting analyses were utilized to determine the composition and cell-cycle status of bone marrow cells. Serum levels of distinct cytokines [G-CSF, vascular endothelial growth factor (VEGF)] were determined by enzyme-linked immunosorbent assay. Stimulation with PTH showed a significant increase of all characterized subpopulations of bone marrow-derived progenitor cells (BMCs) in peripheral blood (1.5- to 9.8-fold) similar to G-CSF. In contrast to G-CSF, PTH treatment resulted in an enhanced cell proliferation with a constant level of lin-/Sca-1+/c-kit+ cells and CD45+/CD34+ subpopulations in bone marrow. A combination of PTH and G-CSF showed only slight additional effects compared to PTH or G-CSF alone^[36]. Interestingly, treatment with PTH resulted in significantly elevated concentrations of G-CSF in serum suggesting an indirect mobilizing effect of PTH *via* stimulation of osteoblasts producing G-CSF. To verify this hypothesis, PTH-stimulated mice were pre-treated with a G-CSF antibody and, thereby, the mobilizing effect could be significantly inhibited^[36]. In a more clinically relevant model Brunner *et al.*^[5] investigated prospectively the effect of primary hyperparathyroidism (PHPT), a condition with high PTH serum levels, on mobilization of BMCs in humans. In 22 patients with PHPT and 10 controls defined subpopulations of circulating BMCs were analyzed by flow cytometry. They found a significant increase of circulating BMCs and an upregulation of SDF-1 and VEGF serum levels in patients with PHPT. The number of these circulating cells positively correlated with PTH serum levels. Interestingly, the number of circulating BMCs returned to control levels measured after surgery^[5].

Because of the therapeutic potential of PTH to activate and increase the number of HSCs in preclinical models, a phase I trial in humans has been conducted. A group of 20 human patients were included who had pre-

viously failed to produce a sufficient number of CD34⁺ HSCs in their peripheral blood following mobilization. Subjects were treated with PTH in escalating doses of 40 µg, 60 µg, 80 µg, and 100 µg for 14 d. On days 10-14 of treatment, subjects received filgrastim (G-CSF) 10 µg/kg. PTH administration was tolerated well and there was no dose-limiting toxicity. Of those patients who previously had a single mobilization failure, 47% met therapeutic mobilization criteria, of those who had previously failed two attempts at mobilization, the post PTH success rate was similar (40%)^[46].

PTH AND STEM CELL HOMING *VIA* SDF1/CXCR4

In light of the promising results showing increased mobilization of BMCs after treatment with PTH, several studies also focused on the migration of different BMCs after PTH pulsing. The main axis of stem cell migration and homing is the interaction between SDF-1a and the homing receptor CXCR-4, which is expressed on many circulating progenitor cells^[47,48]. It has been shown that CXCR4- and SDF-1-deficient mice have a severe migration defect of HSCs from the embryonic liver to the bone marrow by the end of the second trimester. At this period of development, SDF-1 is upregulated in bone marrow and chemoattracts HSCs. Later in life the SDF-1-CXCR4 axis plays a crucial role in the retention and homing of HSCs in the bone marrow stem cell niche^[35]. SDF-1 is expressed by different cell types, including stromal and endothelial cells, bone marrow, heart, skeletal muscle, liver and brain^[49]. Active SDF-1 binds to its receptor CXCR-4 and is cleaved at its position 2 by CD26/dipeptidylpeptidase IV (DPP-IV), a membrane-bound extracellular peptidase^[50-55]. The truncated form of SDF-1 not only loses its chemotactic properties, but also blocks chemotaxis of full length SDF-1^[50]. DPP-IV is expressed on many hematopoietic cell populations and is present in a catalytically active soluble form in the plasma^[56]. In a chimeric mouse model to track BMCs by ubiquitously expression of EGFP under control of the ubiquitin C promoter, Brunner *et al.*^[37] demonstrated reduced migration of CXCR-4+ BMCs associated with decreased expression levels of the corresponding growth factor SDF-1 in ischemic myocardium after treatment with G-CSF. This could be explained by N-terminal cleavage of CXCR4 on mobilized haematopoietic progenitor cells resulting in loss of chemotaxis in response to SDF-1^[57]. In contrast, PTH treated animals revealed an enhanced homing of BMCs associated with an increased protein level of SDF-1 in the ischemic heart^[58,59]. Jung *et al.*^[34] showed recently enhanced levels of SDF-1 in the bone marrow after PTH stimulation. Therefore, our group used an enzymatic activity assay to investigate whether the elevated levels of SDF-1 protein in the ischemic heart after PTH stimulation may be due to changes of DPP-IV activity. Indeed, we were able to demonstrate that PTH inhibited the activity of

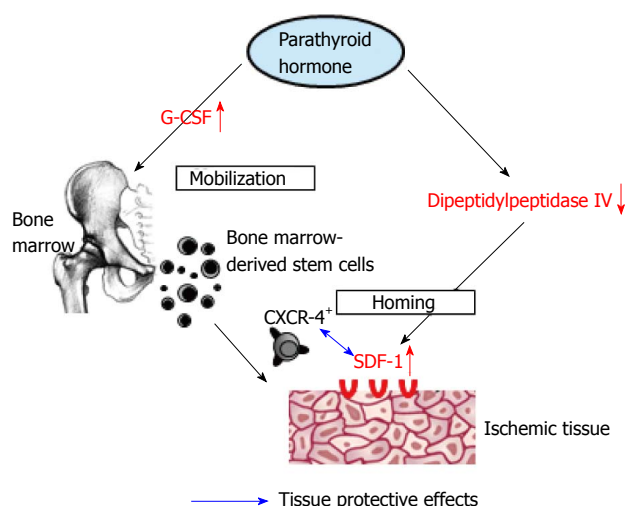


Figure 1 Impact of parathyroid hormone on mobilization and homing of bone marrow-derived stem cells. Left axis: PTH administration results in mobilization of BMCs from bone marrow into peripheral blood via endogenous release of G-CSF. Right axis: PTH results in down-regulation of DPP-IV, which inhibits inactivation of SDF-1 and therefore promotes homing of CXCR4+ BMCs. PTH: Parathyroid hormone; BMCs: Bone marrow-derived stem cells; G-CSF: Granulocyte colony-stimulating factor; SDF-1: Stromal derived factor-1.

DPP-IV *in vitro* and *in vivo*^[58]. In order to exploit whether the observed enhanced stem cell homing after PTH treatment was dependent on an intact SDF-1/CXCR4 axis, the CXCR4 antagonist AMD3100 was injected along with PTH. In fact, the number of CD34+/CD45+ BMCs was significantly decreased in mice treated with PTH and AMD3100 compared to animals treated solely with PTH^[58]. A similar pharmacological concept has been done recently by Zaruba *et al.*^[60]. They used a dual non-invasive therapy based on mobilization of stem cells with G-CSF and pharmacological inhibition of the protease DPP-IV/CD26 and observed enhanced mobilization and migration of different BMC fractions to the ischemic heart^[60,61]. In 2006, a preclinical study with transgenic mice carrying a G-CSF deficiency was done to address the question whether PTH-induced homing of BMCs to the ischemic myocardium is G-CSF-dependent. Corroborating previous studies^[58,59,62], PTH treatment resulted in a significant increase in BMCs in peripheral blood in G-CSF +/+ but not in G-CSF knockout mice. However, a significant increase in SDF-1 levels as well as enhanced migration of BMCs into the ischemic myocardium was observed after PTH treatment in both G-CSF+/+ and G-CSF^{-/-} mice. These data suggest that homing of BMCs is independent of endogenous G-CSF^[63].

In summary, data on preclinical and clinical studies reveal that PTH is a promising substance to enhance migration and homing of BMCs to ischemic tissue due to modulation of the pivotal SDF-1/CXCR4 axis.

PTH FOR THE TREATMENT OF ISCHEMIC DISORDERS

There is a long-lasting interest in the cardiovascular ef-

fects of PTH^[64]. It has been shown that cardiovascular cells, cardiomyocytes and smooth muscle cells are target cells for PTH. PTH is known to induce arterial vasodilation, which is based on the activation of PTH/PTHrP receptor type I. Upon receptor activation, PTH causes an increase of cAMP production leading to a decreased calcium influx resulting in vasodilation^[65,66].

After Calvi *et al.*^[10] established that PTH could alter the HSC niche resulting in HSC expansion and the fact that PTH treatment improved dramatically the survival of mice receiving bone marrow transplants, there was an emerging interest on a potential cardioprotective role of PTH. First, Zaruba *et al.*^[62] exploited the impact of PTH on post-MI survival and functional parameters in a murine model of myocardial infarction. They injected the biological active fragment of PTH [PTH1-34] for up to 14 consecutive days. PTH treatment after MI exerted beneficial effects on survival and myocardial function 6 and 30 d after MI which was associated with an altered cardiac remodelling reflected by smaller infarct sizes. Furthermore, PTH treated animals revealed an augmented mobilization and homing of angiogenic CD45+/CD34+ BMCs associated with an improved neovascularization^[62,67]. In a more recent study, the effect of G-CSF, PTH, and the combination of both was investigated using the innovative pinhole single photon emission computed tomography (SPECT) technique, which allows non-invasive, repetitive, quantitative, and especially intra-individual evaluations of infarct size^[68]. SPECT analyses revealed that PTH treatment resulted in a significant reduction of perfusion defects from day 6 to day 30 in contrast to G-CSF alone. A combination of both cytokines had no additional effects on myocardial perfusion^[59]. To further elucidate the cardioprotective mechanism of PTH, our group focused on the pivotal SDF-1/CXCR4 axis. PTH treatment again significantly improved myocardial function after MI associated with enhanced homing of CXCR4+ BMCs. Homing of BMCs occurred along a SDF-1 protein gradient. Low levels of SDF-1 in the peripheral blood and high SDF-1 levels in the ischemic heart guided CXCR4+ BMCs to the ischemic myocardium. Interestingly, stem cell homing and functional recovery were both reversed by blocking the SDF-1/CXCR4 axis using the CXCR4 antagonist AMD3100^[58]. PTH injections in transgenic G-CSF deficient mice showed that the cardioprotective effects of PTH are independent of endogenous G-CSF release^[63].

That PTH treatment not only exerts beneficial effects in ischemic cardiovascular disorders shows a recent work where PTH therapy was tested after ischemic stroke in mice. PTH treatment significantly increased the expression of cytokines including VEGF, SDF-1, BDNF and Tie-1 in the brain peri-infarct region. Moreover, PTH treatment increased angiogenesis in ischemic brain, promoted neuroblast migration from the subventricular zone and increased the number of newly formed neurons in the peri-infarct cortex. Furthermore, PTH-treated mice revealed better sensorimotor functional recovery com-

pared to stroke controls^[69].

CONCLUSION

In summary, experimental and clinical data suggest a novel function of PTH on modulating the activity of the bone marrow stem cell niche as well as on mobilization and homing of BMCs. PTH is a natural DPP-IV inhibitor and is able to increase SDF-1 protein level in ischemic tissue, which enhances recruitment of regenerative BMCs associated with improved functional recovery. Based on the fact that PTH has already been clinically approved in patients with osteoporosis^[8], the data offer new therapeutic options for PTH in bone marrow and stem cells transplantation as well as in the field of ischemic disorders (Figure 1).

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Mesenchymal stem cells: Emerging mechanisms of immunomodulation and therapy

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Abstract

Mesenchymal stem cells (MSCs) are a pleiotropic population of cells that are self-renewing and capable of differentiating into canonical cells of the mesenchyme, including adipocytes, chondrocytes, and osteocytes. They employ multi-faceted approaches to maintain bone marrow niche homeostasis and promote wound healing during injury. Biomedical research has long sought to exploit their pleiotropic properties as a basis for cell therapy for a variety of diseases and to facilitate hematopoietic stem cell establishment and stromal reconstruction in bone marrow transplantation. Early results demonstrated their usage as safe, and there was little host response to these cells. The discovery of their immunosuppressive functions ushered in a new interest in MSCs as a promising therapeutic tool to suppress inflammation and down-regulate pathogenic immune responses in graft-versus-host and autoimmune diseases such as multiple sclerosis, autoimmune diabetes, and rheumatoid arthritis. MSCs produce a large number of soluble and membrane-bound factors, some of which inhibit immune responses. However, the full range of MSC-mediated immune-modulation remains incompletely understood, as emerging reports also reveal that MSCs can adopt an immunogenic phenotype, stimulate immune cells, and yield seemingly contradictory

results in experimental animal models of inflammatory disease. The present review describes the large body of literature that has been accumulated on the fascinating biology of MSCs and their complex effects on immune responses.

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Key words: Mesenchymal stem cell; Immunosuppression; Immunogenic; Autoimmunity; Cell-based therapy

Core tip: Mesenchymal stem cells (MSCs) comprise a mixture of different stromal cell types that display remarkable pleiotropic properties, including those of anti-apoptosis, angiogenesis, growth factor production, anti-fibrosis, and chemo-attraction. It is because of these diverse biological properties that these cells have been intensively studied in the hopes of their utilization as a platform of cellular therapy in disease settings. Early experimental and preclinical studies focused on their stem cell renewal, differentiation, and regenerative properties for potential use in degenerative diseases of mesenchymal origin. Afterwards, MSCs were found to increase the success of bone marrow transplantation, reduce rejection of engrafted tissues, and display remarkable anti-inflammatory properties. Currently, much work centers on the immune-modulatory facets of MSCs, especially in reducing inflammation and suppressing immune cell function in preclinical injury and autoimmune disease settings. However, emerging reports suggest a multifunctional quality to MSC immune-modulation. This review dissects MSC manipulation of immune responses, which result in either immunosuppression or immuno-stimulation.

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INTRODUCTION

MSCs were originally discovered in the 1950s as the longest surviving cells of human and mouse bone marrow monolayer cell cultures^[1,2]. Friedenstein *et al.*^[3] later noted that these fibroblastic cells were very rare in the bone marrow^[3]. Over time in culture, these sparse colony-forming units divided prolifically and gave rise to expanded populations of fibroblastic clones. These spindle-shaped, fibroblastic cells were plastic adherent and were named MSCs as they could be induced *in vitro* and *in vivo* to differentiate into adipocytes, chondrocytes, connective stromal cells, and osteocytes-cells which all comprise the mesenchyme (Figure 1). MSC differentiation into parenchymal cells of the mesenchyme has become one of the principal criteria of establishing their identity. Additional, though controversial, reports indicate that MSCs may also be induced to transdifferentiate into cells of the endoderm (lung cells, muscle cells, and gut epithelial cells) and the ectoderm (epithelia and neurons)^[4,5].

The pleiotropic nature of MSCs has presented a challenge in their identification. Their functional characteristics of self-renewal and ability to differentiate along with some widely accepted markers together form a profile to help identify them. There is consensus that MSCs, though heterogeneous, share some common features: they are uniformly negative for the expression of key hematopoietic cell markers, including CD34, CD45, CD11b, CD11c, CD14, CD19, CD79 α , CD86, and MHC class II molecules. They express CD90, CD105, CD44, CD73, CD9, and very low levels of CD80. The International Society for Cellular Therapy has designated this expression pattern as the minimal criteria for human MSC discretion, but marker expression panels for MSCs continue to be updated over time^[6,7].

Though MSCs were first isolated from the bone marrow, they have since been harvested from the stroma of multiple organs and tissues, including adipose, tonsils, umbilical cord, skin, and dental pulp^[8-13]. MSCs derived from the marrow continue to be the most frequently studied. The cellular and tissue origins of MSCs have been elusive, but in one landmark study, Crisan and colleagues suggested a pericytic origin for MSCs. Pericytes are perivascular cells that inhabit multiple organ systems^[14]. This group identified pericytes on the basis of CD146, NG2, and PDGF-R β expression from human skeletal muscle, pancreas, adipose tissue, and placenta. They found that these cells expressed markers typical of MSCs and could be differentiated in culture to become myocytes, osteocytes, chondrocytes, and adipocytes. Though the study did not directly track the possible *in vivo* transition of pericytes to MSCs, they identified pericytes as potential progenitor cells to non-bone marrow-derived MSCs.

THE PHYSIOLOGY OF MSCS

MSCs strategically form niches in perivascular spaces in

almost every region of the body. It is thought that such localization allows them to detect local and distant tissue damage, as in wound infliction, and respond by migration to these sites and promoting tissue repair and healing (Figure 2)^[15]. While myriad studies show that exogenously administered MSCs migrate to healthy organs or to injured sites for inflammation suppression and wound healing, there has been sparse data to actually demonstrate *in vivo* mobilization of endogenous MSCs to sites of injury or participation in the wound healing process^[15,16], due in part to lack of unique markers expressed by MSCs.

One of the most insightful reports to address this issue utilizes a natural transplantation model of fetomaternal microchimerism, in which chimeric MSCs take up residence in maternal bone marrow in every pregnancy^[17,18]. Importantly, this study reported that collagen-I-promoter-driven, GFP⁺ MSCs derived from transgenic fetuses homed to wounds inflicted on mothers in as early as 24 h post-infliction^[18]. These cells were still detected 7 d post-infliction, exhibited a fibroblastic appearance, and were marked by vimentin expression, which is indicative of extracellular matrix synthesis and tissue repair. These data implicate endogenous MSCs as capable of travel from the bone marrow to wound sites for healing purposes.

Beyond their role in tissue repair and wound healing, MSCs of the perivascular niche in the bone marrow construct and maintain the hematopoietic stem cell (HSC) microenvironment (Figure 2). MSCs have been demonstrated to migrate and situate in the bone marrow compartment in NOD-SCID mice and differentiate into pericytes, myofibroblasts, endothelia, stromal cells, osteocytes, and osteoblasts^[19]. In bone marrow sinusoids, CD146⁺ MSCs are thought to create the structural framework of the hematopoietic microenvironment, as they are capable of generating this environment at heterotopic sites, along with the establishment of subendothelial cells, upon transfer to miniature bone organs^[20]. These subendothelial cells are important producers of angiopoietin-1, which is known to contribute to HSC sustenance. MSCs in the vicinity that express Nestin are spatially associated with HSCs and may be the primary cells controlling their homeostasis^[21]. Nestin⁺ MSCs produce high levels of HSC-maintenance factors, including CXCL-12, c-kit ligand, angiopoietin-1, IL-7, vascular cell adhesion molecule-1 (VCAM-1), and osteopontin. When HSC mobilization out of marrow is required, these MSCs down-regulate HSC maintenance genes. In response to parathyroid hormone treatment, which promotes osteoblast differentiation and HSC expansion, Nestin⁺ MSCs proliferate and become primed towards osteoblastogenesis. When purified HSCs are transferred to lethally irradiated mice, they only efficiently home to bone marrow that is populated with Nestin⁺ MSCs. In addition, osteoblasts derived from Nestin⁺ MSCs form the endosteal niche that lines the surface of the trabecular bone^[20,22]. This niche, in concert with that formed by perivascular MSCs, regulates HSC survival, proliferation, and quiescent maintenance in

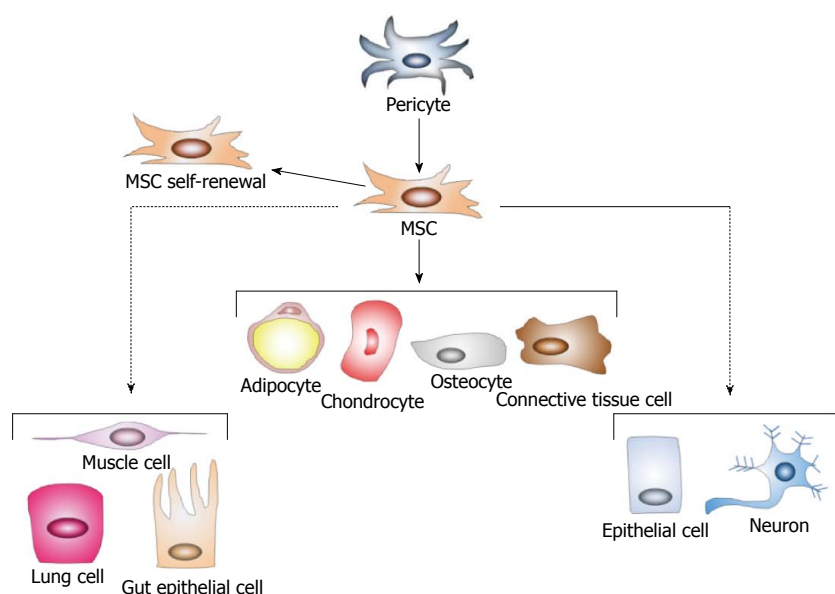


Figure 1 Basic properties of mesenchymal stem cells. Mesenchymal stem cells (MSCs) are a heterogeneous population of stromal cells thought to be derived from pericytes. These cells are defined by self-renewal and the ability to differentiate into the mesodermal cells (solid lines): adipocytes, chondrocytes, osteocytes, and connective tissue cells. Though controversial (dotted lines), they may also transdifferentiate into cells of the endoderm (lung, muscle, and gut epithelial cells) and of the ectoderm (neurons and epithelial cells). Adapted from ref [22].

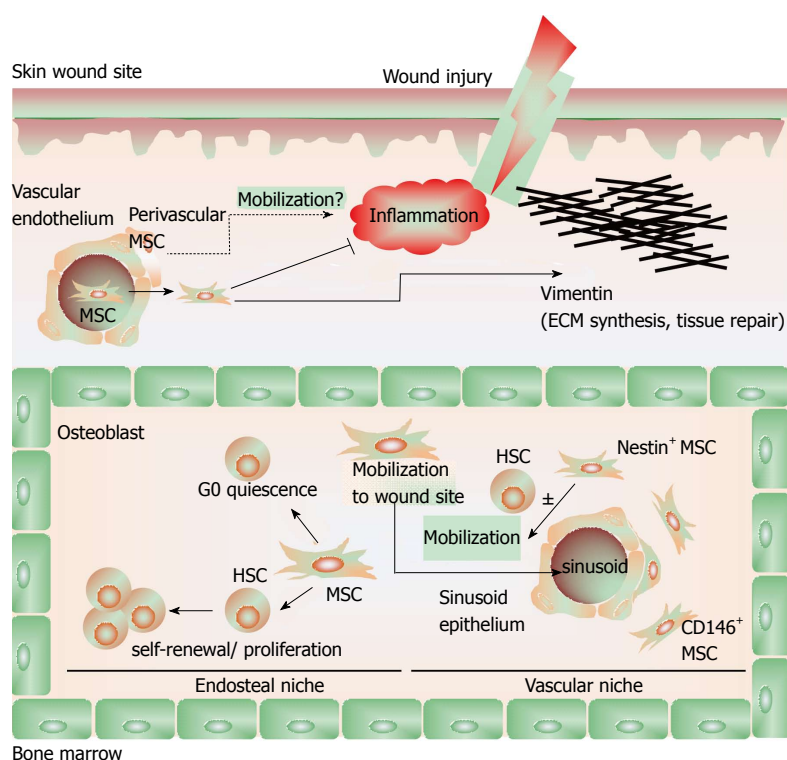


Figure 2 The biology of mesenchymal stem cells. In the bone marrow, mesenchymal stem cells (MSCs) aid in constructing the endosteal niche and regulate the homeostasis of HSCs. MSCs maintain HSCs in a state of quiescence defined by self-renewal and proliferation without differentiation. $CD146^+$ MSCs in the vascular niche also maintain HSC homeostasis and, along with $Nestin^+$ MSCs, regulate the mobilization of HSC into the vascular system. In response to inflammatory cues and chemokine gradients, MSCs mobilize out of the bone marrow and to peripheral sites of injury, where they suppress inflammation to facilitate wound healing. MSCs contribute to tissue reconstruction with the production and deposition of vimentin. It is incompletely understood whether perivascular MSCs may also migrate to sites of injury to contribute to wound healing. Adapted from ref [22].

the G_0 state^[22].

MSCS AND IMMUNOSUPPRESSION

Interest in Immuno-modulatory properties of MSCs

A key method by which MSCs and their stromal derivatives guard the HSC microenvironment is by protecting the niche from inflammatory insults, which could cause inadvertent HSC differentiation and reserve depletion. MSC-derived fibroblasts, which also populate the HSC niche, may exert an anti-inflammatory effect by eliminating survival factors for immune cells, such as T cells, and re-calibrating chemokine gradients, as has been studied

in the context of fibroblast dysfunction in the chronic autoimmune disease rheumatoid arthritis^[23]. This could promote T cell apoptosis and re-direction out of the initial site of inflammation to allow for tissue repair^[23,24]. In addition, MSCs and their derivatives from multiple normal sites within the body, including chondrocytes and fibroblasts from synovial joints, lungs, and skin, suppressed activated T cell proliferation and their cytokine production^[22,25]. MSCs may even influence T cell proliferation indirectly, as splenic stromal cells can induce nitric oxide (NO)-producing dendritic cell (DC) generation in a fibronectin-dependent fashion; these immune-regulatory DCs suppress T cell proliferation^[24,26]. Moreover, it is

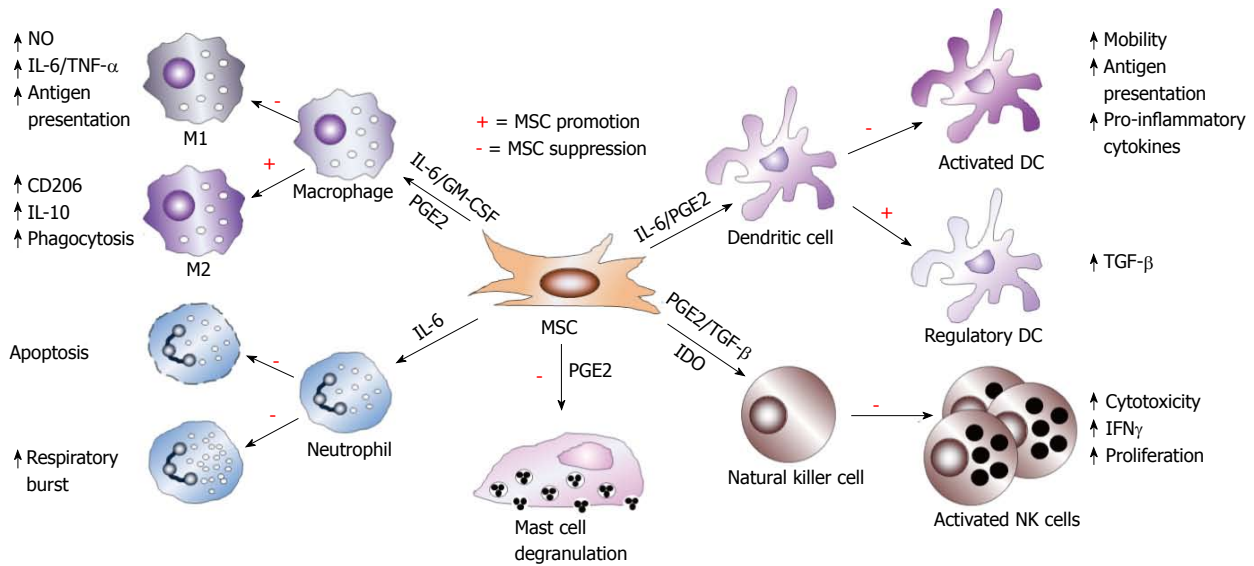


Figure 3 Mesenchymal stem cell immunosuppression of innate immune cells. Mesenchymal stem cells (MSCs) utilize diverse molecular mechanisms to suppress innate immune cells. MSCs suppress macrophage polarization to M1, though favors M2 polarization. MSCs inhibit mast cell degranulation of histamine-containing granules and inhibit NK cell and DC activation, differentiation, and effector functions. MSC-derived PGE2 contributes to all of these effects. MSC-produced IL-6 suppresses neutrophil apoptosis and respiratory burst and also contributes to inhibition of DC function. In the presence of IL-6 and GM-CSF, MSCs also affect macrophage function, while TGF- β and IDO suppress NK cell function. In addition, MSCs also favor the generation of regulatory DCs.

well-established that wound inflictions trigger MSC migration and suppression of inflammation to permit the proliferation of tissue-resident stromal cells, production of reconstructive molecules of the ECM, and wound healing^[15,16].

Mechanisms of MSC suppression of innate immune cells

The discovery of anti-inflammatory properties of MSCs led to investigation of their use as immunosuppressive agents. Innate immune cells have important roles in tissue homeostasis and are the first line of defense against invading pathogens such as viruses and bacteria. Cells of this system respond to pathogens rapidly and do so in a relatively non-specific manner, generally responding to pathogens as a class as opposed to pathogen subtypes and strains. These cells express a multitude of pattern recognition receptors to which they can detect pathogen-associated molecular patterns and respond accordingly (Figure 3).

Macrophages, specifically of the M1 subset, are specialized phagocytes that engulf and digest dead cells and invading microbes such as bacteria. M1 macrophages produce pro-inflammatory cytokines and the anti-microbial molecule nitric oxide (NO), in response to interferon alone or in combination with detection of microbial stimuli such as lipopolysaccharide^[27,28]. However, in the presence of interleukin-4 (IL-4) and IL-13, macrophages differentiate into an alternative, immunosuppressive M2 subset, which is characterized by IL-10 production and decreased expression of IL-12 and tumor necrosis factor- α (TNF- α)^[27,28]. Early work demonstrated that human MSCs antagonize the M1 phenotype and promote M2 polarization, as characterized by increased CD206

expression, increased IL-10 production and phagocytosis, and decreased pro-inflammatory cytokine and NO production^[29]. In transwell cultures, MSCs have also been shown to skew macrophages towards the M2 lineage, which indicates the involvement of soluble, MSC-derived factors that contribute to the polarization^[27]. In addition, MSCs reduce the expression of CD86 and MHCII on macrophages, thus diminishing their stimulatory potency^[30]. In an excisional wound repair model in mice, human gingiva-derived MSCs were shown to migrate to the wound site and polarize M2 for wound repair^[31]. One proposed mechanism is that multiple soluble factors are produced for MSCs to elicit M2 polarization. Prostaglandin E2 (PGE2) was found to be constitutively produced by human MSCs at levels able to suppress IL-6 and TNF- α expression in activated macrophages^[30]. In addition, neutralizing antibodies to IL-6 and granulocyte macrophage-colony stimulating factor (GM-CSF) showed that these cytokines synergistically promote human gingiva-derived MSC-mediated promotion of the M2 phenotype in macrophages^[31].

In addition to macrophages, neutrophils are important phagocytes of the innate immune system. In response to detection of microbial molecules, neutrophils produce a large quantity of microbicidal oxidative products in the so-called oxidative respiratory burst^[32]. Respiratory bursts are also closely associated with neutrophil apoptosis^[33]. MSCs inhibit neutrophil apoptosis, even under IL-8-mediated activation conditions, *via* MSC-derived IL-6^[34,35]. It is thought that MSCs may enact this effect to preserve the non-dividing neutrophil pool found in bone marrow sinusoids. MSCs also prevent respiratory bursts from neutrophils, an effect which aligns with MSC immunosuppression, but had no effect on neutrophil phagocytosis.

sis, matrix adhesion, or chemotaxis^[34].

Mast cells contribute heavily to allergic responses, especially through the release of pro-inflammatory cytokines and histamine-containing granules. Co-culture studies revealed that MSCs suppressed the ability of mast cells to degranulate and produce TNF- α ^[36]. In a passive cutaneous anaphylaxis *in vivo* model, MSCs also reduced inflammation promoted by mast cells. In these experiments, MSC-mediated immunosuppression was dependent on up-regulation of cyclo-oxygenase-2 in MSCs and their production of PGE2, which suppressed mast cells *via* EP4 receptor ligation^[36].

Natural killer cells (NKs) are innate immune cells that, in addition to producing pro-inflammatory cytokines, are cytotoxic toward intracellular pathogen-infected and cancer cells. NK cytotoxicity is regulated by both inhibitory and activating receptors, in addition to target cell MHC expression levels and antibody-dependent cell cytotoxicity. Studies showed that MSCs inhibited NK proliferation activation^[37,38] and reduced the expression of NK activating receptors, including 2B4 and NKG2D^[37]. MSCs also reduced pro-inflammatory cytokine production by NKs. Furthermore, freshly isolated NKs were not cytotoxic towards MSCs, but acquired cytotoxicity after 4 d cultures with IL-15. Neutralization of PGE2 and transforming growth factor- β (TGF- β), both thought to contribute to MSC immunosuppression, overrode MSC-mediated suppression of NK proliferation. Indoleamine-2,3-dioxygenase expression by MSCs has also been found to inhibit NK^[38]. Taken together, these studies indicate that the inhibitory effects of MSCs on NKs may depend on NK culture duration, NK activation state, and time after which MSCs are added to NK cultures.

Dendritic cells (DCs) bridge the innate and adaptive immune systems as they function both as cytokine producers and potent antigen-presenting cells. DCs take up antigen and during maturation and activation up-regulate MHCs, increase the expression of co-stimulatory molecules (*i.e.*, CD40, CD80, CD83 and CD86), and migrate to secondary lymphoid organs and present antigen to T cells for the generation of a primary adaptive immune response. During T cell-priming, DCs also produce a medley of cytokines that affect downstream T cell effector function. MSCs have been shown to affect most of these processes: MSCs inhibit DC endocytosis, up-regulation of MHC, CD40, CD80, CD83, and CD86 during differentiation and prevent further increase of CD40, CD83, and CD86 expression during maturation^[39,40]. They also interfered with DC capacity to produce IL-12 and activate allogeneic T cells^[39,40]. Furthermore, MSCs block the generation of dermal DCs from CD34-derived CD14⁺CD1a⁻ precursors and those derived from immature monocytes^[40]. Monocytes cultured under DC-differentiating conditions in the presence of MSCs fail to proliferate and remain at the G₀ state^[41]. MSC treatment inhibited *in vivo* DC maturation, cytokine secretion, and migration to lymph nodes^[42], which results in insufficient T-cell priming in the lymph nodes. As in previous cellular

contexts, diverse molecular contributions are thought to mediate MSC-modulation of DCs. For example, IL-6 has been shown to at least partially contribute to MSC-mediated inhibition of DC differentiation from bone marrow progenitors^[43], and PGE2 from MSCs has been shown to convert mature CD11c⁺B220⁺DCs into a regulatory subset^[44].

Mechanisms of MSC suppression of adaptive immune cells

Cells of the adaptive immune system, particularly B and T lymphocytes, are composed of billions of unique clones that, as opposed to innate immune cells, recognize highly specific molecules (usually peptides). Each clone expands upon antigen recognition and reaches an effector state in order to eliminate the pathogen present (Figure 4).

B cells are specialized in producing antibodies, which play multiple roles in directly neutralizing pathogens, promoting opsonization for neutralization and phagocytic intake, and activation of other immune cells. Naïve B cells are activated by B-cell receptor (BCR) ligation, CD40/CD40L binding, and Toll-like receptor (TLR) binding of microbial products^[45]. In response to activation, B cells proliferate and differentiate into plasma cells, which produce antibodies. Studies have reported that MSCs inhibit B cell proliferation by arrest at the G₀/G₁ check point, without induction of apoptosis^[45-47]. In addition, MSCs reduced production of IgG, IgM, and IgA during *in vitro* co-culture of B cells^[46]. MSCs also suppressed chemokine receptor expression on B cells^[46]. *In vivo*, MSCs have also been shown to suppress B cell function. In an MRL/Lpr model of systemic lupus erythematosus^[48], a single MSC injection along with cyclophosphamide reduced dsDNA auto-antibodies^[49]. In the context of transplantation, MSC injections led to a reduction of allo-specific antibodies and promoted long-term graft acceptance^[50,51]. In a proteolipid protein (PLP)-mediated form of experimental autoimmune encephalomyelitis (EAE), a murine form of multiple sclerosis^[52], mice given MSCs exhibited an inhibition of PLP-specific antibodies^[53]. Cell-cell contact and soluble factors synthesized by MSCs are thought to suppress B cell function. Programmed death-1 (PD-1)/PD ligand-1 (PD-L1) ligation have been shown to enact B cell suppression by MSCs, with soluble factors largely remaining unidentified^[45,54].

T cells of adaptive immune systems are divided into CD4⁺ and CD8⁺ lineages, both of which can be sub-grouped into different effector subsets. Upon activation through unique T-cell receptors (TCRs) and co-stimulation by APCs such as DCs, T cells rapidly proliferate and differentiate into effector cells. Effector CD4⁺ T cells develop as IFN γ -producing Th1 cells, IL-4- and IL-13-producing Th2 cells, IL-10-producing Treg, and IL-17-producing Th17. CD8⁺ T cells are mainly considered as cytotoxic T lymphocytes (CTLs) and produce cytotoxic granules that kill infected and cancerous cells; however, they can differentiate into many of the same effector

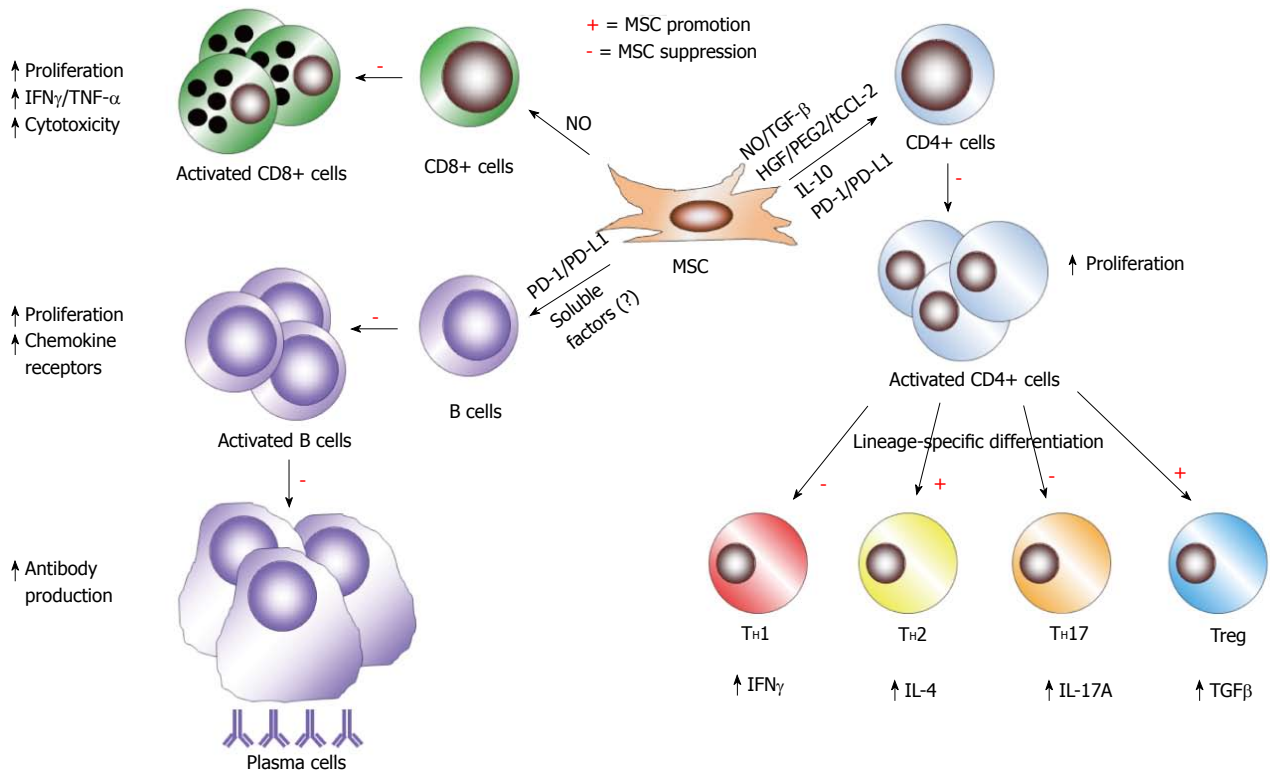


Figure 4 Mesenchymal stem cell immunosuppression of adaptive immune cells. In the context of B cells, mesenchymal stem cells (MSCs) inhibit various facets of B cells activity, including activation, proliferation, chemokine receptor expression, and differentiation to becoming antibody-secreting plasma cells. Unknown soluble factors and PD-1/PD-L1 ligation mediate these effects of MSCs on B cells. MSCs have been shown to induce NO in response to inflammatory cytokine detection to suppress CD8+ T cell proliferation, cytokine production, and cytotoxicity. In response to activation in specific cytokine milieu, CD4+ T cells can differentiate into numerous effector populations. MSCs produce soluble factors (NO, TGF- β , HGF, PGE2, truncated CCL-2, and IL-10) and membrane-bound molecules (PD-1 ligation) to achieve suppression of CD4+ T cell proliferation and the polarization of CD4+ T cells towards TH1 and TH17 cells. MSCs favor the development of TH2 and anti-inflammatory Treg populations.

subtypes as their CD4+ T cell counterparts.

MSCs inhibit T cell proliferation, regardless of stimulus type, by arrest at the G₀/G₁ cell cycle phase^[55-57]. This inhibition is also MHC-independent, as both autologous and allogeneic MSCs exert this same anti-proliferative effect. T cells inhibited by MSCs also exhibit increased survival and less apoptosis, but this state can be partially reverted *via* IL-2^[55]. One study showed that MSCs repressed T cell proliferation *via* up-regulation of inducible nitric oxide synthase (iNOS), which produces the NO which produces such effect^[58]. MSCs also modulated cytokine production of T cells. It was reported that these cells suppressed IFN γ production from TH1, promoted IL-4 secretion from TH2, and increased the proportion of Treg present in culture^[59]. MSCs produce immunomodulatory molecules such as hepatocyte growth factor (HGF), TGF- β , and PGE2, which may enact these cellular effects^[55]. MSCs have also been reported to inhibit TH17 development through various means, including inhibition with the effector molecules PGE2, a truncated peptide of C-C chemokine ligand-2 (CCL-2), IL-10, and PD-1/PD-L1 ligation^[52,60-63]. Importantly, MSCs must be pre-exposed to a combination of effector cytokines, including IFN γ and TNF α or IL-1 β , in order to efficiently suppress T cell function^[58]. Moreover, MSCs have been

shown to suppress the cytotoxicity of CTLs, presumably by a soluble factor^[64]. When administered viral peptides and tumor antigens, the cells suppress CTL killing and were not recognized as targets of infection or foreign cells, despite enhanced MHC-I expression post-IFN γ treatment^[22,65,66].

In vivo, MSCs have been extensively used in pre-clinical experimental disease settings involving pathogenic T cells. Some of the earliest reports show MSC-mediated amelioration of EAE induced by the peptide, myelin oligodendrocyte glycoprotein (MOG) 35-55, which preferentially induces a neuro-inflammatory disease mediated by TH1 and TH17 cells^[52,57]. In this setting, the polarization of these cells was inhibited *in vivo*, and MSC-derived HGF alone suppressed EAE while also promoting a beneficial neurotropic effect^[52,57,67]. MSCs suppressed skin-graft rejection in monkeys, which was associated with T cell suppression of proliferation^[68]. In a model of streptozotocin-induced autoimmune diabetes, MSCs inhibited T-cell mediated destruction of insulin-secreting β -cells in the pancreas^[69]. MSCs also suppressed proliferation of auto-reactive T cells in collagen-induced arthritis, in addition to decreasing TNF- α production and supporting the generation of Treg cells^[70]. These studies demonstrate immense potential for the use of MSCs in modulating

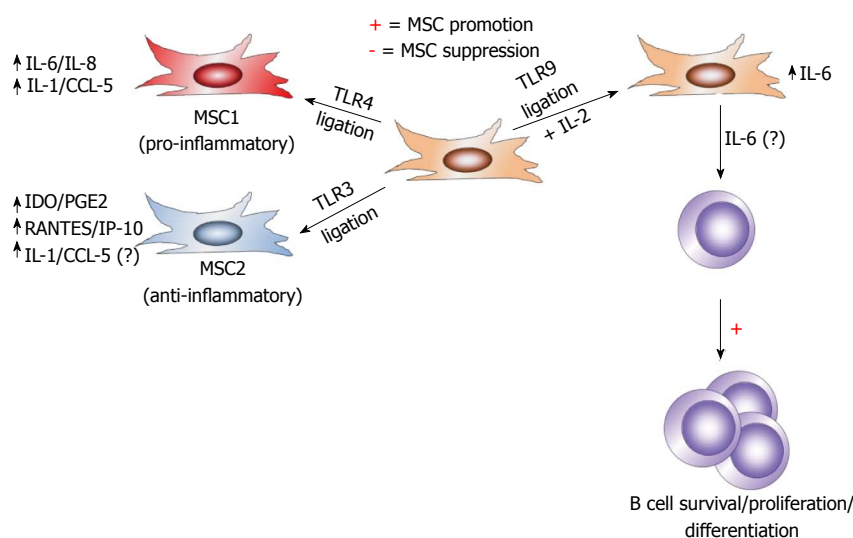


Figure 5 Differential toll-like receptor stimulation affects mesenchymal stem cell immune-modulation. Mesenchymal stem cells (MSCs) are situated throughout the body as sentinels in virtually all organs and the perivascular space and are equipped with pattern-recognition receptors, including Toll-like receptors (TLRs), to detect DAMPs from dying cells and PAMPs from pathogens. In response to TLR3 signaling, MSCs maintain an anti-inflammatory MSC2 phenotype, marked by induction of IDO, PGE2, RANTES, and IP-10 (in addition to IL-1 and CCL-5). However, in response to signaling through TLR3, MSCs adopt the pro-inflammatory MSC1 phenotype and up-regulate IL-6 and IL-8, in addition to IL-1 and CCL-5. In the presence of IL-2 in combination with TLR9 signaling, MSCs have been shown to also produce IL-6, which promotes B cell survival, proliferation, and differentiation, though MSC-derived IL-6 has not been demonstrated to directly exert these effects on B cells.

the immune response in inflammatory settings for therapeutic benefit, especially of autoimmune diseases.

MSCS AND IMMUNOGENICITY

Although the majority of investigations of MSC effects on immune cell function and pre-clinical immunogenic and inflammatory conditions have indicated immunosuppression, other studies have shown immunostimulatory properties, which are discussed next.

Microbial molecule detection

In vivo, MSCs are present in virtually all tissues of the body and express multiple receptor types that permit detection of changes in tissue homeostasis. Differential TLR stimulation of MSCs has been shown to influence the downstream effect of MSCs on immune responses (Figure 5)^[71]. Stimulation of TLR3 with poly (I:C), which mimics viral double-stranded RNA detection, in MSCs causes them to polarize towards an anti-inflammatory phenotype (MSC2 phenotype) characterized by increased production of the immune-regulatory factors IDO and PGE2 and of RANTES and IP-10. However, when MSCs are stimulated with LPS, a TLR4 agonist, they develop a pro-inflammatory MSC1 phenotype in which they up-regulate the pro-inflammatory cytokines IL-6 and IL-8. MSC1, but not un-primed or MSC2, support PBMC activation and proliferation. In opposition to the previous findings, Romieu-Mourez *et al.*^[72] found that stimulation of either TLR3 or TLR4 lead to the production of the pro-inflammatory cytokines IL-6, IL-8, IL-1, and the chemokine CCL-5; however, such differences may be due to differences in stimulation protocols, especially for MSC exposure time differences to TLR agonists^[72]. When MSCs are co-cultured with naïve and transitional B cells in the presence of IL-2 and the TLR9 agonist CpG 2006 (viral/bacterial PAMP mimic), B cell survival, differentiation, and antibody production are enhanced^[73]. Though the effect was cell-contact dependent, the MSCs produced increased IL-6 in co-culture, which is

known to increase B cell proliferation. *In vivo*, MSCs are also postulated to not only support the viability of naïve, but also more differentiated, B cell subsets in the bone marrow^[73].

The rationale for the different MSC polarization types in response to different microbial stimuli detection remains unknown. MSCs are thought to exhibit a homeostatic default immunosuppressive phenotype for the purposes of inhibiting inappropriate HSC differentiation and potential depletion of HSC reserves in the bone marrow. However, outside of the bone marrow, they may adopt the pro-inflammatory MSC1 phenotype to aid in the formation of an immune response in tissues during early tissue damage and/or pathogen invasion. It is interesting to note that tissue necrosis and damage leads to the release of intracellular danger-associated molecular patterns (DAMPs) such as heat shock proteins, high mobility group proteins, and degraded ECM molecules, which trigger stimulation of innate immune cells through TLR4 and TLR2 ligation for resolution of tissue damage^[74]. It is possible that TLR4 stimulation of MSCs, whether derived from PAMP or DAMP, could still lead to the same pro-inflammatory outcome due to the apparent necessity of generating an inflammatory environment for the recruitment and activation of immune cells to respond to either tissue damage and/or pathogen invasion. In contrast, the MSC2 phenotype could be adopted for the down-regulation of immune responses to limit inflammatory damage to tissues and permit ECM reconstruction and healing.

Cytokine milieu

MSCs are pleiotropic cells that are highly sensitive to different microenvironments, especially those containing cytokines. Importantly, cytokines exert immune-suppressive or immunogenic effects on cells and tissues dependent on multiple variables, including cytokine identities, combinations, and concentrations (Figure 6).

In continuation of the differential TLR stimulation on MSC polarization, the downstream effects of TLR

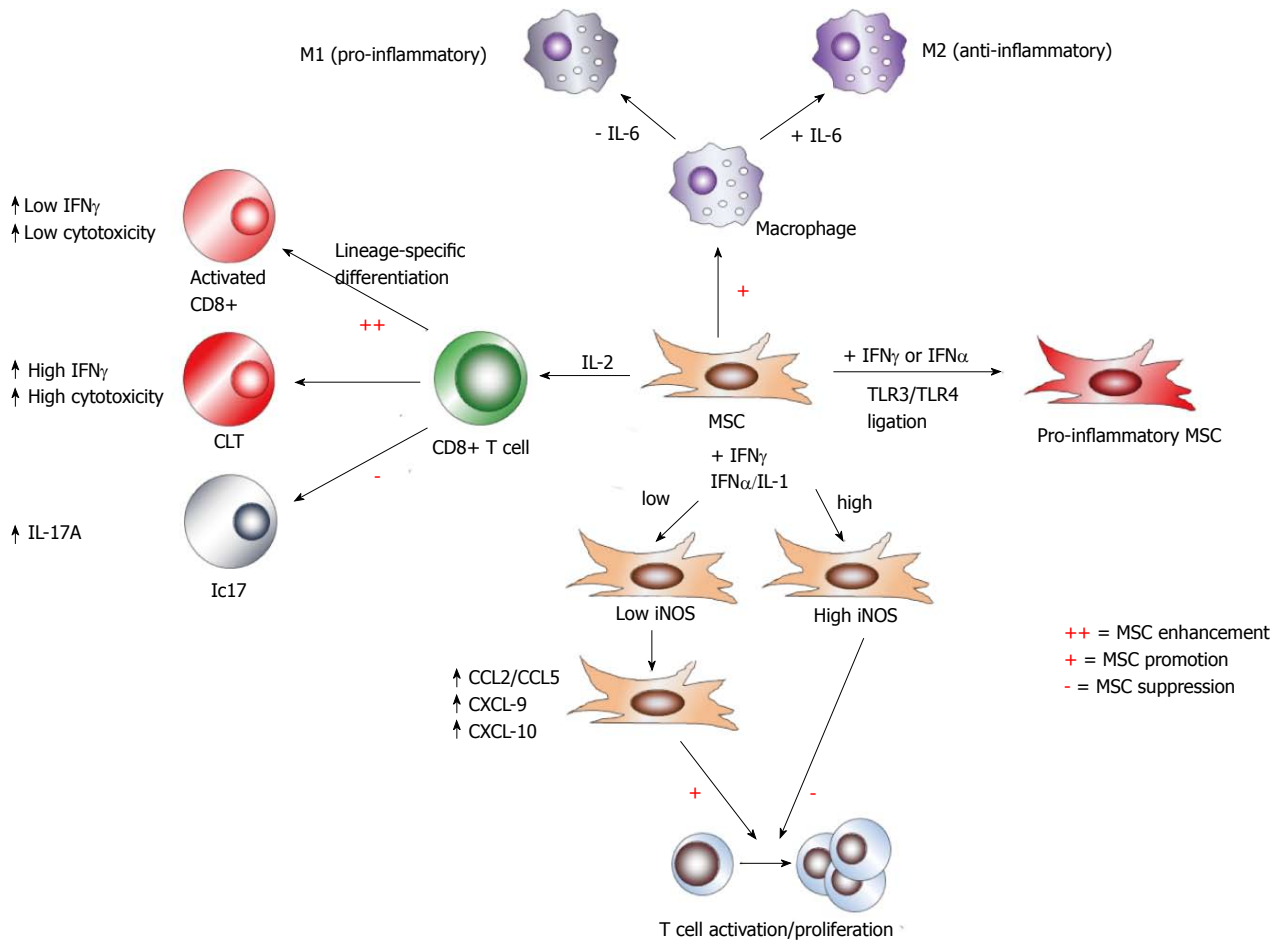


Figure 6 Effects of cytokine milieu on mesenchymal stem cell immune-modulation. Mesenchymal stem cell (MSC) modulation of immune responses is strongly affected by the makeup of cytokine milieus. Toll-like receptor (TLR) ligation in conjunction with interferon signaling drives MSCs down a pro-inflammatory route. While high concentrations of the pro-inflammatory cytokines IFN γ and either tumor necrosis factor- α (TNF- α) or IL-1 have been shown to induce iNOS and NO in MSCs to mediate suppression of T cell proliferation, low concentrations of these factors fail to fully induce iNOS, and instead enhance T cell proliferation, presumably via cytokine-induced chemokines. Furthermore, MSCs differentially affect the polarization of effector CD8+ T cell subsets: through enhanced early IL-2 expression induced by MSCs, activated CD8+ T cells exhibit increased IFN γ expression and cytotoxicity, while fully differentiated cytotoxic T lymphocytes (CTLs) are largely unaffected by MSC action. In contrast, MSCs potently suppress Tc17 development. Moreover, IL-6 signaling acts as a switch for MSC immune-modulation of macrophages. In the presence of IL-6, MSCs retain promotion of M2, but favor M1 polarization in the absence of this cytokine.

stimulation in MSCs can be affected by prior cytokine priming. Initial priming of human MSCs with either IFN- α or IFN- γ synergizes with downstream TLR3 or TLR4 stimulation to enhance the production of pro-inflammatory cytokines by MSCs^[72]. The concentration of inflammatory cytokines has also been postulated to regulate MSC polarization. IFN- γ and IL-1 or TNF- α induction of iNOS and NO production have been demonstrated as an effector mechanism MSCs used for inhibition of T cell proliferation. However, under closer scrutiny, it was discovered that their concentrations must be relatively high, for low/insufficient levels of these cytokines failed to up-regulate iNOS to adequate levels for T cell functional suppression, and led to an induction of T cell responses^[73]. In this scenario, MSCs still retained upregulation of the T-cell activity enhancing chemokines such as CCL2, CCL5, CXCL9, and CXCL10. When iNOS-/-MSCs were injected into normal C57BL/6 mice and challenged with a suboptimal dose of OVA for induction of a delayed type hypersensitivity (DTH) re-

sponse, swelling occurred in injected footpads of mice^[75]. However, when these mutant MSCs were injected into CCR5 -/-CXCR3-/- mice, they could not promote the DTH response, highlighting the importance of chemokine ligation on T cells as an immune-enhancing effect of MSCs in the absence of iNOS induction. Thus high pro-inflammatory cytokine concentrations are thought to promote an MSC2 phenotype while an MSC1 phenotype may result from low level of such cytokines^[76].

As a testament to the importance of the cytokine milieu on influencing MSC function, we recently showed that MSCs differentially affected the generation of different effector CD8+ T cell subsets^[77]. In this study, we found that MSCs had little effect on the functions of IL-2 and IL-12-generated CTLs, increased cytokine production and cytotoxicity of non-polarized, activated CD8+ T cells, and potently suppressed IL-17A-producing, Tc17 development. IFN γ -producing CD8+ T cells were also cytotoxic towards MSCs, which was associated with heavily increased MHC-I expression on MSCs. These effects

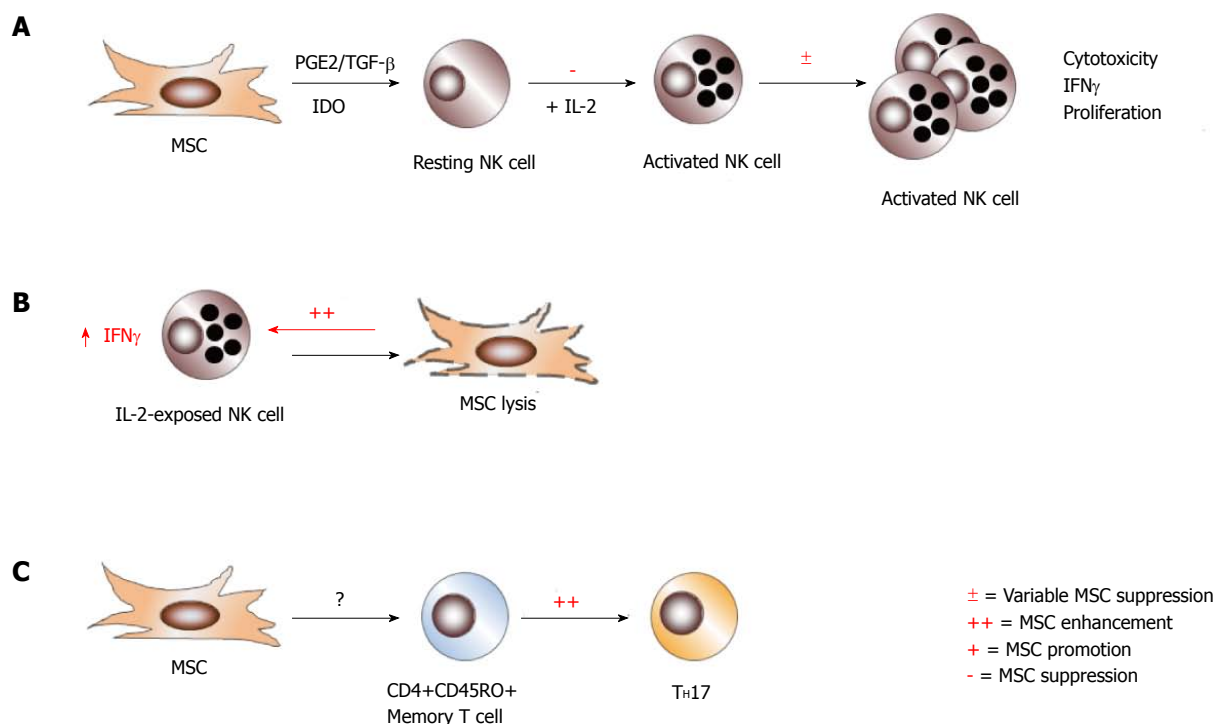


Figure 7 Effects of immune cell activation state on mesenchymal stem cell immune-modulation. The differentiation state of immune cells can render them susceptible or refractory to mesenchymal stem cell (MSC) action. Though MSCs efficiently inhibit the activation and downstream cytotoxicity of resting NK cells, they exert variable suppression on IL-2-activated NK cells, which is partially ratio dependent (A). MSCs themselves may become targets of activated NK cells for lysis, and enhance NK cell production of IFN_γ in the process (B). Interestingly, MSCs promote TH17 differentiation from CD4+CD45RO+ memory T cells, but no other CD4+ or CD8+ T cell population (C).

were associated with the early enhancement of IL-2 production, which is known to promote CTLs but antagonize the IL-17-producing program. In a the MOG³⁷⁻⁵⁰ model of EAE, which is mediated by pathogenic CD8+ T cells, MSCs exacerbated the disease and increased the CD8+ T cell presence in the brains of diseased mice. Here, the MSCs appeared to alter the activation program of the developing T cells, but the precise mechanisms of MSC-induced IL-2 production and downstream effector function remain undefined.

In another report of MSC modulation of neuro-inflammatory autoimmune disease, MSCs were found to ameliorate mild MOG-induced EAE, but worsen the severe form, with intracerebroventricular (ICT) injection into mice^[78]. In almost two-thirds of severe-EAE animals, these MSCs migrated into the parenchyma and formed masses characterized by focal inflammation, demyelination, axon loss, and collagen and fibronectin deposits. Importantly, these MSCs do encounter an inflammatory environment when injected ICT, and may undergo a polarization similar to the aforementioned MSC1 type, which could be dependent on the cytokine and molecular milieu.

In addition to the pro-inflammatory cytokines mentioned above, production and detection of IL-6 also acts as a switch for MSCs during immune responses^[76]. This molecule, which is constitutively produced by MSCs, polarized macrophages towards the M2 type upon cell-cell contact^[79]. This polarization was also dependent upon MSC production of IDO and PGE2. However, in the

absence of IL-6, MSCs induced polarization of macrophages towards the M1 phenotype, which is characterized by IFN_γ, TNF-α, and CD40L expression^[76]. In contrast, a positive correlation with IL-6 *in vivo* production and MSC administration in mice exhibiting collagen-induced arthritis was reported to worsen this disease^[80]. The molecular milieu that governs the production of IL-6 from MSCs in the context of macrophage polarization has not been determined, but may involve pre-exposure to certain cytokine combinations that influence MSCs in a concentration-dependent manner, as in the case of iNOS. The *in vivo* milieu must also be taken into account, for increased IL-6 production could theoretically enhance inflammation by promoting effector immune cell differentiation, as in the case of IL-17A-producing T cells.

Immune cell differentiation state

Upon activation through cell-specific receptor signaling, immune cells undergo successive stages of differentiation towards a terminal phenotype characterized by optimal effector function, usually before subsequent apoptosis or transition into memory status. The specific stage of an immune cell's differentiation may render it susceptible or refractory to any MSC action (Figure 7).

NK cells are generally in a resting state, but upon IL-2 activation, proliferate and differentiate into activated cytolytic and cytokine-producing cells capable of efficient lysis of target cells. MSCs robustly prevented resting NK cell activation and proliferation, but were only partially

Table 1 Effects of mesenchymal stem cells on preclinical disease models

Disease	Species	Route of administration	Effect	MSC mechanism of action	Ref.
Skin-graft rejection	Monkey	Systemic	Prolonged skin graft survival	Inhibition of T cell proliferation	[68]
Skin-graft rejection	Mouse	Systemic	Increased rejection	Induction of memory T cell response	[85]
Skin-graft rejection	Mouse	Systemic	Increased rejection	Potential increased T cell alloreactivity	[48]
Graft-v-Host disease	Mouse	Systemic	Disease prevention	Cytokine-induced iNOS to inhibit T cell proliferation	[58]
Graft-v-Host disease	Mouse	Systemic	No clinical benefit	Lack of suppression of donor T cell proliferation	[86]
Skin wound	Mouse	Systemic	Wound healing	M2 polarization, decreased inflammation, increased IL-10	[31]
Acute lung injury	Mouse	Systemic	Protected lungs from injury	Blockade of TNF α and IL-1 production	[87]
Acute lung injury	Mouse	Local	Decreased severity	Down-regulation of inflammation, increased IL-10	[88]
Melanoma	Mouse	Local	Increased tumor growth	Inhibition of tumor-specific T cell response	[89]
MOG ³⁵⁻⁵⁵ EAE	Mouse	Systemic	Disease amelioration	Inhibition of CD4 ⁺ T cell proliferation	[57]
MOG ³⁵⁻⁵⁵ EAE (severe)	Mouse	Local	Disease worsening	Focal cell mass formation and increased inflammation	[78]
MOG ³⁷⁻⁵⁰ EAE	Mouse	Systemic	Disease worsening	Increased pro-inflammatory CD8 ⁺ T cell frequency in CNS	[77]
Experimental autoimmune neuritis	Mouse	Systemic	No clinical benefit	Unknown	[90]
Rheumatoid arthritis	Mouse	Systemic	Prevention	Reduced T cell proliferation, decreased inflammation, Treg induction	[70]
Rheumatoid arthritis	Mouse	Systemic	No clinical benefit	Accentuation of TH1 response	[91]
Systemic lupus erythematosus	Mouse	Systemic	Multi-organ dysfunction reversal	Suppression of TH17 and induction of Treg	[92]
Type-I-diabetes	Mouse	Systemic	Delayed onset	Promotion of TH2 response	[93]
Inflammatory bowel disease	Mouse	Systemic	Prevention	Decreased neutrophil infiltration	[94]

MSC: Mesenchymal stem cell; MOG: Myelin oligodendrocyte glycoprotein; EAE: Experimental autoimmune encephalomyelitis; TNF- α : Tumor necrosis factor- α .

capable of suppressing this process on NK cells that have been pre-exposed to IL-2^[38]. Moreover, the extent of MSC suppression of NK cell proliferation in the latter case was ratio dependent, with decreasing suppression with increasing NK:MSC ratio. IL-2-pre-exposed, but not resting, NK cells also efficiently lysed autologous and allogeneic MSCs, and exhibited increased IFN γ production with MSC co-culture. Interestingly, IFN γ -pre-exposed MSCs had a better capacity of inhibiting pre-activated NK cell activity, presumably due to increased MHC-I expression on MSCs in response to inflammatory cytokine signaling, which negatively affects NK cell function.

Under the arm of adaptive immunity, MSCs have been extensively shown to suppress TH17 and Tc17 development, but less work has addressed MSC effects on memory T cells. Hsu and colleagues showed that MSCs specifically enhanced IL-17 expression in CD4⁺CD45RO⁺ memory T cells, but not in any other populations of CD4⁺ or CD8⁺ T cells^[81]. These TH17 subsequently enhanced neutrophil function. It is thought that, since these memory T cells rapidly react to a pathogen challenge *in vivo*, they could interact with MSCs at peripheral sites to enhance their function and increase the T cell response for efficient pathogen elimination. Thus immune cell activation state is an important factor in influencing outcome with MSC interactions.

THERAPEUTIC CONSIDERATIONS AND CONCLUSION

Initial pre-clinical animal models of inflammatory con-

ditions suggested that MSCs exerted a beneficial effect for a range of diseases and ushered in their potential use in controlling human diseases, especially autoimmune disease (Table 1). However, additional studies also indicate an exacerbation of disease symptoms, thus raising points to consider regarding the safe use of these cells in humans^[82,83]. Importantly, MSCs represent a highly heterogeneous and pleiotropic population of stem cells. The intrinsic variability in the cellular make-up may influence multiple properties of how MSCs affect immune cell function and disease. Therefore, an intensified focus on further characterizing the subtypes of MSCs is desperately needed. The heterogeneity in the isolation, culturing, and expansion of MSC populations are known to affect the phenotype of MSCs^[84]. For potential clinical use, a more thorough standardization for isolating and culturing these cells is needed along with the ability to project the specific immune-modulatory effects of a given MSC population depending on its subtype make-up.

When injected systemically, MSCs accumulate in the lungs and capillary beds of other tissues, which could decrease the number of MSCs migrating to target areas for treatment. Several lines of genetic and chemical engineering research are already working to improve cell delivery^[82]. There still remains a dearth of information on the long-term engraftment of MSCs in target organs, which is important in light of their initial lung entrapment. Importantly, more research is necessary for a better understanding of the fate of injected MSCs, to determine whether they maintain their primary phenotype or differ-

entiate, depending on the molecular milieu and microenvironment encountered.

The use of MSCs for immune-modulation represents an exciting new step in cellular therapy. However, a number of considerations and further characterizations of the precise nature of these cells will improve their future use in a number of different settings. The conditions of culture can greatly impact the phenotypes of the cells, which is a consideration of *in vitro* culture of cells for therapy. As the MSCs respond to their environments, a more difficult variable to control will be the *in vivo* setting in which they are introduced; cells introduced into an inflammatory environment may respond differently from those introduced into a suppressive environment, for example.

Thus, future studies that further address these questions and are geared toward a more precise characterization of MSC populations and how they respond to these different pathological settings may help promote safe and effective clinical utility of these cells.

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Current applications of adipose-derived stem cells and their future perspectives

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ferentiation; Transplantation; Cell-based therapy

Core tip: Adult stem cells have a great potential for reconstructive and regenerative medicine. Particularly, adipose-derived stem cells (ADSCs) are a promising useful cell source for cell-based therapy because of their capability of expansion and differentiation into special cell types. In this review, the current status of ADSC isolation, differentiation and their therapeutic applications are discussed.

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Abstract

Adult stem cells have a great potential to treat various diseases. For these cell-based therapies, adipose-derived stem cells (ADSCs) are one of the most promising stem cell types, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). ESCs and iPSCs have taken center stage due to their pluripotency. However, ESCs and iPSCs have limitations in ethical issues and in identification of characteristics, respectively. Unlike ESCs and iPSCs, ADSCs do not have such limitations and are not only easily obtained but also uniquely expandable. ADSCs can differentiate into adipocytes, osteoblasts, chondrocytes, myocytes and neurons under specific differentiation conditions, and these kinds of differentiation potential of ADSCs could be applied in regenerative medicine *e.g.*, skin reconstruction, bone and cartilage formation, *etc.* In this review, the current status of ADSC isolation, differentiation and their therapeutic applications are discussed.

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Key words: Adipose-derived stem cells; Isolation; Dif-

INTRODUCTION

Stem cells include embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and postnatal adult stem cells. ESCs are capable of self-renewal and differentiation into any cell type in the body. Induced PSCs are genetically reprogrammed somatic cells and have characteristics of ESCs but it is still unknown what the differences between ESCs and iPSCs are. Because of ethical and political concerns, it is difficult to apply ESCs in clinical research and practice but iPSCs and postnatal adult stem cells do not have such problems. Among postnatal adult stem cells, adipose-derived stem cells (ADSCs) are one of the most promising stem cell types. They can be easily obtained from liposuction aspirates or subcutaneous adipose tissue fragments and expanded *in vitro* and there are no ethical concerns like human ESCs for their use in diverse clinical applications.

ADSCs are found in any type of white adipose tissue, including subcutaneous and omental fat^[1]. To obtain

adipose tissue for ADSC isolation, liposuction is a safe process with a low complication rate^[2]. Shiffman *et al*^[3] reported that 90%-100% of adipocytes from lipoaspirate are intact after autologous fat transplantation. The isolated ADSCs can be expanded vigorously until they enter into the differentiation process to specific cell lineages. ADSCs are capable of differentiating into adipocytes, osteoblasts, chondrocytes, myocytes *etc.*, *in vitro* and genetically stable in long-term culture. Thus, ADSCs would be a valuable stem cell source for clinical use, with fewer restrictions compared to other cell sources.

ISOLATION AND CULTURE OF ADSCs

Stem cells derived from adipose tissue show higher yields compared with other stem cell sources. Currently, ADSCs could be isolated not only manually but also automatically using automatic centrifuge for cell isolation specialized in cells from adipose tissue.

To isolate stem cells from adipose tissue, current methods rely on a collagenase digestion followed by centrifugal separation. They display a fibroblast-like morphology and lack intracellular lipid droplets seen in adipocytes. Isolated ADSCs are typically expanded in a monolayer on standard tissue culture plastics with a basal medium containing 10% fetal bovine serum^[4].

DIFFERENTIATION POTENTIAL

ADSCs are multipotent and can differentiate into adipocytes^[5-7], osteoblasts^[5,8], chondrocytes^[5], myocytes^[5,9] and neuronal cells^[10].

For induction of adipogenic differentiation, dexamethasone, insulin and isobutylmethylxanthine are needed^[5]. Adipogenic differentiation status can be evaluated by Oil Red O staining.

Differentiation into osteoblasts can be induced by dexamethasone, ascorbic acid and glycerophosphate and identified using Alizarin red which stains calcified extracellular matrix in the osteoblasts or alkaline phosphatase^[11].

Induction of chondrocyte differentiation is carried out by the addition of insulin, transforming growth factor beta 1 and ascorbic acid. The chondrocyte differentiation can be assessed by safranin O or toluidine blue staining.

ADSCs differentiate into myocytes in media supplemented with hydrocortisone and dexamethasone usually. ADSCs can also differentiate in a medium which is composed of control medium supplemented with horse serum and hydrocortisone and expresses myoD1 and myosin heavy chain^[12,13]. The differentiated cells form myotubes and express myosin light chain kinase in addition to other markers characteristic of the myocyte lineage^[13].

Recently, ADSCs have also been induced to differentiate into neuronal cells. The composition of the neuronal induction medium is basal medium with butylated hydroxyanisole, retinoic acid, epidermal growth factor and basic fibroblast growth factor. The differentiated cells express neuronal markers for immature and mature neu-

rons, such as β III-tubulin, microtubule-associated protein 2, neuron specific enolase, synaptophysin and TAU^[10].

CELL-BASED THERAPEUTIC APPLICATIONS OF ADSCs

Due to multipotency of the ADSCs, they can be used widely in various clinical applications. Unlike ESCs, ADSCs lack the ability to form all tissues or organs of the body and regenerate an entire living organism. Inducing differentiation of ADSCs requires potent doses of growth factors *in vitro*. ADSCs do not easily transform to mature cell types without strong signaling and they tend to resist differentiation *in situ*. The mechanisms for signaling ADSC differentiation to a mature adipocyte within a native adipose deposit are not well understood^[14,15].

Adipocytes derived from ADSCs have uses in soft tissue defects, postmastectomy repair, lipodystrophy and soft tissue cosmetic applications, like anti-contour defects and anti-wrinkles. For soft tissue regeneration, autologous fat grafts have been widely used; however, several limitations still remain. One of the limitations is the poor long-term graft retention. The transplanted fat grafts can lose its volume over time due to tissue resorption that can result in the loss of 20%-90% of the original transplanted grafts volume^[16]. The soft tissue regeneration would be more effective if the defect volume is filled. To fill the soft tissue defects, vasculature for supplying nutrition to the grafted tissue is needed. ADSCs could help the neovascularization by vascular endothelial growth factor (VEGF) secretion and adipocyte and fibroblast regeneration by their differentiation potential.

Chondrocytes differentiated from ADSCs express extracellular matrix components which are localized in cartilage and maintain their phenotype *in vivo*^[17]. Chondrocyte derived ADSCs show practical possibilities for applications in repair of articular cartilage defects, such as osteoarthritis, in the future.

For bone repair, isolated ADSCs can be induced to differentiate into osteoblasts which are able to mineralize their extracellular matrix and express genes and proteins associated with a bone phenotype^[18]. Osteoblasts or precursors of osteoblasts derived from ADSCs are able to be applied not only as cell materials, but also in combination with scaffold to a bone defect site^[19].

Various cardiovascular diseases are the leading causes of mortality worldwide. Growing evidence indicates that injection of ADSCs improves cardiac function *via* the differentiation into cardiomyocytes and vascular cells and through paracrine pathways. Paracrine factors secreted by injected ADSCs enhance angiogenesis, reduce cell apoptosis rates, and promote neuron sprouts in damaged myocardium^[20-23]. Danoviz *et al*^[22] showed the effects of co-injecting ADSCs with biopolymers on cell cardiac retention, ventricular morphometry and performance in a rat model of myocardial infarction. They could confirm that intramyocardial injection of ADSCs mitigates the negative cardiac remodeling and preserves ventricular

function post myocardial infarction. These findings suggest important implications for the design of future cell therapy strategies for cardiac repair.

For treatment of neurodegenerative diseases, various stem cell types are under investigation. Stem cells are able to differentiate into neurons^[24,25] and glial cells^[26,27]. Similarly to other stem cell types, ADSCs have been known to have a differentiation potential into neuronal and glial cells^[28,29] and are capable of promoting neuronal healing by secretion of some nerve growth factors. ADSCs express a significantly high proportion of nestin, which is a marker for neural progenitor cells^[30]. ADSCs can secrete angiogenic factors such as VEGF^[31] and some neuro-protective factors such as insulin-like growth factor 1, the major factor that mediates protection against serum and potassium deprivation-induced apoptosis of cerebellar granule neurons^[32]. Limitations in protocols to establish homogeneous populations of neural progenitors and stem cells still need to be resolved for effective therapy for neurodegenerative diseases like Parkinson's disease, multiple sclerosis and Alzheimer's disease^[33].

PERSPECTIVES

Stem cells would be a useful tool for cell-based therapies for diverse diseases. A number of challenges still remain for cell-based therapies using stem cells. Safety issues in clinical use of stem cells expanded *in vitro*, development of differentiation protocol and *in vivo* delivery method, and problems of immune response in allogeneic transplantation are some to be overcome.

Because ADSCs can be harvested in large numbers and have shown evidence of safety and efficacy, their use is currently increasing in clinical fields. For ADSC culture, a whole adipose-derived stromal vascular fraction is usually used which is a heterogeneous mixture of various cell populations, including ADSCs. However, suitable cell surface markers can identify an ADSC population and the positively marked ADSCs can be separated by a cell sorting experiment. ADSCs purified by the specific cell surface markers would differentiate more efficiently into targeted cell types and make it easier to evaluate their influences on the therapeutic effects. Development of culture media compositions without animal origins is also an important aspect. This problem could be resolved by technology of recombinant proteins and cryopreservation methods of ADSCs over long time periods would be also useful.

Recently, many of these aspects have been considered and investigated and the progress to overcome such limitations would lead to applying stem cells, including ADSCs, widely in clinical practice in the future.

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WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Intestinal stem cells and celiac disease

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Abstract

Stem cells (SCs) are the key to tissue genesis and regeneration. Given their central role in homeostasis, dysfunctions of the SC compartment play a pivotal role in the development of cancers, degenerative disorders, chronic inflammatory pathologies and organ failure. The gastrointestinal tract is constantly exposed to harsh mechanical and chemical conditions and most of the epithelial cells are replaced every 3 to 5 d. According to the so-called Unitarian hypothesis, this renewal is driven by a common intestinal stem cell (ISC) residing within the crypt base at the origin of the crypt-to-villus hierarchical migratory pattern. Celiac disease (CD) can be defined as a chronic immune-mediated disease that is triggered and maintained by dietary proteins (gluten) in genetically predisposed individuals. Many advances have been achieved over the last years in understanding of the pathogenic interactions among genetic, immunological and environmental factors in CD, with a particular emphasis on intestinal barrier and gut microbiota. Conversely, little is known about ISC modulation and deregulation in active celiac disease and upon a gluten-free diet. Nonetheless, bone marrow-derived SC transplantation has become an option for celiac patients with complicated or refractory disease. This manuscript summarizes the “state of the art” regarding CD and ISCs, their niche and potential role in the de-

velopment and treatment of the disease.

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Key words: Intestinal stem cells; CD133; Lgr5; Celiac disease; Paneth cells; Gut microbiota; Gut barrier

Core tip: The intestinal epithelium has a high turnover rate since most of the epithelial cells are replaced every 3 to 5 d. This renewal is driven by intestinal stem cells residing within the crypt base at the origin of the crypt-to-villus hierarchical migratory pattern. Many aspects of the pathogenesis of celiac disease have been elucidated over the last years regarding the interactions among genetic and immunological factors, intestinal barrier and gut microbiota. Conversely, little is known about intestinal stem cell modulation and deregulation in celiac disease. The current knowledge regarding celiac disease and intestinal stem cells, and the potential role of stem cells in the development and treatment of the disease are summarized.

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“Enthusiasm is that temper of the mind in which the imagination has got the better of the judgment” - William Warburton.

STEM CELLS AND THEIR POTENTIAL

Stemness can be defined as the capability of extensive self-maintenance and differentiation^[1,2]. Stem cells (SCs) are undifferentiated cells able to give rise to diverse mature progenies and to self-renew through the alternation of symmetrical and asymmetrical divisions. SCs play a central role in tissue genesis, regeneration and homeosta-

sis by providing differentiated cells that can increase tissue mass during pre- and post-natal growth and replace cell loss due to senescence or damage^[3,6].

SCs possess a hierarchy of potentialities: from the totipotency of the zygote and its immediate progeny, to the pluripotency of embryonic stem cells (ESCs), up to the multi/unipotency of adult SCs (ASCs)^[7].

ESCs are pluripotent cells derived from the inner cell mass of the blastocyst that can generate any differentiated phenotype of the three primary germ layers (endoderm, mesoderm and ectoderm), as well as germ cells. ESCs might constitute an easily available source to obtain a large number of transplantable cells for regenerative treatments. Nevertheless, ethical concerns and the possibility of immune rejection and teratoma/teratocarcinoma formation are major obstacles to the feasibility and safety of ESC clinical applications^[8].

Pluripotent stem-like cells could also derive from non-pluripotent cells-typically an adult somatic cell-by inducing a “forced” expression of specific genes. These induced pluripotent stem-like cells (iPS cells) are similar to ESCs in many aspects, such as the expression of certain SC genes, potency and differentiability, formation of embryoid bodies, teratomas and viable chimeras, even if the full extent of their relationship to natural pluripotent SCs is not fully elucidated; as a consequence, they cannot be currently considered a reliable and feasible source of SCs^[9,10].

Another population of SCs with high differentiation potential is represented by cells established from placental/cord tissues, which do not tend to form teratomas/teratocarcinomas and have a higher proliferation and differentiation potential than ASCs. In particular, the plasticity and accessibility of umbilical cord blood SCs (CBSCs) have given the rationale for the creation of CBSC unit banks where these cells can be collected and stored for future use^[7].

The least differentiation potential is possessed by ASCs, which persist indefinitely in the tissue of origin, allowing for local tissue regeneration and renewal^[11]. Despite the paradigm of unidirectional cell determination, recent studies have shown that ASCs are endowed with an unexpected plasticity as circulating adult progenitor cells can differentiate into mature cells of other tissue types^[5]. A particularly high degree of plasticity is shown by hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs).

HSCs are responsible for the renewal of blood cells^[12]. Commonly used markers for HSCs identification and isolation include two membrane phosphoglycoproteins: CD34 and AC133 (CD133, or “prominin1” in rodents)^[13]. It is generally accepted that the most primitive and long-term human HSCs are characterized by the expression of CD133, Thy1 (CD90) and VEGFR2 and by a variable expression of CD34 and CD38^[14,15]. Bone marrow (BM) resident HSCs can be mobilized into the peripheral blood under specific stimuli such as tissue injury or administration of mobilizing agents^[1]. *In vitro* cul-

ture and *in vivo* transplantation assays have demonstrated that HSCs are able to give rise to a wide array of phenotypes, including blood, cartilage, fat, tendon, lung, liver, muscle, brain, heart and kidney cells^[1]. Moreover, it has been demonstrated that the number of circulating HSCs expressing early markers for muscle, nerve and hepatic differentiation increases following treatment with mobilizing agents. This phenomenon has led to speculation about the existence of BM-derived circulating pluripotent SCs which could migrate from the peripheral blood into every tissue and contribute to normal turnover and repair following injury^[16].

MSCs, also called “stromal stem cells”, “stromal precursors”, “mesenchymal progenitors” and “colony-forming unit-fibroblast cells”, are highly proliferating, adherent cells which reside in a perivascular niche within the BM and also in the wall of blood vessels within most organs^[17]. MSCs can differentiate into a variety of mesodermal cell lineages, including osteoblasts, chondroblasts, adipocytes, myocytes and cardiomyocytes, as well as non-mesodermal cells, such as hepatocytes and neurons^[18]. In addition to BM, MSCs have been isolated from various adult tissues, including muscle, adipose tissue, connective tissue, trabecular bone, synovial fluid and from perinatal tissues (umbilical cord, amniotic fluid and placenta). The presence of MSCs in peripheral blood is still being debated as some authors identified a circulating fibroblast-like population, whereas others failed^[19].

SCs colocalize with supporting cells in a physiologically limited and specialized microenvironment or niche that varies in nature and location depending upon the tissue type^[20]. The reciprocal interactions between SCs and their microenvironment, through cell-cell and cell-matrix connections as well as the secretion of soluble factors, influence SC behavior, regulating the balance between quiescence and dividing state under specific pathological or physiological conditions^[5]. Understanding the molecular signals which regulate SC behavior is critical for their therapeutic applications. In fact, the exogenous stimulation with specific growth factors or cytokines may be used to activate SCs *in vivo* and *in vitro*.

DEVELOPMENT AND TURNOVER OF THE INTESTINAL EPITHELIUM

The gastrointestinal tract surface derives from the endoderm. The embryonic stratified endodermal epithelium is subsequently converted into a monolayer overlying nascent villi while dividing cells segregate to the intervillous region. Intestinal crypts develop during the early postnatal period, becoming the niche for gastrointestinal SCs^[21]. Once completely structured, the epithelium along the gut is characterized by a heterogeneous cell population, in terms of morpho-functional properties and proliferation kinetics, reflecting the various functions of the different gastrointestinal components^[7]. The adult mammalian gut can be broadly segregated into two functionally distinct parts: the small intestine and the colon,

which present with marked architectural differences, reflecting their different functions. In particular, in the small intestine, the crypts of Lieberkuhn are associated with the intestinal villi that maximize surface area, endowing the small intestine with an excellent capacity to absorb dietary nutrients from the lumen. In contrast, the absence of villi within the colonic epithelium translates to a flatter morphology, highlighting its predominant role in stool compaction^[22].

As a consequence of its role in digestion, nutrient absorption and waste excretion, the gastrointestinal tract is constantly exposed to harsh mechanical and chemical conditions. Therefore, the intestinal tract has evolved mechanisms to cope with these assaults *via* a highly regulated process of self-renewal^[23]. Mucosal proliferation plays a fundamental role in the maintenance of the gut integrity. Most of the epithelial cells are replaced every 3 to 5 d which is a high proliferation rate, second only to the hematopoietic system^[7]. According to the so-called “Unitarian hypothesis”, first proposed by Cheng and Leblond in 1974^[24], this epithelial renewal is driven by a common intestinal stem cell (ISC) residing within the crypt base at the origin of the well established crypt-to-villus hierarchical migratory pattern^[25,26]. From their niche, ISCs give rise to transit-amplifying (TA) cells that migrate upwards and progressively lose their proliferative capability and mature to become fully-differentiated villous epithelial cells (absorptive enterocytes or secretory cells which include goblet cells, enteroendocrine cells, Paneth cells and Tuft cells). Each adult crypt harbors approximately 5 to 15 ISCs that are responsible for the daily production of about 300 cells; up to 10 crypts are necessary to replenish the epithelium of a single villus^[23]. Crypt-derived epithelial cells generally reach the villus tip after 3-5 d when they die and are exfoliated into the lumen^[27], except for Paneth cells (PCs) that evade this upward migration program, instead forcing their way to the base of the crypt^[28]. PCs are confined to the small intestine where they can live for up to 8 wk^[29]. PCs are also unique in that they appear after birth during crypt emergence^[30]. PCs secrete defensins, lysozyme and phospholipase A2 and play a central role in host defense against enteric pathogens; moreover, the antimicrobial peptides secreted by PCs shape the composition of gut microbiota and protect from bacterial translocation^[29]. In addition, crypts supply less common cell types such as the M cells and cup cells, although their lineages are poorly understood^[23].

ISCS AND THEIR NICHE

ISC hierarchy

Since the 1970s, several studies have supported the concept of ISCs. The ability of SCs to regenerate gut epithelium has been investigated in various animal models of intestinal injury. Such studies have led to the hypothesis of an ISC hierarchy organized in three main compartments and progressively recruited at various degrees of

damage in order to ensure an effective crypt regeneration^[7].

The initial location for the ISCs was deemed to be the fourth cell position from the bottom of the crypt (+4) where slowly cycling cells that show label-retention of BrdU (the so-called “+4 label retaining cells”, LRCs) were described by Potten *et al*^[31] in 1974.

A second theory regarding the location of the ISCs was formulated in the same year by Cheng and Leblond^[24]. In a series of electron microscopy studies on the small intestinal crypts, these authors described slender, immature, cycling cells wedged between PCs at the positions 1-4 of the crypt base. Upon ³H-thymidine treatment, these “crypt base columnar” (CBC) cells were able to phagocytose close damaged cells; subsequently, phagosome-labeled cells were found in all intestinal epithelial lineages, suggesting the role of CBCs as ISCs.

In 2007, a Wnt-target gene encoding a leucine-rich orphan G protein-coupled receptor named *Lgr5* was identified to specifically label CBCs in the mouse small intestine^[27]. Through a lineage tracing approach, Sato *et al*^[32] demonstrated that CBCs are able to give rise to all intestinal epithelial lineages and are a self-renewing population of multipotent SCs. Further proof that *Lgr5*+ cells are ISCs derived from *ex vivo* culture assays, where single *Lgr5*+ cells were able to form self-renewing epithelial organoids highly reminiscent of crypt/villus epithelial units *in vivo*, while cells that expressed low or no *Lgr5* were unable to form such structures. Unlike LRCs, *Lgr5*+ CBCs are resistant to radiation and are rapidly proliferating, thus challenging the previously held belief that all ASCs are quiescent or slowly cycling. In 2009, lineage tracing studies also showed that some *Lgr5*+ cells co-express prominin-1 (or CD133) and these CD133+ cells can generate the entire intestinal epithelium^[33,34].

In addition to *Lgr5* and CD133, other potential ISC markers have been identified in the last years, including *musashi1* (*MSI1*), expressed by both LRCs and CBCs; *olfactomedin 4* (*OLFM4*), expressed by *Lgr5*+ cells; *PTEN*, *AKT1*, *mTERT* and *BMI1*, predominantly expressed in LRCs (for extensive revision on this topic, see^[23,35]) (Figure 1).

In 2008, Scoville *et al*^[36] proposed the coexistence of two types of ISCs: the LRCs at the +4 location that are a “reserve pool” in a prolonged quiescent state and the actively cycling CBCs able to respond to stimulating signals from their microenvironment and to provide progenitor cells on an everyday basis. To support this hypothesis, Sangiorgi *et al*^[37] found that *Bmi1*+ cells corresponding to 4+ LRCs can self-renew, proliferate, expand and like CBCs give rise to all the differentiated lineages of the small intestine epithelium. The authors concluded that +4 LRCs and CBCs are ISCs in different niches, able to migrate from one to the other^[37]. Recently, two independent groups showed a dynamic interplay between both cell populations: Tian *et al*^[38] demonstrated that *Lgr5*+ cells are dispensable for gut homeostasis and that *BMI1*+ cells are able to replenish the *Lgr5*+ cell compartment after its experimental ablation; Takeda *et al*^[39] suggested

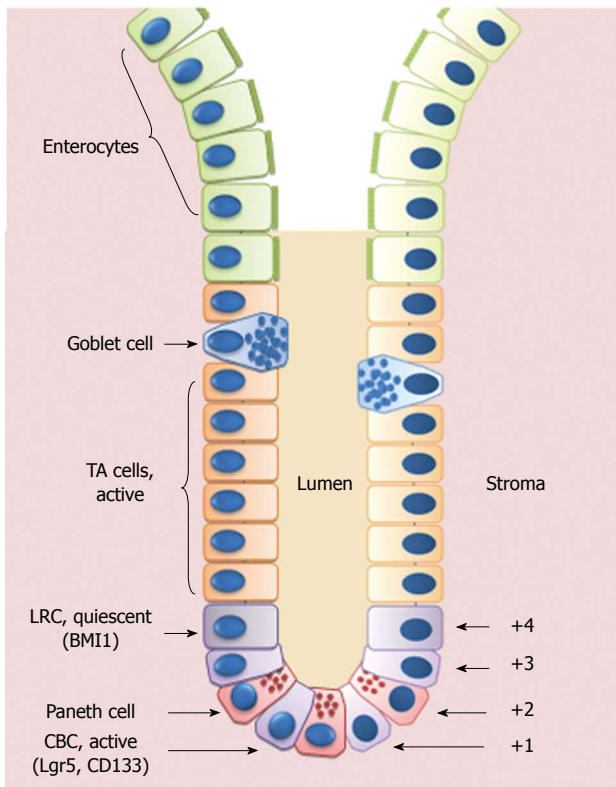


Figure 1 Schematic representation of the crypt/villus axis. Putative intestinal stem cells (ISCs) reside either at the crypt base, between Paneth cells, as Crypt Base Columnar Cells (CBCs), or in position +4 from the bottom of the crypt, as Label Retaining Cells (LRCs). ISCs give rise to Transit Amplifying (TA) cells that are able to migrate upwards and progressively mature losing their proliferative capability to become fully-differentiated villous epithelial cells.

a more complicated bidirectional relationship between *Lgr5*⁺ cells and +4 LRCs, the latter being able to either originate from or give rise to *Lgr5*⁺ cells. Whether the +4 LRCs and the CBCs truly are two distinct ISC populations and whether this is an intrinsic quality or the result of the different location within the ISC niche is still a matter of debate^[40].

A third potential source of ISCs is represented by circulating multipotent SCs of BM origin that can colonize the intestinal epithelium and contribute to its turnover and regeneration^[41-43]. BM stem cells may participate in gut repair by giving rise to ISCs through direct differentiation and also by providing supporting elements within the ISC niche, as demonstrated in different experimental models^[44-47]. However, the reduced levels of engraftment and the low rate of differentiation into intestinal cells reported in most of these studies discouraged the practical application of these cells in a clinical setting. Recently, efforts have been made to develop strategies to enhance the levels of engraftment. Zhang *et al.*^[48] demonstrated that transplantation with BM SCs genetically modified to express CXCR-4 resulted in levels of engraftment able to ameliorate radiation enteritis. Colletti *et al.*^[49] identified a marker (EphB2) for isolating and culturing an expandable subpopulation of human BM-derived SCs with enhanced intestinal homing and contribution to ISC region.

ISC niche

ISCs reside in a physiologically limited and specialized niche that dictates the mechanisms of tissue turnover and regeneration through cell-cell interactions and molecular signals^[5,50].

Traditionally, the underlying stromal cells (pericryptal myofibroblasts, enteric neurons, endothelial cells and intraepithelial lymphocytes) have been considered to constitute the niche for ISCs. Recently, it has been suggested that PCs are an essential component of the *Lgr5*⁺ ISC niche^[51]. Much evidence sustains this hypothesis. *In vivo*, the absence of PCs compromises the recovery ability, resulting in complete loss of the intestinal epithelial integrity^[52]. *In vitro*, the presence of PCs significantly increases the generation of epithelial organoids by *Lgr5*⁺ cells^[51]. PCs produce many growth factors involved in ISC maintenance and activation, including epidermal growth factor, Wnt3 and transforming growth factor- α ^[23]. The intimate relationship between PCs and ISCs seems to be involved in the response to nutritional status of the organism. Indeed, PCs can act as a “sensor” for nutritional status and enhance ISC function in response to caloric restriction^[53]. Finally, PCs seem essential to regulate ISC self-renewal by neutral competition between symmetrically dividing ISCs and a limited PC-defined niche within the crypt base^[54]. Thus, PCs serve as multifunctional guardians of ISCs by secreting bactericidal products and by providing essential niche signals. As a consequence, despite the fact that SC niches are typically portrayed as pre-existing sites to which SCs migrate^[55], ISCs are unique since they also receive niche support from their own specialized progeny of PCs.

The main molecular pathways involved in ISC regulation are Wnt, Notch, Hedgehog, Bmp and PTEN-PI3K-Akt.

Wnt signaling: Wnt signaling is based on the autocrine and paracrine interaction of secreted cysteine-rich Wnt-glycoproteins with a transmembrane Frizzled receptor (Fz). Binding of Wnt to its receptor activates the canonical pathway with stabilization and nuclear translocation of beta-catenin or the non-canonical pathway that encompasses the planar cell polarity and the Wnt/ Ca^{2+} pathway. The canonical pathway is the best characterized and most relevant in SC signaling: the binding of secreted Wnt-proteins to Fz induces nuclear translocation of beta-catenin that triggers Wnt-target gene transcription. Many studies have shown the importance of this pathway in the proliferation and differentiation of the gastrointestinal epithelium (revised in^[40,56]). Wnt signaling has different effects in different cell types, also depending on its localization along the crypt/villus axis.

Direct evidence of Wnt-activity in ISCs is their unique expression of *Lgr5*, a Wnt-target gene^[27]. Other Wnt-target genes associated with proliferation of TA-cells include *c-myc* and *cyclin D1*^[57,58]. R-spondins, glycoproteins likely secreted by enteroendocrine cells, amplify Wnt signaling, induce a proliferative response in human

intestinal epithelium, and are responsible for the expansion of organoid cultures^[59-61].

Wnt signaling is necessary for ISC proliferation and maintenance of the ISC phenotype. The previously reported PC-dependence of single Lgr5+ cells in plating efficiency can be overcome by the addition of Wnt-3 in culture^[51]. Conversely, a decreased Wnt signaling results in the loss of the proliferative compartment. Over-expression of Kruppel-like factor 4 (Klf4, a negative regulator of Wnt signals) induces cell cycle arrest, while its deletion leads to increased proliferation^[40,62,63].

Wnt signaling plays a pivotal role in cell differentiation: an overactive Wnt signaling impedes ISC differentiation and induces mislocalization of PCs, impaired goblet cell and enterocyte maturation; on the other hand, an underactive Wnt signaling induces depletion of progenitor cells, leading to the absence of properly differentiated cells^[40,62]. The development of PCs is also directly dependent on Wnt signaling^[64].

Wnt signaling is indispensable for intestinal morphogenesis and normal cell migration. Indeed, beta-catenin ensures the correct positioning of epithelial cells along the crypt/villus axis by regulating the expression of members of the Ephrin and Ephrin receptor (Eph) families^[65]. Ephrins and Eph, both membrane-bound proteins, are differentially expressed in intestinal mucosa, with Eph localized in the intestinal crypt region, while Ephrin proteins colonize the villi^[66,67]. A direct influence of EphB-signaling on ISC proliferation has been shown^[68] and it has been demonstrated that EphB3 is essential for PC downward migration^[69]. In addition to their role in promoting cell proliferation of the intestinal epithelium, tissue repair, acceleration of wound closure and maintenance of homeostasis of the intestinal barrier in adults, Ephrin/Eph signaling has been recognized to function as tumor suppressors by controlling cell migration and inhibiting tumoral invasive growth^[70-72].

Given its pivotal role within the ISC niche, it is not surprising that alterations in Wnt signaling play a pivotal role in the development of non-neoplastic gastrointestinal disorders, such as chronic inflammatory bowel disease and intestinal cancers (as reviewed elsewhere^[40,73]).

Notch signaling: Notch signaling is known to control cell fate decisions in the development of many tissues. The ligands Delta or Jagged bind the Notch receptor, thereby inducing its proteolytic cleavage; NCID, a cleavage fragment of Notch, translocates to the nucleus where it acts as a transcription factor, thus inducing the activation of molecular pathways involved in the control of proliferation and differentiation^[74]. Manipulations of the Notch signaling in experimental models revealed its role in intestinal epithelial differentiation. Hes1, the major Notch-target gene, colocalizes with Msi1 in both the CBCs and the +4 LRCs^[75,76].

Notch signaling plays a central role in preserving self-renewal in the intestinal progenitor cells by suppressing Atoh1^[40]. Notch signaling seems to trigger proliferation

of crypt progenitor cells in TA-cells and a regulated reduction of notch signaling in cooperation with activation of specific transcription factors (such as Atoh1 and neuroD) induces specific differentiation into the intestinal epithelial lineages^[40,56].

Hedgehog and BMP pathways: The morphogens Sonic Hedgehog (Shh) and Indian Hedgehog (Ihh) are secreted by epithelial cells, while their receptor, Patched (PTCH), is expressed by subepithelial myofibroblasts. In the intestinal epithelium, Ihh is mainly expressed at the base of the villi^[77]. Given the importance of the stromal-epithelial interactions in the regulation of the epithelial cell fate, hh signaling is indirectly involved in the ISC fate through its modulation of the maturation and localization of the underlying stromal cells that in turn generate signal molecules responsible for the maintenance of the ISC niche^[78]. Ihh down-regulates expression of TCF4 and beta-catenin, restricting Wnt signaling to the crypt base^[79]. Ihh also promotes maturation of the tolerogenic immune cells in the small intestine and is critical to the ability of the gut to respond to pro-inflammatory stimuli: disruption in the hh pathway may contribute to the pathogenesis of autoimmune diseases^[40,80].

Disturbed hh signaling results in severe developmental defects, enhancement of Wnt signaling, increased proliferation and structural abnormalities of crypts and villi. Such effects are mainly due to the reduced expression of bone morphogenetic proteins (BMPs) by stromal cells, which is normally triggered by hh^[81].

BMPs regulate differentiation, apoptosis and cell growth depending upon the specific cellular context. BMPs bind to BMP receptors, leading to phosphorylation of SMADs, which upon heterodimerization translocate to the nucleus and act as transcriptional factors^[82]. BMP pathway participates in the control of ISC numbers and self-renewal: active BMP signaling is found predominantly in differentiated intestinal epithelial cells, while its inhibition seems to confer intestinal stemness properties^[81]. Physiological inhibitors of BMP signaling, Noggin and Gremlin, induce Wnt signaling activation and are produced by myofibroblasts at the crypt base, ensuring a “BMP-free” ISC niche^[83]. Mesenchymal cells are the main target of BMP signaling which in turn down-regulates epithelial proliferation^[40]. Of note, the BMP pathway has a direct role in the differentiation of the intestinal epithelium toward secretory lineages (especially enteroendocrine cells), while it does not affect the absorptive phenotype^[84].

Mutations involving BMP signaling are associated with juvenile polyposis^[81]. BMPs stabilize PTEN, thereby leading to reduced Akt activity and subsequent reduction of nuclear beta-catenin accumulation^[56].

PTEN-PI3K-Akt pathway: PI3k activation leads to phosphorylation and subsequent activation of the kinase Akt, which induces cell survival, growth and proliferation programs. PTEN is a negative regulator of this pathway,

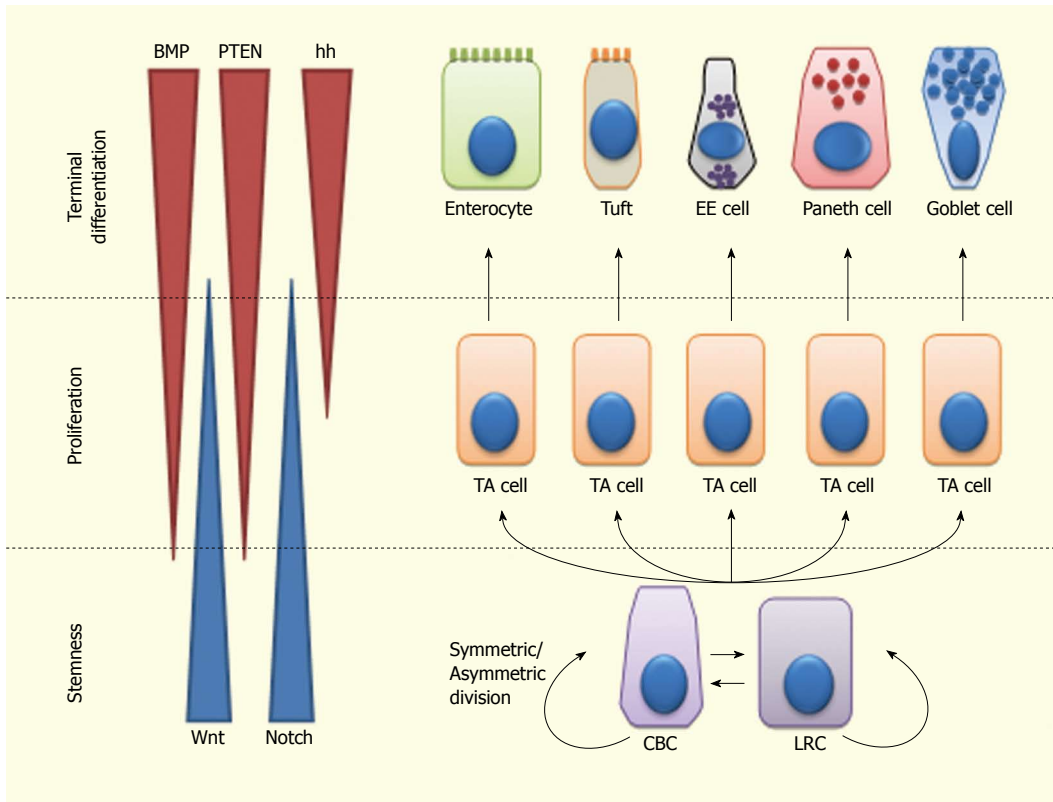


Figure 2 Lineage specification of intestinal stem cells. Intestinal stem cells (ISCs)-Crypt Columnar Cells (CBCs) and Label Retaining Cells (LRCs)-can divide asymmetrically or symmetrically to maintain the stem cell compartment. ISCs give rise to Transit Amplifying (TA) cells which actively proliferate and can further differentiate into enterocytes, tuft cells, enteroendocrine (EE) cells or goblet cells. Wnt signaling maintains the stem-like phenotype of ISCs, while Notch signaling maintains the proliferation of progenitor cells. In the upper crypt region, hedgehog (hh) triggers BMP expression in stromal cells which activates PTEN expression; all these factors inhibit Wnt signaling in the ISC niche.

thereby inhibiting Akt function^[85].

This pathway is activated in many human tumors, mainly as a consequence of PTEN inactivation^[86]. PTEN inherited mutations are responsible for hamartomatous polyps (Cowden syndrome)^[81].

As for the role of the PI3K pathway in ISC regulation, it has been demonstrated that it enhances ISC self-renewal, probably because p-Akt can increase the transcriptional activity of beta-catenin, the main effector of the canonical Wnt pathway^[87]. Moreover, PTEN might be involved in the restriction of the strong Wnt signaling to the crypt base^[88].

Overall, ISC fate is regulated by a complex balance among signals controlling stem cell maintenance, proliferation and differentiation^[40,56,89]. Wnt and Notch are mainly involved in ISC self-renewal and expansion. Moreover, Notch is involved in ISC differentiation, independently from Wnt. Notch inhibition leads to differentiation to a secretory phenotype, while Notch activation leads either to self-renewal within the ISC compartment or to differentiation towards an absorptive phenotype. The Wnt pathway is also implicated in PC differentiation and regulates cell migration along the crypt-villus axis, *via* Eph/Ephrin signaling. Hh effects on the ISCs are mainly indirect and occur through regulation of the BMP pathway. BMP signaling inhibits proliferation of ISCs, an-

tagonizing the Wnt pathway; this suggests a homeostatic function of BMP in keeping self-renewal within the ISC niche. Most likely, this interaction is mediated by PTEN inhibition of Akt, which in turn inhibits Wnt signaling. BMPs also support the differentiation of secretory cell lineages (especially of enteroendocrine cells) (Figure 2).

ISCs in GI diseases

Observations that mutations in the pathways involved in ISC maintenance occur in most colon cancers have led the majority of the research on ISC biology in humans. Alterations of ISC pathways have also been reported in inflammatory bowel diseases. In particular, decreased expression of TCF4 (Wnt target gene, correlated with defensin production) has been described in ileal Crohn's disease^[90]; increased activation of Notch and PC dysfunction have been reported in both ulcerative colitis and Crohn's disease^[29,40].

A better knowledge of ISC function and dysregulation in gastrointestinal diseases will help to understand the pathophysiology of such disorders and might also offer new insight into the development of SC-based therapies.

Theoretically, ISCs would be the best source for intestinal regeneration. Although ISCs can be expanded for multiple passages in the form of organoids, most of the

culture conditions provide little control over their self-renewal and differentiation. As a consequence, the inability to efficiently expand Lgr5+ SCs has so far considerably limited the translation to therapies as well as the study of intestinal epithelial biology. However, very recently Yin and co-workers identified small molecules (CHIR99021 and valproic acid) that target Wnt, Notch and BMP pathways to maintain the self-renewal of Lgr5+ ISC, resulting in nearly homogeneous cultures with high colony-forming efficiency and preservation of the multilineage differentiation ability^[91]. This might be a promising SC source for regenerative medicine, tissue engineering and drug screening.

So far, the only SCs that have “left the bench and reached the bedside” in gastroenterology are BM-derived SCs. BM SC transplantation has become an option for the treatment of selected cases of inflammatory bowel disorders (IBD). Experimental and clinical studies have suggested that both allogeneic and autologous BM SC transplants may be effective in inducing IBD remission^[92-94]. The mechanisms underlying this beneficial effect are still under investigation; they might include local immune-modulation and a direct contribution to tissue repair^[95,96].

BM SCs might also be used to cure other gastrointestinal pathologies, such as gastric ulcers or motility disorders, like gastroparesis, achalasia and chronic constipation^[97,98].

Finally, a promising application for SC-based therapy is celiac disease (CD). The following chapters will attempt to summarize the body of knowledge regarding CD physiopathology and clinical manifestations, as well as the status of the ISC compartment during the course of the disease and the possible SC-based treatments.

CELIAC DISEASE: FROM PATHOGENESIS TO CURRENT TREATMENT

CD likely first developed after the last ice age in the fertile crescent of the Middle East with cultivation of grains^[99]. The major breakthrough for the modern understanding of CD was the observation that bread shortages during World War II resulted in a dramatic decrease in death rate from celiac disease^[100]. Also known as “nontropical sprue”, “celiac sprue” and “gluten-sensitive enteropathy”, CD can be defined as a chronic immune-mediated disease that is triggered and maintained by dietary proteins (gluten) in genetically predisposed individuals. Patients affected by the disease display a specific autoantibody response, various degrees of intestinal inflammation and a broad range of clinical symptoms^[101,102].

Once considered a rare small bowel disease of childhood, CD is now recognized as a relatively common, systemic disease that may manifest at any age. CD affects 0.6%-1% of the population worldwide. The prevalence is up to 3-fold higher in women than in men; moreover, first-degree relatives of CD patients (10%-15%), individuals affected by autoimmune diseases, particularly type

1 diabetes (3%-16%) and Hashimoto's thyroiditis (5%), IgA deficiency (9%), Down's syndrome (5%) and Turner's syndrome (3%) are at increased risk of developing the disease. The disease is less common in Hispanic Americans and it is thought to be rare in central Africa and east Asia; the frequency of CD is increasing in many developing countries because of many factors, such as increased awareness of the disease, changes in wheat production and preparation and westernization of the diet. Interestingly, serological screening studies have shown that only a small proportion of cases of CD (up to 20%) are clinically recognized^[103].

Genetic background plays a pivotal role in the predisposition to CD: results from genetic linkage studies showed that CD is strongly associated with HLA-DQ genes (COELIAC1 locus, on chromosome 6p21). In particular, up to 90% of CD patients carry a variant of DQ2 (haplotype DQA1*0501/DQB1*0201), while about 5% of CD patients carry a variant of DQ8 (haplotype DQA1*0301/DQB1*0302); almost all of the remaining 5% of celiac patients have at least one of the two genes encoding DQ2^[101]. DQ2 and DQ8 haplotypes are necessary for the development of CD: DQ2 and DQ8, expressed on the surface of antigen-presenting cells, can bind activated (deaminated) gluten peptides, triggering an abnormal immune response. However, DQ2 is carried by approximately a third of the general population, thus suggesting that HLA is only partly the cause of the condition.

So far, more than 30 genes, mostly involved in inflammatory and immune response, have been linked to a CD predisposition^[104]. Non-HLA genes associated with CD include COELIAC2 (5q31-33) that contains cytokine gene clusters, COELIAC3 (2q33), encoding for the negative costimulatory molecule CTLA4, and COELIAC4 (19p13.1) that harbors an unconventional myosin able to alter cytoskeleton remodeling^[105,106].

Almost all patients with CD develop immunoglobulin IgA autoantibodies to the enzyme tissue transglutaminase 2 (TG), which is expressed by many cell types and is associated with the extracellular matrix (endomysium or reticulin fibers). TG targets certain glutamine residues in some extracellular and intracellular proteins, usually tethering them to a lysine residue of a second protein that results in cross-linking of both proteins. Alternatively, TG merely deaminates glutamines to negatively charged glutamine acid residues. Gluten proteins are preferred substrates for TG and once deaminated, they bind more strongly to HLA-DQ2 or DQ8 on the surface of antigen presenting cells^[105].

Serological tests are fundamental for CD screening. In patients with positive serology, a biopsy of the small intestine showing typical CD characteristics (increased number of intra-epithelial lymphocytes (IELs), elongation of the crypts and villous atrophy) is required to confirm the diagnosis. However, according to the most recent European guidelines, the confirmation biopsy is no longer required in children with predisposing HLA-genotypes, typical symptoms and a higher titer of anti-

TG (>10 times the upper limit of normal range)^[107].

CD is a “unique” model of autoimmune disease in that the key genetic components (HLA DQ2 and/or DQ8) are present in almost all patients, the autoantigen (TG) has been identified and the environmental trigger (gluten) is known. The central role of gluten in this cascade of events explains how the cornerstone of therapy for CD is a “gluten-free” diet (GFD).

Gluten is a protein complex composed of gliadins and glutenins that is responsible for the baking properties of wheat. Analysis of gliadin has identified more than one hundred components that can be grouped into four main types (omega5-, omega1, 2-, alpha/beta- and gamma-gliadins). The immunogenicity and toxicity of several gliadin epitopes has been established; although several gluten epitopes are immunostimulatory, an immunodominant peptide of 33 amino acids identified from the alpha-gliadin fraction has functional properties attributable to many proline and glutamine residues. Proline gives increased resistance to gastrointestinal proteolysis and causes a left-handed helical conformation which strengthens binding with DQ2 and DQ8 molecules on antigen-presenting cells. Additionally, glutamine residues are a preferred substrate for tissue transglutaminase-mediated deamination, which confers an enhanced immunogenicity. Storage proteins (prolamines), with similar amino acid composition to the gliadin fraction of wheat, have been identified in barley (hordeins) and rye (secalins) and show a close correlation to the taxonomy and toxic properties of wheat cereal^[102].

Gluten peptides can be transported across the intestinal epithelium either paracellularly, especially in presence of an impaired gut barrier, or *via* transcytosis or retrotranscytosis of secretory IgA through the transferrin receptor. Gluten can elicit an innate immune response in professional antigen-presenting cells (monocytes, macrophages and dendritic cells) that activates both IELs and intestinal epithelial cells. This immediate reaction might favor the development of adaptive immunity to gluten in HLA-DQ2 or DQ8 carriers^[108]. Innate immune activation of IELs by gluten induces expression of the non-classic class I molecule (MICA) on intestinal epithelium, which can in turn activate natural killer-like IELs, gamma-delta T cells and a subset of CD4+ and CD8+ T cells^[109]. Epithelial MICA and production of IL-15 by epithelial cells, macrophages and dendritic cells lead to enhanced proliferation of IELs and cytokine secretion in CD patients; moreover, IL-21, produced by CD4+ Th1 cells, acts in concert with IL-15 as an additional driving force of innate immunity in CD pathogenesis^[110].

Deamination or cross-linking of gluten by TG enhances the binding to HLA-DQ2 or DQ8 expressed by antigen presenting cells, leading to a more rigorous gluten-specific CD4+ Th1 T-cell activation^[105]. Activated gluten-reactive CD4+ T cells produce high levels of pro-inflammatory cytokines, thus inducing a Th1-pattern dominated by interferon (IFN)- γ . Th-1 cytokines promote extracellular matrix degradation and increase cyto-

toxicity of IELs and NK cells. Additionally, IFN-alpha released by dendritic cells perpetuates the inflammatory reaction by inducing CD4+ T cells to produce IFN- γ . Finally, the production of Th2 cytokines by activated CD4 T cells drives the clonal expansion of B cells and subsequent production of antigliadin and anti-TG antibodies that can form deposits in the basement membrane region of the mucosal layer, leading to cytoskeleton remodeling and subsequent epithelial damage^[102].

Clinical presentations of CD are extremely variable, reflecting the systemic nature of the disease. CD can be divided into 5 clinical subcategories: major (or classic), minor (or atypical), asymptomatic (or silent), latent and potential^[102,111].

Major CD has three distinctive features: malabsorption (diarrhea, weight loss, vitamin and nutrient deficiencies), positive serology and pathological findings of villous atrophy. A rare life-threatening manifestation of CD is the so-called “celiac crisis”, mostly observed in children that manifests with profuse diarrhea, hypoproteinemia, metabolic and electrolyte imbalances.

Minor CD may present with only trivial, transient and apparently unrelated symptoms (fatigue, anemia, abdominal discomfort, dyspepsia, altered bowel habits, cryptic hypertransaminasemia, osteoporosis, infertility, peripheral and central neurological disorders, short stature, dental enamel defects, dermatitis herpetiformis) or isolated symptoms of associated autoimmune diseases. Most of these patients are biopsied after a positive search for anti-TG and/or anti-endomysial antibodies.

Asymptomatic CD is recognized on biopsy specimens of patients with positive serology but without symptoms of disease.

Potential CD includes subjects with positive serology but normal small bowel mucosa on a gluten-containing diet in whom CD may develop later in life. Finally, the term “latent” has been attributed to a “preclinical state” of CD, usually recognized retrospectively, or to patients with an earlier presentation of CD who recover on a GFD and later remain silent when gluten is reintroduced into the diet. Potency and latency might be transient and these patients should be followed clinically since some degree of villous atrophy with variable symptoms may develop in the future in about 80% of cases^[102,111].

The only current treatment for CD involves a strict and life-long adherence to a GFD. With maintenance of a GFD, symptoms and serum celiac antibodies gradually disappear and healing of the intestinal damage typically occurs within 6 to 24 mo after initiation of the diet.

Refractory CD is diagnosed when there are persistent or recurrent malabsorptive symptoms and signs with villous atrophy detected on biopsy despite the maintenance of a strict GFD for more than 12 mo. Complications associated with untreated and/or refractory CD include ulcerative jejunoileitis, splenic hypofunction, enteropathy-associated T cell lymphoma and adenocarcinoma of the jejunum^[101].

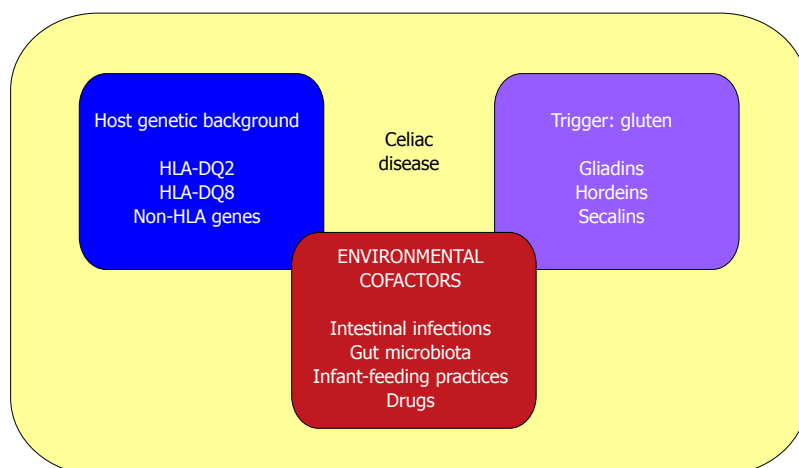


Figure 3 Causative factors in celiac disease. The pathogenesis of celiac disease involves: (1) host genetic background (HLA-DQ2 or DQ8 and other non-HLA genes); (2) an external trigger (gluten); and (3) environmental cofactors (such as intestinal pathogens, altered gut microbiota composition, infant-feeding practices and some immune-modulatory drugs).

GLUTEN EFFECTS ON EPITHELIAL BARRIER AND INTESTINAL HOMEOSTASIS

The presence of gluten in the mucosa is a prerequisite for the activation of gluten-reactive T-cells and the ensuing inflammation. However, gluten also affects the intestinal mucosa by non-immune mediated mechanisms.

It has been demonstrated that gliadin-derived cytotoxic peptides can induce oxidative stress, rearrangement of actin cytoskeleton, impairment of epithelial tight junction assembly and deregulation of the epithelial homeostasis in experiments on cultured epithelial cells and celiac mucosa^[112,113].

The oxidative stress induced by gliadin in epithelial cells might be responsible for the increased nuclear factor (NF)- κ B activity and subsequent interleukin (IL)-15 transcription that is present in the small intestinal mucosa of celiac patients^[114]. Epithelial NF- κ B activation in healthy hosts is normally suppressed by anti-inflammatory cytokines produced by underlying T lymphocytes, such as transforming growth factor (TGF)- β and IL-10. In active CD, the status of chronic inflammation and the direct toxic effects of gluten worsen the epithelial layer damage, thus causing activation of NF- κ B which leads to a vicious cycle of aberrant immune response, mucosal inflammation, increased mucosal permeability and impairment of the regenerative potential of the intestinal epithelium.

As for the alterations of the epithelial barrier, it is speculated that dysregulation of zonulin in many diseases may involve loss of cell junction integrity^[115]. The endogenous zonulin, which is functionally and immunologically related to zonula occludens toxin from *Vibrio cholera*, has been found to disassemble intercellular tight junctions *via* interaction with cell membrane receptors. Serum zonulin is up-regulated in active CD and decreases following GFD, suggesting a role for a “leaky gut” in the development of autoimmunity^[116]. Some gliadin peptides have

been shown to bind to the chemokine receptor CXCR3 on the surface of epithelial cells and induce tight junction permeability and zonulin release^[117].

Gliadin peptides can interfere with endocytic vesicle maturation and promote cell proliferation by prolonging epidermal growth factor receptor (EGFR) activation, which may correlate with the histological finding of crypt hyperplasia in CD^[118]. Interestingly, p31-43 gluten peptide stimulation on proliferation of epithelial cells *in vitro* is dependent on IL-15 activity^[108,118].

Furthermore, some toxic gliadin peptides have been reported to induce enterocyte apoptosis *via* the Fas-Fas ligand (FasL) pathway^[119]. IL-15 has also been shown to induce enterocyte MICA expression in CD patients and to trigger the anti-apoptotic pathway in human IELs, which can kill intestinal epithelial cells^[120].

ENVIRONMENTAL COFACTORS IN CELIAC DISEASE: GUT MICROBIOTA

Environmental cofactors that participate in the development and maintenance of CD include: intestinal pathogens that could enhance gluten immunogenicity and toxicity (*i.e.*, rotavirus infections^[121]); altered gut microbiota composition^[122,123]; infant-feeding practices (with a reported 50% lower risk among infants who are still being breast-fed at the time of gluten introduction^[124]); and some immune-modulatory drugs (*i.e.*, IFN- α ^[125]) (Figure 3).

In CD, the homeostatic mechanisms that allow coexistence of the host organism and the commensal microbiota are disrupted. Several studies have shown that celiac patients are characterized by a different composition of the gut microbiota when compared to healthy individuals. Rod shaped bacteria adhering to the small intestinal mucosa were frequently seen in patients with CD during the “Swedish CD epidemic”^[126]. Nadal *et al*^[127] demonstrated a higher proportion of total and gram-negative

bacteria, also including potentially pro-inflammatory bacteria (*Bacteroides-Prevotella* and *E. coli*), in active CD children *vs* symptom-free patients and controls. Schippa *et al*^[128] found a distinctive “microbial signature” in celiac patients, irrespective of the disease status. The duodenal mucosa of CD patients showed a higher diversity of associated bacteria population; *Bacteroides vulgatus* and *E. coli* were detected more often in celiacs than in controls^[128].

The changes detected in gut microbiota of CD patients could be either a consequence or a cause of the disease. In the first scenario, the damaged mucosa covered by immature enterocytes would facilitate gram-negative bacterial colonization to the detriment of gram-positive bacteria. In the second case, the predominant colonization of gram-negative bacteria in genetically predisposed individuals would contribute to the loss of tolerance to gluten. Indeed, changes in resident microbiota composition seem to precede the onset of the disease and as such, they might be a risk factor for the development of celiac disease in susceptible individuals. Of note, interplay has been observed between HLA genes and milk feeding practice for microbial colonization that could influence the manifestation of the disease. The PROFICEL study demonstrated that infants at high genetic risk have higher numbers of *B. fragilis* and *Staphylococcus spp.* and reduced numbers of *Bifidobacterium spp.*; breast-feeding promoted colonization of *Bifidobacteria*, while formula-feeding promoted that of *Bacteroides fragilis* and *E. coli*, among others. In breast-fed infants, the increased genetic risk was associated with increased *C. leptum* group numbers, while in formula-fed infants it was associated with increased *Staphylococcus* and *B. fragilis* group numbers. Finally, breast-feeding reduced the genotype-related differences in microbiota composition, which could partly explain the protective role attributed to breast milk in this disorder^[129].

Through a process of “cross-talk” with the mucosal immune system, gut microbiota negotiates mutual growth, survival and inflammatory control of the intestinal ecosystem. The intestinal mucosa is equipped with transmembrane and intracytoplasmic receptors referred to as pattern/pathogen recognition receptors (PRRs) that are defined by their ability to specifically recognize and bind distinctive microbial macromolecular ligands (microbial-associated or pathogen-associated molecular patterns, MAMPs or PAMPs), such as LPS, flagellin, peptidoglycans and formylated peptides. Subsequent signaling consists of an intricate and inter-relational pathway which determines the signaling output based on the initial perception of the triggering organism. Output can be a protective response to commensal microbiota, an inflammatory response to pathogenic organisms, or a trigger for apoptosis. Intestinal epithelial cells express high levels of the Toll-like receptor (TLR) inhibitor and Toll-interacting protein (TOLLIP). Expression of TOLLIP has been shown to correlate with the *in vivo* luminal bacterial load and is highest in healthy colonic mucosa; this inhibitory molecule is important in maintaining microbial homeo-

stasis.

Many studies have demonstrated that the expression of TLRs is deregulated in active CD, suggesting that microbiota-associated factors may be important in the development of the disease. Higher densities of TLR4+ cells were found in active CD patients *vs* controls^[130]. Recently, Kalliomäki *et al*^[131] demonstrated that expression of IL-8 mRNA (marker of intestinal inflammation) and TLR-2 mRNA significantly increased in duodenal biopsies of active celiacs compared with treated celiacs and controls, while expression of TOLLIP mRNA was down-regulated.

The CD-associated bacteria and the dysbiosis they might cause in the resident microbiota through TLR/PAMP interactions might contribute to the Th1 pro-inflammatory milieu characteristic of CD. Medina *et al*^[132] showed that gut microbiota from both active and treated CD patients increased TNF- α and IFN- γ production and decreased IL-10 production and CD4 expression in peripheral blood mononuclear cells compared with control samples. Interestingly, probiotics (*Bifidobacterium* strains) suppressed this pro-inflammatory cytokine pattern and increased IL-10 production. Similar beneficial effects of *B. longum* were found in an animal model of gliadin-induced enteropathy^[133].

ISC MODULATION IN CD

Despite the many achievements in understanding of the pathogenic interactions among genetic, immunological and environmental factors in CD, little is known about ISC modulation and deregulation during the course of the disease.

In the last years it has been observed that ISC differentiation towards PCs and goblet cells may be disturbed in active CD. This may result in a defective antimicrobial and mucus barrier which enables the intestinal bacteria to invade the mucosa and trigger the inflammation. Indeed, the expression of natural antibiotics such as defensins is limited in CD. In particular, it has been demonstrated that some beta-defensins are underrepresented among celiac patients and that their expression correlated negatively with the degree of villous atrophy and rose on GFD; this suggests that increased copy numbers could protect from CD, possibly by impeding bacterial infiltration more efficiently and preserving gut epithelial integrity^[134-136].

As for PC deregulation in CD, a number of studies have reported conflicting results. The earliest reports described the disappearance of PCs in patients with refractory CD and a significant decrease in patients with untreated and treated CD. However, later studies did not confirm a numeric reduction of celiac PCs and some authors even hypothesized that PCs would be increased in active CD given the high level of α -defensins found in untreated celiac mucosa^[137]. Di Sabatino *et al*^[137] adopted a multiple histochemical approach and showed no change of PC numbers in uncomplicated treated or untreated CD *vs* normal controls, while they observed a signifi-

cant decrease of PCs in patients with complicated CD; of note, this decrease did not correlate with the degree of mucosal damage or with the duration of GFD. The proliferative pattern of PCs was not statistically different among the various groups, while crypt enterocyte proliferation was significantly higher in uncomplicated, untreated CD in comparison with treated CD and control cases^[137]. More recently, Rubio found that in active CD patients, the normal production of PCs in the crypts is replaced by lysozyme-producing mucus cells. The author speculated that in CD, ISCs are re-programmed as an antimicrobial adaptation to signals generated by pathogenic duodenal bacteria^[138]. The molecular mechanisms behind the abrogation of PCs in duodenal crypts and their substitution with lysozyme-producing mucus cells in CD remain to be elucidated. Further studies are needed to clarify the exact entity of PC deregulation in CD, the underlying molecular pathways and its implications in terms of ISC fate.

The intriguing hypothesis that PC secretion might be involved in the control neoplasia, thus accounting for the low incidence of neoplasms in the small bowel, encourages further investigation of the relationship between PC deficiency and premalignant and malignant complications of CD, as well as other inflammatory bowel disorders.

Regarding the deregulation of goblet cells in CD, Cinova *et al*^[139] observed that gliadin fragments and/or IFN- γ were able to reduce the number of PAS-positive goblet cells and increase mucin secretion in rat intestinal loops; interestingly, these changes were more pronounced in the presence of potentially pathogenic enterobacteria, while the decrease in PAS-positive goblet cells by gliadin was reversed by probiotics (*B. bifidum* LATA-ES2).

The molecular mechanisms underlying the deregulation of ISC differentiation in CD are still being elucidated. Capuano *et al*^[140] assessed the miRNA-based modulation of gene expression in the celiac small intestine for genes involved in intestinal differentiation and proliferation. They found a downregulation of the Notch pathway and KLF4 signals in celiac patients, whereas more nuclear beta-catenin staining (a sign of Wnt signaling activation) and more Ki67 staining (a sign of cell proliferation) were present in crypts from celiacs than in controls. Moreover, they documented a reduction of the number of goblet cells in the small intestine of children with active CD and in those on a GFD compared to controls. The authors postulated that the Notch pathway could be constitutively altered in CD and that it could drive the increased proliferation and the decreased differentiation of ISCs towards the secretory goblet cell lineage^[140].

Another reported ISC niche alteration in CD regards the mucosal vasculature in the small intestine of active celiac patients that differs considerably from normal. Indeed, in celiac mucosa the capillary tufts are totally missing and the entire vasculature is disorganized. Myrsky *et al*^[141] reported that IgA and anti-TG from CD patients disturb several steps of angiogenesis (sprouting and migration of endothelial and vascular mesenchymal cells)

and also induce disorganization of the actin cytoskeleton *in vitro*. This disturbance of the angiogenic process could lead *in vivo* to the disruption of the mucosal vasculature seen in active CD^[141].

Finally, little is known about the contribution of BM-derived SCs in CD. Mastrandrea *et al*^[142] showed an increased traffic of circulating CD34+ HSCs in active CD patients *vs* healthy controls, but no correlation was found with anti-TG levels or histological severity. The authors postulated that this increased traffic of HSCs was more related to a defect shared by chronic inflammatory diseases than to a gliadin-specific Th1 local reaction. They hypothesized that the prevalence of apoptotic *vs* survival programs leading to excessive cell death in active CD might induce the mobilization of BM multipotent SCs as a supplementary source of ISCs for intestinal repair^[142].

The potential contribution of extra-intestinal SCs in gut regeneration offers new insights into the development of SC-based treatments against CD.

NOVEL THERAPIES FOR CD: A ROLE FOR STEM CELLS?

Adherence to a strict GFD might restrict social activities and limit nutritional variety. Additionally, it is costly and difficult to maintain in many countries. In the last years, alternative therapeutic strategies have been tested. These include intraluminal therapies (genetic modification of wheat and/or pretreatment of flours to reduce immunotoxicity, oral enzyme therapy, intraluminal binding of gluten peptides, neutralizing gluten antibodies), transepithelial treatments (inhibition of intestinal permeability through zonulin receptor antagonists) and subepithelial actions (TG inhibitors, gluten peptides that downregulate innate responses, HLA-DQ2 inhibitors, CCR9 and integrin antagonists, IL-15 antagonists, anti-IFN- γ antibody, anti-CD3, anti-CD4 and anti-CD25 antibodies). Such approaches have been tested in experimental models and in small clinical trials with inconclusive results overall in terms of efficacy; moreover, some of these treatments have a poor safety profile and their hypothetical use should be reserved for complicated forms of CD^[102,105].

Novel treatments for CD might derive from SCs. Indeed, the advancements in SC biology have led to the concept of regenerative medicine which is based on SC potential for therapies aimed at facilitating the repair of injured tissues^[143]. Such therapies require a deep knowledge of the dynamics underlying SC compartment regulation, both in physiological and pathological conditions.

A potential therapeutic avenue for CD is the discovery of epithelial mitogens that stimulates mucosa growth. Recently, R-spondin-1 has been shown to stimulate crypt cell growth, accelerate mucosal regeneration and restore intestinal architecture in experimental colitis in mice^[144]. In CD, the infusion of such mitogens might help to accelerate intestinal healing.

Another potential SC-based therapy for CD is transplantation of multipotent extraintestinal SCs of BM

origin that can contribute to intestinal repair. In the last two decades, BM-derived SC transplantation has become an option for patients with severe autoimmune diseases refractory to conventional treatments. Such a therapy has recently found an application in gastroenterology for the treatment of selected cases of complicated CD. Bishton *et al*^[145] reported the efficacy of autologous HSC transplantation preceded by conditioning in patients with enteropathy-associated T cell lymphoma: 4 out of 6 patients remained in a sustained complete remission for up to 4 years. Kline *et al*^[146] treated one celiac patient affected by acute myelogenous leukemia with allogeneic HSC transplant preceded by conditioning and achieved correction of CD despite the reintroduction of a gluten-containing diet. Similarly, Hoekstra *et al*^[147] reported that in one patient with severe aplastic anaemia and CD, allotransplant of SCs resulted in the cure of CD even after the return to a free diet. Recently, Ciccocioppo *et al*^[148] showed that in 2 patients affected by CD and β -thalassemia major who underwent successful myeloablative allogeneic HSC transplantation for the latter condition, the introduction of a gluten-containing diet did not cause the reappearance of clinical, serological and histological markers of CD in up to 5 years of follow-up. Al-Toma *et al*^[149] subjected patients with refractory CD type II to autologous peripheral blood SC transplant after conditioning: 6 out of 7 patients obtained a significant reduction in aberrant T cells in duodenal biopsies, associated with clinical improvement. Similar results were obtained by Tack *et al*^[150] in refractory type-II CD patients who showed an impressive clinical improvement upon auto-transplant of HSCs.

Despite these encouraging results, further studies and longer follow-up periods are required to confirm the efficacy of HSC infusion in refractory and complicated CD.

The molecular mechanisms underlying the beneficial effects of HSC transplantation in CD are still largely unknown. It has been postulated that the immune system ablation followed by HSC transplantation provides a reset of the host immune system imbalance; this effect is likely to be more pronounced and prolonged in cases of allogeneic transplantation when HSCs that do not carry any CD-predisposing polymorphism are infused upon conditioning. Moreover, BM cells might contribute to tissue repair by differentiating into epithelial cells and myofibroblasts and by facilitating the neoangiogenesis^[92,95].

CONCLUSION

SCs are the key to tissue genesis and regeneration. Given their central role in homeostasis, SC dysfunctions are involved in the pathogenesis of virtually all diseases, from cancers to degenerative disorders to chronic inflammatory pathologies. Ten years ago, SC research was compared to a “Pandora’s vase”, the opening of which could make it possible to clarify the nature and the pathophysiology of all human disease. If SCs are the source of all pathology, they can also be the ultimate cure; this is the pre-condition of regenerative medicine which is based on SC

potential for tissue renewal and regeneration^[2,7].

Despite the efforts made during the last 30 years, the body of knowledge of the physiology of ISC and their involvement in bowel disorders appears fragmentary, incomplete and sometimes contradictory. As for CD, the role of ISCs and their niche in the development and maintenance of the disease is far from being elucidated and the clinical applications of SC-based treatments for CD are limited to a few case reports and uncontrolled trials, with small numbers of subjects affected by complicated disease.

Nonetheless, the expectations of the general population for SC-based therapies against CD are very high. Among the families that collect and bank cord blood for private storage, potential treatment of CD is the second most common motivation (19.7%)^[151]. This imposes a careful consideration: further studies are needed to clarify the complex interplay among gluten, gut microbiota, gut barrier, immune system and ISC modulation and deregulation in CD. Such knowledge should be the basis for any potential clinical application of SCs against CD in order to avoid an “excess of enthusiasm” that might get “the better of judgment”.

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Adipose-derived stem cells: Implications in tissue regeneration

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Abstract

Adipose-derived stem cells (ASCs) are mesenchymal stem cells (MSCs) that are obtained from abundant adipose tissue, adherent on plastic culture flasks, can be expanded *in vitro*, and have the capacity to differentiate into multiple cell lineages. Unlike bone marrow-derived MSCs, ASCs can be obtained from abundant adipose tissue by a minimally invasive procedure, which results in a high number of cells. Therefore, ASCs are promising for regenerating tissues and organs damaged by injury and diseases. This article reviews the implications of ASCs in tissue regeneration.

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Key words: Mesenchymal stem cells; Adipose-derived stem cells; Differentiation; Growth factors; Tissue engineering; Clinical trials

Core tip: This review article provides an overview on adipose-derived stem cells (ASCs) for implications in

tissue regeneration. ASCs are obtained in high yields from abundant adipose tissue in the body and have multi-lineage differentiation ability. This article focuses on ASC characterization, growth factor secretion from ASCs, differentiation ability *in vitro* and *in vivo*, and the potential clinical applications.

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INTRODUCTION

Mesenchymal stem cells (MSCs) are adult stem cells that were originally identified in bone marrow as multi-potent cells^[1,2]. Stem cells are characterized by their self-renewal ability and multi-potency. Bone marrow-derived stem cells are most broadly studied for therapeutic potentials since their discovery in the 1960s^[1]. After the discovery of bone marrow-derived MSCs, MSCs have been isolated from nearly every tissue in the body^[3], for example, adipose tissue^[4], umbilical cord blood^[5], peripheral blood^[6], dental pulp^[7], dermis^[8], and amniotic fluid^[9], and even in tumors^[10]. Adipose-derived stem cells (ASCs) were first identified as MSCs in adipose tissue in 2001^[11], and since then adipose tissue has been studied as a cell source for tissue engineering and regenerative medicine. There are multiple terms for stem cells derived from adipose tissue, for example, preadipocytes, adipose-derived stromal cells, processed lipoaspirated cells, adipose-derived mesenchymal stem cells, adipose-derived adult stem cells. In 2004, the consensus was reached the term as ASCs.

There are several types of adipose tissue, with subcutaneous as the most clinically relevant source. ASCs can be isolated from subcutaneous adipose tissue of the abdomen, thigh, and arm. Because adipose tissue is typi-

cally abundant in the human body, ASCs can potentially be isolated in high numbers. The multi-lineage capacity of ASCs offers the potential to repair, maintain or enhance various tissues. This review article will focus on source, isolation, and characterization of ASCs, secretion of growth factors from ASCs, *in vitro* and *in vivo* differentiation ability of ASCs, and the potential clinical application.

SOURCE, ISOLATION, AND CHARACTERIZATION OF ASCS

There are mainly two types of adipose tissue: white adipose tissue and brown adipose tissue. They are morphologically and functionally different. Brown adipose tissue much less abundant than white adipose tissue, but can be found in the neck, mediastinum, and interscapular areas in neonates. However, brown adipose tissue undergoes a morphologic transformation with aging. The appearance of brown adipose tissue is literally brown. Brown adipocytes are multilocular and retain small lipid vacuoles compared to white adipocytes. Vascularization is obvious because brown adipose tissue requires much more oxygen consumption compared to other tissues. Brown adipocytes have no known correlation with insulin resistance. The main function of brown adipose tissue is thermogenesis^[12,13]. Brown adipose tissue contains a large number of mitochondria and expresses uncoupling protein 1 (UCP1). UCP1 is a brown adipose tissue-specific marker, not expressed within white adipose tissue. UCP1 is expressed in the inner membrane of mitochondria, mainly regulated by adrenergic signaling through sympathetic innervations, and this signaling is responsible for thermogenesis^[12,13]. Brown adipose tissue is activated by thyroid hormone, cold temperatures, thiazolidinediones, and activated brown adipose tissue is inversely correlated with body mass index, adipose tissue mass and insulin resistance.

White adipose tissue is found throughout the body, representatively in subcutaneous and visceral adipose tissue. The appearance of white adipose tissue is yellow or ivory. White adipocytes are unilocular and contain large lipid vacuoles. White adipose tissue function is to store excess energy in the form of triglycerides, and its hyperplasia causes obesity and dysfunction of metabolic pathways as insulin resistance. UCP1 is not expressed in white adipocytes but the isoform UCP2 is expressed in parts of white adipocytes.

Recently, beige adipocytes have been discovered within white adipose tissue, especially inguinal white adipose tissue^[14]. Beige adipocytes have the characteristics of both brown and white adipocytes. Beige adipocytes contain both unilocular large and multiple small lipid vacuoles. Its function is adaptive thermogenesis. In response to cold temperature exposure, beige cells transform into cells which have brown adipose tissue-like characteristics, such as UCP1 expression and small lipid vacuoles^[15]. It is still controversial whether the beige adipocytes arise through

the transdifferentiation of white adipocytes or by *de novo* adipogenesis from a subgroup of precursor cells^[16,17].

ASCs isolated from white adipose tissue have different characteristics from those isolated from brown adipose tissue, just as ASCs from different anatomical areas have different characteristics. Subcutaneous tissues are easily obtained *via* lipoaspiration and usually discarded after the surgery. The lipoaspiration technique does not affect function of ASCs, but the vacuum process does damage mature adipocytes^[18].

Zuk *et al.*^[4] developed a widely used method for isolating ASCs from white adipose tissue in 2001. Adipose tissues are minced and then undergo enzymatic digestion with collagenase type II. After centrifugation, the resulting pellet is called the stroma vascular fraction (SVF). Approximately 2 to 6 million cells in SVF can be obtained from one milliliter of lipoaspirate^[19]. SVF contains ASCs, endothelial cells, endothelial progenitor cells, pericytes, smooth muscle cells, leukocytes, and erythrocytes^[20]. ASCs are obtained as the plastic-adherent population after overnight culturing.

Stem cell yield is higher from adipose tissue than bone marrow—both for aspirated and excised adipose tissues. One gram of aspirated adipose tissue yields approximately 3.5×10^5 to 1×10^6 ASCs. This is compared to 5 hundred to 5×10^4 of bone marrow-derived MSCs (BM-MSCs) isolated from one gram of bone marrow aspirate^[21]. However, ASC yield from lipoaspirated adipose tissue has been reported to be approximately one half that isolated from whole, excised adipose tissue^[22]. ASCs are isolated from the SVF after plating, as ASCs adhere fairly quickly to the surface of tissue culture-treated flasks. ASCs are easily cultured and expanded *in vitro*; average doubling time of cultured ASC varies between 2 to 5 d, depending on passage number and culture medium^[23,24]. ASCs can be easily cryopreserved in a media containing serum and dimethylsulfoxide. Proliferation and differentiation of ASCs are not affected by cryopreservation^[25]. The morphology of ASCs is spindle-shaped, very similar to BM-MSCs.

One notable characteristic of ASCs is that they are not homogenous population^[11,26]. Many studies have attempted to characterize ASCs using cell surface markers *via* flow cytometry analysis, but a unique single marker has yet to be identified. ASCs have a positive expression of CD34 at the first passage of culture, but CD34 expression decreases after passaging^[20,23]. ASCs express typical mesenchymal markers such as CD13, CD29, CD44, CD63, CD73, CD90, and CD105, and ASCs are negative for hematopoietic antigens such as CD14, CD31, CD45, and CD144^[4,23,27]. After culturing and passaging, ASC's surface markers can change with passaging. The expression of hematopoietic markers such as CD11, CD14, CD34, and CD45 dissipates or are lost^[28]. On the other hand, the expression level of CD29, CD73, CD90, and CD166 increase from the SVF to passage 2^[23]. Passaging is considered to select cell population with more homogenous cell surface markers compared to SVF.

Further characterization of the heterogeneous ASC

population has been recently reported. Li *et al.*^[26] categorized four ASC subpopulations: pericytes as CD146⁺/CD31⁻/CD34⁻, mature endothelial cells as CD31⁺/CD34⁻, premature endothelial cells as CD31⁺CD34⁺, and preadipocytes as CD31⁻/CD34⁺. The highest subpopulation was preadipocytes with 67.6%, premature endothelial cell was the second highest subpopulation with 5.2%, and the percentage of pericytes and mature endothelial cells were less than 1%. The cells with CD31⁻/CD34⁺ expression demonstrated the greatest proliferation and highest adipogenic differentiation. The localization of ASCs within adipose tissue is not totally clarified yet, but the niche of ASCs is suggested to be in the vasculature of adipose tissue^[29]. Histological analysis also suggested that ASCs reside within adipose tissue in a perivascular location^[30,31]. Traktuev *et al.*^[31] concluded that the location of the ASCs in the vessel is at interface between endothelium and adipocytes, and ASCs have the ability to both support vascular structure and generate adipocytes.

ASC GROWTH FACTOR SECRETION

ASCs are considered to be a mediator of tissue regeneration through the secretion of specific soluble factors. ASCs secrete multiple growth factors, including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1, hepatocyte growth factors (HGF), and transforming growth factor (TGF)- β ^[32]. The expression of cytokines renders ASCs promising therapies for transplantation and ischemia patients. Transplanted tissues and organs are exposed to hypoxia soon after transplantation due to a lack of initial vasculature and tend to undergo apoptosis^[33]. The levels of VEGF, bFGF, and HGF secreted by ASCs are reported to be upregulated by hypoxia. VEGF was secreted at the concentrations of 70.17 and 200.17 pg/mL under normoxia and hypoxia, respectively. Basic FGF was secreted at the concentrations of 10.62 and 24.75 pg/mL under normoxia and hypoxia, respectively^[34]. Similarly, Rehman *et al.*^[35] reported that ASCs secreted significant levels of VEGF and HGF under hypoxia, which induced the healing mice hindlimb ischemia. ASCs are considered to be a cell source that induces angiogenesis, which is actually used for human ischemia treatment^[36].

In addition to growth factor secretion, ASCs are responsive to growth factors, including enhancing proliferation by bFGF, and platelet-derived growth factor (PDGF). Basic FGF is released from an injured extracellular matrix^[37]. PDGF is released from activated platelets on bleeding^[38]. When ASCs are exposed growth factors, tissues can be regenerated more effectively. Kaewsuwan *et al.*^[39] studied effect of six growth factors on the proliferation of ASCs, and found that PDGF-BB had the highest stimulatory effect at the concentration of 10 ng/mL. PDGF receptors α and β are expressed in ASCs, and PDGF-BB and PDGF receptor β signaling is involved in the stimulation of ASCs^[39]. Besides PDGF receptors α and β , ASCs express VEGF, HGF, epidermal growth factor (EGF), and bFGF receptors^[40,41]. VEGF increases

migration and promotes chondrogenic differentiation^[41], and HGF promotes hepatogenic differentiation of ASCs *in vitro*^[42]. EGF inhibits ASC adipogenic differentiation, and bFGF increase ASC proliferation, promotes adipogenic and chondrogenic differentiation, and inhibit osteogenic differentiation *in vitro*^[43-47].

ASCs possess unique paracrine characteristics. ASCs secrete growth factors that stimulate recovery of damaged tissue. Furthermore, ASCs express several kinds of growth factor receptors and are sensitive to growth factors. Therefore, ASCs mediate tissue regeneration.

IN VITRO DIFFERENTIATION ABILITY OF ASCS

ASCs can be differentiated into multiple lineages under culturing with specific conditions^[11], which results in the potential of ASCs for multiple clinical applications. The induction of ASC differentiation *in vitro* is achieved by culture with media containing selective lineage-specific induction factors. ASCs have been shown to be differentiated into cells of ectodermal, endodermal and mesodermal origin^[4,48,49]. Less controversial is the differentiation of ASCs into adipogenic, chondrogenic, and osteogenic cells, because ASCs are of mesodermal origin. With a combination of morphological observation, immunofluorescence, and polymerase chain reaction (PCR) analysis *in vitro*, adipogenic, osteogenic, and chondrogenic potentials of ASCs has been reported^[4,11]. As mentioned above, MSCs from different anatomical sources demonstrate some differences. ASCs have prominent adipogenic differentiation ability compared to BM-MSCs *in vitro*^[50,51]. BM-MSCs have been shown to have higher osteogenic differentiation ability compared to ASCs^[51-53].

ASCs can be differentiated into adipocytes when cultured in adipogenic differentiation media, which typically contains isobutyl-methylxanthine, insulin, and indomethacin^[54]. ASCs develop multiple lipid droplets about 7 d following exposure to the induction media, and the number of lipid droplets gradually increases. By 2 to 3 wk, the lipid droplets begin to form a unilocular lipid. During differentiation into mature adipocytes, ASCs express several types of extracellular matrix (ECM) proteins, including fibronectin, laminin, and various types of collagen. During adipogenesis, a fibronectin network develops first, and a type- I collagen network is formed last^[55]. These ECMs allow ASCs to differentiate into mature adipocytes. ASCs show promise for soft-tissue applications. Lipid droplets contain triglycerides, and can be easily confirmed histologically using Oil red O and Sudan III staining. Gene expression that is specific to mature adipocytes includes peroxisome proliferator activated receptor (PPAR)- γ 2, leptin, aP2, and glucose transporter type 4^[56]. The real-time PCR study showed that the expression levels of PPAR- γ 2 in ASCs isolated from female mice were higher than in those from male mice, suggesting that adipogenic differentiation of ASCs is closely related to gender^[57].

When ASCs are cultured in osteogenic differentiation media, which may contain 1,25-dihydroxyvitamin D3, ascorbate-2-phosphate, and bone morphogenetic protein-2 (BMP)-2, for 2 to 4 wk, the cells differentiate into osteoblast-like cells *in vitro*^[58]. After differentiation, the osteoblast-like cells start to produce calcium phosphate within the ECM which can be assessed with Alizarin Red or von Kossa staining to reveal osteocytes. Alkaline phosphatase, type I collagen, osteopontin, osteocalcin, bone sialoprotein, Runx-1, BMP-2, BMP-4, parathyroid hormone receptor, BMP receptor 1 and 2 are common genes that are up-regulated during osteogenesis^[56]. Furthermore, male ASCs differentiate into bone more rapidly and more effectively than female ASCs^[59].

For chondrogenic differentiation, cells typically require a 3D environment, such as an "aggregate culture" or "micromass pellet culture". The micromass pellet culture model mimics precartilaginous condensation during embryonic development, which increases the cell-to-cell interaction and leads to the production of a cartilage-like matrix^[60]. Chondrogenic differentiation requires the use of a defined media supplemented with TGF- β 1, insulin, dexamethasone, ascorbate-2-phosphate, and BMP-6. Basic FGF can be used to expand ASCs, and at the same time, down-regulate chondrogenic markers during cell expansion^[61]. Differentiated chondrocytes express type II collagen, type IV collagen, aggrecan, prolyl endopeptidase-like, and sulfate-proteoglycan^[62]. Alcian blue and collagen type II staining indicate chondrocytes.

Although somewhat controversial, ASCs may possess ectodermal differentiation capacity, *e.g.*, neurogenesis. Many studies have been reported^[48,63,64]. Under culture conditions with media containing butyric acid, valproic acid, and insulin, ASCs become morphologically similar to neurons, and express markers of both neuronal (neuron-specific enolase, nestin, and NeuN) and glial lineages [S100, p75, nerve growth factor (NGF), receptor, and NG2]^[48]. The differentiation of ASCs into Schwann cells that are capable of myelinating peripheral neurons has been reported^[48]. Human ASCs form nestin-positive neurospheres and express Schwann cell markers including S100, glial fibrillary acidic protein, and the p75 NGF receptor after dissociation.

In addition to mesodermal and ectodermal capacity, the endodermal differentiation of ASCs has been reported. Numerous studies reported differentiation of ASCs into hepatocytes and beta islet cells^[42,65,66]. In an environment with the differentiation factors activin-A, exendin-4, HGF, and pentagastrin, ASCs were demonstrated to differentiate into insulin-producing cells *in vitro*^[66]. Meanwhile, adding HGF, oncostatin M, and dimethyl sulfoxide in the culture media resulted in the ability of ASCs to gain hepatocytic functions *in vitro*, including albumin and alpha-fetoprotein expression and urea production^[42].

IN VIVO DIFFERENTIATION ABILITY OF ASCS

While *in vitro* differentiation of ASCs into multiple phe-

notypes has been reported, the *in vivo* translation can be challenging. While ASCs have many advantages as a cell source, (*e.g.*, easily harvested, abundant, and easy to culture), there remains the challenge of cell survival *in vivo*. Poor cell survival after *in vivo* injection or implantation is common. This is in part due to the hypoxic environment, particularly if cells are transplanted into ischemic tissues. ASCs have been shown to survive in ischemic tissues, whereas mature adipocytes die easily under ischemic conditions^[67], ASCs also secrete angiogenic factors under hypoxic conditions^[34,35]. For certain clinical applications, ASC implantation may require suitable biomaterial scaffolds that support cell attachment, proliferation, and differentiation. The scaffold should be selected based on numerous characteristics, such as porosity, bioactivity, mechanical integrity, biodegradability, and low immunogenicity. Ideal scaffolds can provide cells with an environment suitable for cell survival^[68]. The environment immediately following implantation can be severe for the cells to survive because oxygen and nutrients are insufficient. Implanted cells need to survive until effective angiogenesis occurs. As described above, ASCs secrete significant levels of angiogenic factors under hypoxia. ASCs can survive in an ischemic environment, and provide a reservoir of growth factors that are necessary for angiogenesis.

ASCs have immense potential in wound healing applications. Altman *et al.*^[69] grafted an acellular dermal matrix construct seeded with human ASCs into a murine injury model, and found that ASCs enhanced the wound healing at day 7. Most of the ASCs were viable 2 wk after the engraftment. An appropriate scaffold contributed to ASC homing and surviving. ASCs grafted in the wound can result in the augmentation of the local blood supply and in an improvement of regeneration capacity.

As ASC differentiation into adipocytes is well established, adipose tissue regeneration using ASCs *in vivo* has been investigated. Clinical applications include soft tissue augmentation after injury, surgical resection, and congenital malformations. Among the strategies to generate adipose tissue are the combination of ASCs and scaffolds, the use of acellular scaffolds, and the addition of drugs or growth factors to the scaffolds that have been examined include type I collagen, fibrin, silk fibroin, alginate, hyaluronic acid, and matrigel^[70-72]. Injectable scaffolds are an attractive option, as minimally invasive therapies would be widely adapted by surgeons. Methods of drug delivery include using polymeric microspheres to control the release of factors such as bFGF, insulin, and dexamethasone^[73-75].

Regarding osteogenic potential, ASCs show promise for bone tissue regeneration after injection or congenital malformations. Since ASCs were discovered to have osteogenic potential, many *in vivo* studies have combined ASCs with biodegradable scaffold materials to promote bone growth. Immuno-deficient animal models for nonweight-bearing bone formation have become a common model to assess human ASC osteogenic potential *in vivo*. Because bone is composed of hydroxyapatite (HA)

crystals, bioceramics such as HA and beta-tricalcium phosphate are used for bone regeneration. The ceramic biomaterials used in these applications should mimic the natural bone architecture to ensure ASC attachment and migration within porous materials. In addition, ceramic biomaterials should be absorbed overtime or integrated with the surrounding tissue and eventually replaced by new or existing host tissue. As collagen is the other main component of bone tissue, it has been widely studied as a natural biomaterial scaffold for bone regeneration. In contrast, synthetic polymers such as poly (L-lactic acid-co-glycolic acid), and poly-ε-caprolactone (PCL), can also be utilized for bone engineering. The advantage of these polymers is that they are readily reproducible, and have flexible mechanical, chemical, and biological properties that allow them to be tailored to suit specific functions^[76].

There has been much interest in examining ASCs for the cartilage tissue engineering required to remedy osteoarthritis (OA), which affects millions of patients all over the world. A cure for OA remains elusive, because, in part, while the cartilage ECM is maintained by a sparse population of chondrocytes, it exhibits little capacity for self-repair owing to the lack of a tissue blood supply. Researchers have investigated a variety of scaffold materials including alginate, agarose, fibrin, gelatin, and chondroitin sulfate to evaluate their ability to support chondrogenic differentiation of ASCs *in vivo*^[77-79]. Several studies have demonstrated that ASCs were able to differentiate into chondrocytes *in vivo* when seeded within any of these scaffolds, but different construct materials can significantly influence the differentiation of ASCs and functional properties of the tissue-engineered construct^[77,78].

Finally, ASCs also have potential in neural applications. Peripheral nerves can be regenerated if injuries are small, and bioengineering strategies are focused on alternatives to the nerve autograft^[80]. Properties of the ideal nerve conduit should include biodegradability, controlled release of growth factors, incorporation of support cells, electrical activity, intraluminal channels, and oriented nerve substratum. Santiago *et al*^[81] was among the first to report that implanted ASCs into the lumen of PCL-based nerve conduits in a rat sciatic nerve defect model was shown to promote the formation of a more robust nerve. However, both the endodermal and ectodermal transdifferentiation of ASCs remains to be validated.

CLINICAL APPLICATIONS

A number of clinical applications using ASCs can be found through searches and on clinical trial websites. ASCs are mainly used for cell-based therapy, and the combination of ASCs with biomaterials or drugs is still to be studied. Most studies use adipose tissue as the scaffold. Garcia-Olmo *et al*^[82-86] performed phase I - III clinical trials to investigate the efficacy and safety of expanded ASCs in the treatment of complex perianal fistulae including Crohn's disease. Autologous ASCs were mixed with fibrin glue then injected into the fistulous tract. As a result,

patients who received ASCs demonstrated a better rate of healing compared to the patients who received fibrin glue without ASCs. ASCs with fibrin glue therapy were determined to be a safe and effective for treating complex perianal fistulae. Two mechanisms of ASCs to treat fistulae are speculated: one was that ASCs induced immunosuppressive activity, and the other was that ASCs might help healing through the expression of matrix proteins^[83].

One of the first clinical reports using stem cells derived from adipose tissue in a patient was reported in 2004. Lendeckel *et al*^[87] reported a case of a 7-year-old girl suffering from widespread calvarial defects after severe head injury with multifragment calvarial fractures. This is among the first reports of bone tissue engineering using autologous stromal vascular fraction and fibrin glue, although it was a case study. Fibrin glue was manufactured from the patient's plasma 2 d prior to the surgery. SVF was kept in place using autologous fibrin glue, and computed tomography scans showed new bone formation 3 mo after the reconstruction. It was noted that ASCs have a great advantage in the point of cell yield compared to BM-MSCs especially for pediatric patients. Indeed, 295×10^6 mononuclear cells were extracted from 42.3 g adipose tissue, and about 2%-3% of these cells are expected to be stem cells^[87].

The disadvantages associated with the implantation of synthetic materials or autologous fat grafts could be overcome by engineered adipose tissue. Stillaert *et al*^[88] attempted adipose tissue engineering in 12 volunteers. Hyaluronic acid-based scaffolds were implanted in the subumbilical area with and without ASCs. Unlike successful results with nude mice^[89], the hyaluronic acid-based scaffolds didn't support ASC survival and were not inductive towards adipose tissue formation in humans. Meanwhile, ASC enriched lipotransfer has been studied for facial lipoatrophy and breast augmentation^[90,91]. Yoshimura *et al*^[92] enrolled 15 patients, transplanted SVF containing lipoaspirate after removing artificial breast implants, and followed for 12 mo. It was concluded that ASC-rich lipotransfer is effective to enhance the volume of injected adipose tissue^[90-92]. The increased volume of adipose tissue may not be due to ASC differentiation but paracrine support of the tissue through the secretion of angiogenic and adipogenic factors. However, the interaction between ASCs and cancer cells are not fully elucidated. ASCs may promote cancer growth and metastasis through paracrine properties, epithelial-mesenchymal transition^[93,94], and immunosuppressive mechanisms^[95,96]. Higher risk of local recurrence was observed in early stage breast cancer patients following lipoinjection^[97]. ASCs have not only bright side for regenerative medicine but dark side as cancer promotion.

In the field of wound healing, Rigotti *et al*^[98] showed ASCs are effective on severe symptoms such as atrophy, retraction, fibrosis, or ulcers induced by radiation therapy. Twenty patients were recruited and received lipoaspirate containing ASCs repeatedly, and followed-up to 31 mo. Patients demonstrated an improvement of ultrastructural

tissue characteristics with neovessel formation as well as significant clinical improvements. The authors concluded that the treatment with ASC-containing lipoaspirates is potentially extended to other forms of microangiopathies.

Regarding the potential of ASCs to generate immune tolerance for transplant patients, ASCs have been reported to have an immunomodulatory effect^[99]. It has been shown that ASCs don't possess human leucocyte antigen class II antigens, and ASCs can suppress inflammatory cytokines, stimulate anti-inflammatory cytokine interleukin-10, and induce antigen-specific regulatory T cells^[100]. In a case study, the intravenous infusion of allogenic ASCs in treating severe refractory acute graft-versus-host disease has proven to be effective^[101]. Fang *et al.*^[102,103] treated patients with hematologic and immunologic disorders such as idiopathic thrombocytopenic purpura and refractory pure red cell aplasia, with allogenic ASC infusions, and reported significant improvements with these patients. From these results, ASCs are suggested to have immunomodulatory.

CONCLUSION

ASCs have prominent implications in tissue regeneration due to their high cell yield in adipose tissue, the ability to differentiate into multiple lineages and secrete various cytokines, and immunomodulatory effects. A large number of clinical trials using ASCs have already performed and many of them are ongoing. However, very few phase III clinical studies have been published. ASCs are a promising cell source for regenerative medicine, and more research is needed to warrant the safety of ASCs and the efficacy of tissue engineering using ASCs.

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Connexin mutant embryonic stem cells and human diseases

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Core tip: Numerous gap junction-encoding connexin (Cx) mutant mice have been established as models of human diseases. Although these analyses have facilitated current understanding of native Cx functions and the pathogenesis of related diseases, care must be taken when extrapolating findings from mice to humans, and vice versa, because there can be striking diversity in tissue organization and Cx expression patterns between these species. Recently, the use of human induced pluripotent stem cells (iPSCs) allowed further direct approaches for studying human diseases. According to the studies using mutant mouse embryonic stem cells, Cx mutant human iPSCs may become a useful model.

Abstract

Intercellular communication *via* gap junctions allows cells within multicellular organisms to share small molecules. The effect of such interactions has been elucidated using mouse gene knockout strategies. Although several mutations in human gap junction-encoding *connexin* (Cx) have been described, Cx mutants in mice do not always recapitulate the human disease. Among the 20 mouse Cxs, Cx26, Cx43, and Cx45 play roles in early cardiac or placental development, and disruption of the genes results in lethality that hampers further analyses. Embryonic stem cells (ESCs) that lack Cx43 or Cx45 have made analysis feasible in both *in vitro* differentiated cell cultures and *in vivo* chimeric tissues. The success of mouse ESCs studies is leading to the use of induced pluripotent stem cells to learn more about the pathogenesis of human Cx diseases. This review summarizes the current status of mouse Cx disruption models and ESC differentiation studies, and discusses their implication for understanding human Cx diseases.

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Key words: Embryonic stem cells; Induced pluripotent stem cells; Gap junction; Human diseases; Genetic models; Differentiation; Chimera

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INTRODUCTION

Gap junctions consist of arrays of intercellular channels between adjacent cells. The channels are formed by the head-to-head docking of hexameric hemichannels called connexons, whose subunit proteins are encoded by the *connexin* (Cx) gene family in mammals (Table 1)^[1,2]. Most cell types communicate with each other *via* gap junctions, which require cell-cell contacts, to maintain their homeostasis. This is likely a critical mode of communication in multicellular animals because Cx expression is highly conserved. In contrast, intercellular communication is performed *via* membrane-lined channels called plasmodesmata in plants and fungi^[3]. Unique combinations of Cx isoforms are expressed in each animal tissue, thereby regulating cell-type specific homeostasis^[4]. A classical experiment revealed that trophoblasts in the blastocyst are linked by gap junctions to other trophoblasts, as well as to cells in the inner cell mass, in preimplantation em-

bryos; those cells that are linked by gap junctions to both trophoblasts and cells in the inner cell mass cells probably form the polar trophoctoderm^[5,6]. However, shortly after implantation the intercellular communication between trophoblasts and inner cell mass cells is lost^[7]. Another typical example occurs in the cardiac conduction system. In ventricular cardiac myocytes (CM), Cx43 is the main gap junction protein, whereas Cx40 expression predominates within the core conduction system. Although Cx43 and Cx40 both have high conductance, Cx45 forms low conductance and voltage-sensitive gap junctions between the ventricular CM and the core conduction system^[1,8,9]. It is believed that this expression pattern effectively insulates the conduction system while also maintaining proper contacts between the conductive and ventricular CM.

Approximately 20 Cx isoforms have been reported in mice and humans^[1]. They are expressed in most tissues at varying levels and stoichiometry. One gap junction is composed of two hexameric connexons: 12 Cxs form a single channel. Many types of Cxs can be assembled into one connexon^[4]. Because a single cell usually expresses multiple Cx isoforms, theoretically there can be a plethora of different gap junction channels between cells, each with unique properties. Recent *in vivo* studies elucidated how the expression of a multitude of Cxs results in specific biological functions using mouse mutagenesis, as well as the molecular cloning of Cx mutations related to human diseases.

MOUSE GENETIC MODELS

Cx gene knockout (KO) strategies in mice were first applied to Cx43 by Reaume *et al.*^[10] in 1995. Subsequently, mouse mutants have been reported for all of the Cxs, except for Cx23 and Cx33 (Table 1). Some Cx-KO strains show specific abnormalities. For example, Cx37 forms a unique gap junction between oocytes and granulosa cells in mice. Accordingly, Cx37-KO mice show impaired oocyte development and female infertility^[11]. Cx45 is thought to confer unique characteristics on peristaltic contractions in the early developing heart. Therefore, Cx45-KO embryos show lethality that is caused by a conduction block in early cardiogenesis^[12,13]. The placenta is dependent on Cx26, Cx31, and Cx31.1, and each KO strain shows placental dysmorphogenesis^[14-16]. Similarly, the lens epithelium co-expresses Cx46 and Cx50, and both Cx46-KO and Cx50-KO mice experience cataracts^[17-19]. Cx46 and Cx50 have a redundant role in lens development, but individual roles in overall growth. Specifically, the targeted replacement of Cx50 with Cx46 prevented cataracts, but did not restore microphthalmia, which was apparent only in the Cx50-KO mice^[20]. Thus, a specific individual Cx does not seem to possess one-to-one association with a unique cell type *in vivo*. Instead, most cells express multiple Cxs to maintain intercellular communication. This might partly explain why the lack of two Cxs results in phenotypes that were not present in

each individual KO^[21-29]. In the heart, CMs express Cx30, Cx30.2, Cx40, Cx43, Cx45, and Cx46, and their expression is regulated both temporally and regionally^[30-33]. Each Cx-KO strain exhibits developmental and electrophysiological abnormalities that are closely related to their expression patterns and channel properties (Table 1). As a result, three Cx-KO strains are shown to be lethal: Cx26-KO mice with defective transplacental glucose uptake, Cx43-KO mice with cardiac malformation, and Cx45-KO mice with blocked conduction in early cardiogenesis. Because these constitutive KO mice are embryonically lethal, other approaches are required to obtain insights into the role of these Cxs in adult tissues.

HUMAN DISEASES

Mouse Cx mutants do not always exhibit the same phenotype as would be expected from human Cx diseases (Table 1). The most divergent one is probably that of the placenta, whose structure is highly variable among mammalian species. The mouse fetomaternal barrier consists of three trophoblast layers (two syncytiotrophoblastic and one cytotrophoblastic layers), whereas that of humans has two (one syncytiotrophoblastic and one cytotrophoblastic layer). Moreover, the Cx isoforms expressed in the placenta differ among species^[34]. These structural and expression differences are probably a reason why placental defects are prevalent in Cx mutant mice. Accordingly, KO of the human deafness and skin disease-associated genes Cx26 and Cx31, together with Cx31.1, which is not a known human disease-related gene, causes placental dysfunction. Because of the striking diversity in Cx expression in placental structures, care must be taken when extrapolating findings from one species to another. The lethality of Cx26-KO mice was overcome using Cre/loxP technology to create tissue-specific Cx26-KO mice. For example, knocking out Cx26 in the mouse inner ear epithelium caused cell death in the cochlear epithelial network and sensory hair cells, which greatly enhanced our understanding of the pathogenesis of deafness^[35].

Cx37-KO mice show complete female infertility^[11]. Although this finding provides an important insight into oogenesis, no human diseases that cause female infertility have been linked to Cx37. Cx32 is the causative gene of human X-linked Charcot-Marie-Tooth disease^[36,37]. Although Cx32-KO mice exhibit peripheral neuropathy similar to that observed with the abovementioned disease, they also show liver dysfunction, which has not been described in humans^[38-40]. Generally, interspecies differences in Cx expression and organogenesis make loss-of-function phenotypes somewhat divergent. In addition, minor phenotypes in Cx-KO mice might not yet have been described as symptoms of human diseases.

In contrast, the major spatio-temporal expression patterns of Cxs in the heart appear to be relatively conserved among mammalian species^[9]. A detailed comparison of the expression of Cx40, Cx43, and Cx45 in

Table 1 *Connexin* knockout studies and human diseases

Mouse gene ^[1]	Mouse KO phenotypes	Human disease	Ref.
Cx23 ¹			
Cx26	Embryonic lethality due to defective transplacental glucose uptake	Deafness; Skin disease	[14,68-70]
Cx29	No phenotype		[71]
Cx30	Hearing impairment; accelerated heart rate	Deafness; Skin disease	[31,68,72]
Cx30.2	Accelerated atrioventricular nodal conduction		[73]
Cx30.3	Difference in behavioral reactivity to vanilla scent	Skin disease	[69,74]
Cx31 ²	Partial embryonic lethality due to a defect in early placental development	Deafness; Skin disease	[15,68,69,75]
Cx31.1 ²	Partial embryonic lethality due to impaired placental development; Changed gene expression in the central nervous system		[16,76]
Cx32	Liver dysfunction; High incidence of liver tumors; Peripheral neuropathy	Charcot-Marie-Tooth disease	[36-38,40,77,78]
Cx33 ¹			
Cx36	Loss of electrical coupling in interneurons of the neocortex; Disrupted rod pathways; Altered spontaneous firing patterns in the retina; Alterations in insulin secretion	Juvenile myoclonic epilepsy	[25,79-84]
Cx37	Female infertility; High bone mass		[11,85]
Cx39	Accelerated myogenesis and regeneration of skeletal muscle		[86]
Cx40	Cardiac conduction abnormalities; High incidence of cardiac malformations	Atrial standstill; Atrial fibrillation	[23,42,43,45,87,88]
Cx43	Early postnatal lethality due to cardiac malformation; Osteoblast dysfunction	Oculodentodigital dysplasia; Visceroatrial heterotaxia; Hypoplastic left heart syndrome; Atrial fibrillation	[10,52,55,67,89-94]
Cx45	Embryonic lethality due to cardiovascular defects; Altered spontaneous firing patterns in the retina		[12,25,44,53]
Cx46	Cataracts; Reduced heart rate and aberrant conduction along the His bundle branches	Cataract	[17,33,69]
Cx47	Myelin abnormalities	Pelizaeus-Merzbacher-like disease	[24,95-98]
Cx50	Microphthalmia and cataract	Cataract	[18,19,69]
Cx57	Reduction in horizontal cell receptive fields		[99,100]

¹No knockout (KO) studies have been reported for *connexin* (Cx) 23 and Cx33. Notably, however, the mouse small-eye mutant Aey12 has a point mutation in the Cx23 locus^[101]. For Cx33, there is no orthologous gene in the human genome^[1]; ²About 60% (Cx31) and 30% (Cx31.1) of the embryos were lost in utero; the surviving adult mice were observed to have no morphological defects.

developing mouse and human hearts indicated that their expression paralleled one another^[41]. Although no null mutations have been reported in human Cx40, Cx43, and Cx45, the loss of Cx40 blocked atrioventricular conduction and caused a high incidence of cardiac malformations in mice. Cx43-KO mice exhibited neonatal lethality due to cardiac malformation; Cx45-KO mice experienced a lethal conduction block in early cardiogenesis^[10,12,23,42-45]. It is possible that null mutations in human Cx40, Cx43, and Cx45 exist, but that the development of the fetus could be aborted. However, several missense mutations in Cx40 and Cx43 have been described in human heart diseases, and attempts have been made to create mice with the Cx43 missense mutations related to oculodentodigital dysplasia in humans (Table 1)^[46,47]. In addition to CM with missense mutations, adult mice with Cx-KOs are required to understand why or how Cx30, Cx30.2, Cx40, Cx43, Cx45, and Cx46 are expressed differentially in the heart and also to extrapolate human Cx functions from mouse studies. Adult CM cannot be obtained from lethal Cx43-KO and Cx45-KO mice. Therefore, attempts have been made to mutate a unique Cx isoform in a tissue-specific manner.

EMBRYONIC STEM CELLS LACKING Cx43 OR Cx45

A widely accepted approach to circumvent the lethality of constitutive KOs is the tissue-specific deletion of a gene using Cre/*loxP* technology (Figure 1). In this method, the target gene is flanked by *loxP* sequences, and the tissue-specific expression of Cre recombinase deletes the gene of interest. The embryonic lethal genes Cx26, Cx43, and Cx45 have all been analyzed using this method. They were all deleted specifically in adult tissues, for example in the inner ear epithelium, CM, and neurons^[13,35,48-51].

The use of ESCs lacking Cx43 or Cx45 has advantages in addition to those afforded by Cre/*loxP* technology (Figure 1)^[52,53]. The CM-specific deletion of Cx43 slowed conduction and caused sudden arrhythmic death^[49]. Similarly, the CM-specific deletion of Cx45 was embryonic lethal, similar to constitutive Cx45-KO mice^[13]. In both these examples, Cre recombinase was used to delete the genes in most of the CM. Because Cx is a gap junction protein, understanding what happens at the borders between Cx-positive and -negative cells has been of great interest. Chimeric mice, which are formed from mutant

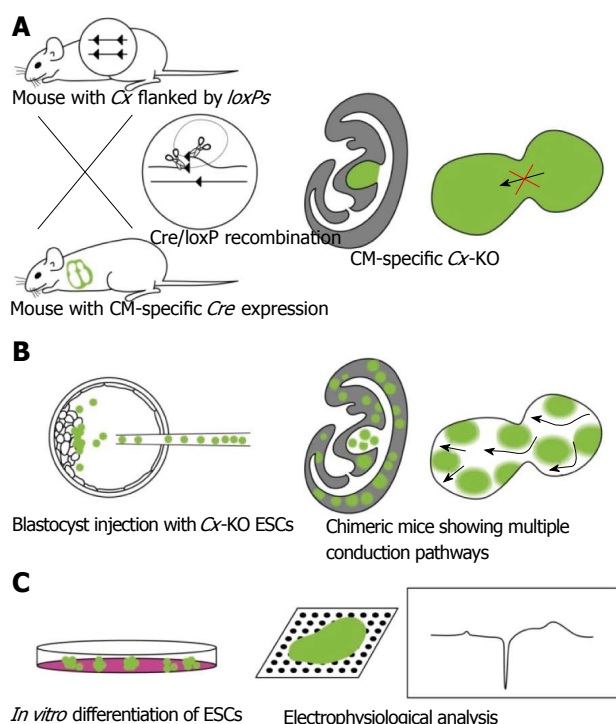


Figure 1 Cre/loxP-mediated tissue-specific knockout mouse models and analysis of embryonic stem cell differentiation. Mutant cells and regions are shown in green. Mouse and heart drawings, respectively, constitute the middle and right pictures in (A) and (B). A: In the Cre/loxP model shown here, the *connexin* (*Cx*) gene, which when lost causes lethality, is deleted specifically in the CM. This results in relatively consistent delay or block in conduction^[13,49]; B: Chimeric mice containing embryonic stem cell (ESCs) lacking the *Cx43* gene. The example shown here reveals multiple conduction pathways in the heart^[62]; C: ESCs can be differentiated *in vitro*. In this example, the induced CMs are subjected to planar multielectrode array analyses (middle); a typical extracellular recording data is shown in the right graph^[53,67]. KO: Knockout

ESCs and recipient blastocysts, allow these experiments to be performed. Mouse ESCs express *Cx31*, *Cx43*, and *Cx45* proteins^[54]. *Cx43*-KO ESCs were used to form chimeric tissues with wild-type cells, and the chimeric heart showed conduction defects and diminished cardiac performance^[52]. This study supports the concept that tissue mosaicism in different *Cx* isoforms might be responsible for reentrant arrhythmias. Indeed, in humans, atrial tissue genetic mosaicism in a loss-of-function *Cx43* mutation was reported to be associated with sporadic lone atrial fibrillation^[55]. *Cx43* chimeric mice form a model of atrial fibrillation, which might facilitate the development of therapeutic approaches for modifying the function of cardiac gap junctions.

Research using ESCs that lack *Cx45* developed very differently from those lacking *Cx43*. *Cx45*-KO ESCs cannot be integrated into chimeras, because they never mix with the inner cell mass of the recipient^[53]. Innate *Cx45* is expressed abundantly in early embryos, suggesting that it might play a role in cell adhesiveness during early development. Irrespective of their incompatibility with chimera production, *Cx45*-KO ESCs differentiate into the three germ layers *in vitro*. CMs induced from *Cx45*-KO ESCs showed conduction abnormalities^[53]. Constitutive

Cx45-KO mice were reported initially by two laboratories independently^[12,44]. One group reported heart abnormalities, whereas the other focused on vascular abnormalities. Later, as described above, the CM-specific *Cx45*-KO mice were shown to be similar to the constitutive *Cx45*-KO mice^[13]. Taken together, the heart abnormalities are expected to be the primary defect associated with the loss of *Cx45* in developing embryos.

INDUCED PLURIPOTENT STEM CELLS AND BEYOND

Induced pluripotent stem cells (iPSCs) have similar potential to ESCs, and can differentiate into many cell types including germ cells^[56,57]. Importantly, iPSCs can be derived from adult somatic cells, including from individuals with genetic diseases^[58]. Human iPSCs from patients might provide unlimited supplies of specific tissues, and the use of human cells is more important than creating mouse genetic models for the understanding of human diseases^[59]. Theoretically, chimeric human tissue formed from diseased and normal iPSCs could be generated *in vitro*. As studies performed using mouse ESCs indicate, this approach might be particularly useful for studying human junction proteins including *Cxs*. Even minor tissues such as endocrine cells can be supplied in unlimited amounts in rare diseases, and biological specimens of uniform quality will improve reproducibility greatly, which is often problematic in human studies. The future of iPSC technology also seems very promising in mouse studies because iPSCs can be derived from many mouse genetic models. For example, attempts have been made to improve disease conditions by the transplantation of tissues differentiated *in vitro*. The transplanted tissues were derived from autologous iPSCs in which the specific genetic disorder had been corrected^[60]. Although establishing iPSCs with multiple targeted mutations might require breeding different mutant mice, this is likely far easier than performing multiple gene targeting using ESCs. Therefore, the use of iPSCs might allow the unique and redundant contributions of *Cxs* in intercellular communication to be elucidated further.

CONCLUSION

Cx mutant mouse strategies have revealed detailed *in vivo* functions of intercellular communication carried out by individual *Cx* species. The use of *Cx* mutant ESCs and iPSCs has additional advantages. Especially, iPSCs can be obtained from individuals with genetic diseases. Analysis of chimeric and *in vitro* differentiated tissues is useful for understanding the molecular target in human *Cx* diseases. To date, some reagents are known to modulate gap junctional intercellular communication and are used in clinical trials for the treatment of wound, arrhythmia, migraine, and cancer^[61-66]. Reproducibility in the stem cell-based experimental systems will be a great advantage for the

development of such therapeutic drugs.

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Early B lymphocyte development: Similarities and differences in human and mouse

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Core tip: There are several species differences between human and mouse, while the mouse studies precede those of human. Recent progresses of experimental techniques have made it possible to understand the biology in human B lymphopoiesis deeply. Various phenotype markers, which can define the distinct developmental stages, and requirement of cytokines are distinguishable. More common issues are observed in the role of signaling molecules, including transforming growth factor- β superfamily, Wnt family, and Notch family, which have been known the high conservation among mammals. The knowledge on niches for human hematopoietic stem cell and B cell development is still limited.

Abstract

B lymphocytes differentiate from hematopoietic stem cells through a series of distinct stages. Early B cell development proceeds in bone marrow until immature B cells migrate out to secondary lymphoid tissues, such as a spleen and lymph nodes, after completion of immunoglobulin heavy and light chain rearrangement. Although the information about the regulation by numerous factors, including signaling molecules, transcription factors, epigenetic changes and the microenvironment, could provide the clinical application, our knowledge on human B lymphopoiesis is limited. However, with great methodological advances, significant progress for understanding B lymphopoiesis both in human and mouse has been made. In this review, we summarize the experimental models for studies about human adult B lymphopoiesis, and the role of microenvironment and signaling molecules, such as cytokines, transforming growth factor- β superfamily, Wnt family and Notch family, with point-by-point comparison between human and mouse.

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Key words: Human B lymphopoiesis; B cell cultures; IL-7; Microenvironment; Wnt signaling

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INTRODUCTION

B lineage cells develop from hematopoietic stem cells (HSCs) in adult bone marrow (BM) through several well-characterized stages before migrating to secondary lymphoid tissues such as a spleen and lymph nodes. Once HSC divides asymmetrically into one stem cell and one differentiating cell, it gives rise to progenitor cells that undergo lineage commitment and the production of specific lineage blood cells starts. Multipotent progenitors (MPP), which lose the reconstituting capacity, differentiate sequentially into lymphoid-committed progenitors, and B lineage-restricted progenitors originate from the lymphoid-primed multipotent/ early lymphoid progenitors (LMPP/ELP), followed by common lymphoid progenitors (CLP), pro-B cells, pre-B cells and immature B cells (Figure 1). Immunoglobulin gene rearrangements

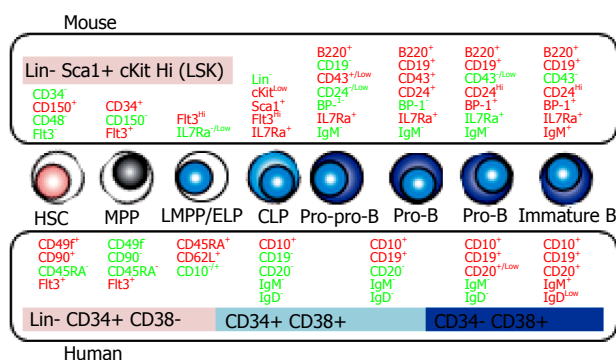


Figure 1 B cell development in bone marrow. B lineage cells are differentiated from hematopoietic stem cells (HSC) through the several steps defined by the distinct surface phenotypes of positive (red) or negative (green) expression. The comparison between mouse (upper side) and human (lower side) is shown. MPP: Multipotent progenitors; LMPP/ELP: Lymphoid-primed multipotent/ early lymphoid progenitors; CLP: Common lymphoid progenitors; IL7Ra: IL-7 receptor alpha.

are required for the process of B lymphopoiesis^[1-3]. The activation of the recombination enzymes, such as recombination-activating gene (RAG)-1, RAG-2 and terminal deoxynucleotidyl transferase, promotes the D-to-J and V-to-DJ rearrangements in the immunoglobulin heavy (IgH) chain locus during the differentiation from CLP to pro-B stage. Signaling through the pre-B-cell antigen receptor (pre-BCR), composed of IgH chains and surrogate light (L) chains, induces VJ_L rearrangements and allelic excision at IgH chain locus leading the functional BCR expression on immature B cells. This rearrangement machinery is precisely regulated by several transcription factors including PU.1, E2A, early B cell factor (EBF) and Pax5^[2,3]. For example, Pax5 activates the expression of Cd19, Cd79a, Blnk, Igl15 (lambda5) and VpreB1 involving in the pre-BCR signaling. Although it was believed that the fate decision of B cell commitment would occur after becoming CLP, recent studies have shown the lineage skewing begins earlier than previously expected^[3-7]. The expression of lymphoid-lineage priming genes like Satb1 and Ikaros in HSC is recognized^[8,9]. During the differentiation from HSC to CLP, lymphopoiesis proceeds in asynchronous ways. These developmental procedures are regulated by signaling molecules, transcription factors, epigenetic changes and the microenvironment^[6,7,10,11].

It has been known that HSC are extremely heterogeneous. Those can be subdivided to long-term and short-term HSC based on reconstitution time periods in transplantation assays^[12-16]. Recent studies suggest that HSC compartment also contains distinct subtypes with different developmental preferences^[15-18]. Myeloid-biased HSC produce greater numbers of myeloid than lymphoid lineage cells and tend to be quiescent. On the other hand, lymphoid-biased HSC generate more lymphoid cells and have shorter duration of reconstitution than myeloid-biased HSC. In aged mice, which reduce production of B and T cells and diminish function of mature lymphocytes, the number of myeloid-biased HSC increases^[17,19,20]. The distribution of HSC subsets is at least partly respon-

sible for homeostasis of B lymphopoiesis.

The evidences about hematopoietic biology have been accumulated from murine experiments and primary deficiencies in humans. However, recent advances in biological analysis techniques including xenotransplantation model, *in vitro* clonal assays and flow cytometric analysis and sorting made great progress for understanding normal hematopoiesis in human. Mouse and human are obviously different in size, ecology, and lifespan. It has been known that human B lymphopoiesis differs from that in mice with requirement of cytokines and the role of microenvironment. To apply the findings about the regulation of B lymphopoiesis for clinical settings, studies in human are necessary.

In this article, we focus on common and distinct features in human and mouse early B lymphopoiesis. First we discuss the differences of adult B cell development from HSC between these two species. In the late sections, we describe the role of microenvironment in BM including the cellular components and signaling molecules, especially about members of TGF- β superfamily, Wnt family, and Notch family, which have been known the importance in regulating proliferation, differentiation, and survival.

HUMAN AND MOUSE B LYMPHOPOIESIS IN BONE MARROW

Methodological advances in human B lymphopoiesis studies (Figure 2)

As we mentioned above, there are several species differences in B cell lymphopoiesis between human and mouse. The development of human study has been relatively slow with several reasons. The most critical one is the lack of adequate experimental models for evaluating molecular mechanisms *in vivo* and *in vitro*. For murine studies, various *in vitro* assays, such as Whitlock-Witte long-term cultures, cultures of BM cells with or without stromal cell lines, and colony assays for IL-7-responding progenitors are available^[21,22]. However, cultures to generate human B lymphocyte have not been well established. Although murine stromal cell lines can support human B cell development from hematopoietic stem/progenitor cells (HSPC), the species differences make the precise evaluation about some necessary cytokines and interaction with the microenvironment difficult^[23-26]. The establishment of new culture systems reported from our group and others hampered this problem^[27-29]. We established co-culture with human mesenchymal stem cells (MSC) and stromal cell-free culture systems. Our co-culture or stromal cell-free culture systems in the presence of stem cell factor (SCF) and Flt3 ligand (Flt3L) are successfully produced CD10⁺ CD19⁺ B cells within 4 wk from human umbilical cord blood (CB) CD34⁺ CD38⁻ HSC. Surface IgM⁺ immature B cells begin to appear after 4 wk of co-cultures. Although lymphocyte production from adult BM-derived HSC in the stromal cell-free culture is much more difficult than CB cells, both are responsive to granulocyte

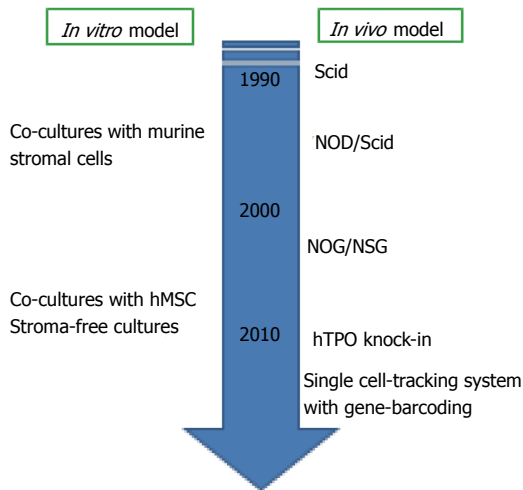


Figure 2 Experimental models for human B lymphopoiesis. Experimental techniques for studying human B lymphopoiesis have incredibly advanced within these two decades. Now several culture systems with human mesenchymal stem cells (hMSC) or without stromal cells are available. For *in vivo* studies, the generation of humanized mice has been developed after the discovery of severe combined immune-deficient mouse (Scid). NOG, nonobese diabetic (NOD)-Scid mouse with truncation in the IL-2 receptor common gamma chain; NSG, NOD-Scid mouse with deletion in the IL-2 receptor common gamma chain; hTPO knock-in, RAG-2^{-/-} NSG mouse with humanization of thrombopoietin.

colony stimulating factor (G-CSF). Our data showed that human MSC can efficiently support commitment and differentiation of human HSC into B lymphocytes, and human does not require the direct interactions with stromal cells for B cell generation.

Concerning about *in vivo* studies, humanized mouse models were established around 1990s with the discovery of the severe combined immune-deficient (Scid) mouse lacking B and T cells^[30,31]. Since then, a variety of xenograft models including nonobese diabetic (NOD)-Scid mice and NOD-Scid with either truncation (NOG) or deletion (NSG) in the IL-2 receptor common gamma chain have been generated to improve the efficiency of human HSC engraftment and long-term reconstitution^[32,33]. With humanized model, we can observe multi-lineage reconstitution from human HSC *in vivo*. Newer generation of transplantation methods are now being developed. To elucidate the role of cytokines which are not cross-reactive, transgenic mice producing human cytokines such as thrombopoietin, IL-3 and GM-CSF, have been generated^[34]. The viral integration site tracking system and the use in combination with massively parallel sequencing make it possible to track human HSC clones in transplanted Scid mice^[35].

Another obstacle to studying human lymphopoiesis is genetic and biological diversity. Human BM samples are all different in age, sex, body size, genetic and epigenetic background and health condition when samples are collected. The development of highly purifying techniques with flow cytometry, single-cell assay methods and gene sequencing would help this problem solved^[36].

Markers of hematopoietic stem cells and B progenitors

HSC is an extremely rare subset. The frequency of HSC

in human BM is only 1 in 10⁶ cells^[37]. In mice, lineage (Lin)⁻/Low Sca-1⁺ c-Kit^{Hi} (LSK) fraction contains multipotent cells such as HSC and MPP^[38]. Using CD34, Flt3, SLAM family markers (CD150, CD48, CD229 and CD244) and Hoechst 33342 efflux, HSPC in LSK cells can be resolved into several subsets with distinct level of reconstituting potential and lineage preference^[12,39-41]. According to c-Kit intensity decline, lymphoid committed cells are differentiated. Kondo *et al.*^[42] defined CLP in mice as Lin⁻ IL-7 receptor alpha (IL-7Ra)⁺ Sca-1⁺ c-Kit^{Low} cells that appear to produce mainly B, T and natural killer (NK) cells. B lineage-restricted progenitors are fractionated based on the developmental stage and surface expression of CD45R/B220, CD19, CD24 (heat-stable antigen), CD43 and BP-1^[43,44]. Mouse lymphopoietic hierarchy with cell surface markers is shown in Figure 1.

In human, HSPC markers are quite different from murine ones (Figure 1). Unlike mice, human HSPC can be enriched with CD34 expression although a very rare subset of HSC are devoid of that^[45-47]. Other phenotypes of HSC are Flt3⁺, CD38⁻ and CD150⁻, in great contrast with the expression on murine one^[48,49]. CD133 helps the isolation of human HSPC and the rare CD34⁻ HSC subset^[46,50]. Recently, Dick and colleagues subdivided human Lin⁻ CD34⁺ HSPC into long-term HSC, short-term HSC/MPP, and 6 lineage progenitor subsets on the basis of expression of the markers CD34, CD38, CD90 (Thy-1), CD49f, CD135 (Flt3), CD45RA, CD10, and CD7^[51,52].

CD10 and CD45RA are often used as human-specific markers of lymphoid progenitors at early stages. Doulatov *et al.*^[51] described CD34⁺ CD38⁻ CD45RA⁺ CD10⁺ fraction as multilymphoid progenitor and CD34⁺ CD38⁺ CD45RA⁺ CD10⁻ fraction as granulocyte and monocyte progenitor. It is known that CD34⁺ CD10⁺ cells have a strong bias toward B cell development with relatively little T or NK cell potential^[53,54]. We previously reported CD34⁺ early lymphocyte progenitors differ in CD10 expression^[53]. CD34⁺ CD10^{Hi} and CD34⁺ CD10^{Low} populations have unique patterns depending on their sources; CB, BM and G-CSF mobilized peripheral blood, and increasing level of CD10 corresponds to expression of B lymphoid related transcription factors and markers, as well as loss of proliferative potential. Recently, Kohn *et al.*^[55] reported that L-selectin (CD62L) is expressed at the earliest stage of lymphoid priming before starting CD10 positive. CD34⁺ CD45RA⁺ CD62L^{Hi} CD10⁻ cells showed lymphoid skewing although they produced both of myeloid and lymphoid cells in transplanted NSG mice. The differentiation potential and gene profiling indicated that CD34⁺ CD45RA⁺ CD62L^{Hi} CD10⁻ cells are placed between HSC and CD34⁺ CD10⁺ lymphoid progenitors.

Requirement of cytokine signaling (Figure 3)

In mice, two cytokines, IL-7 and Flt3L, are known to be essential for adult B lymphopoiesis^[56-58]. The loss of these receptors completely blocks B cell development. The up-regulation of IL-7Ra with Flt3 signaling induces EBF expression in B lineage progenitors, and that al-

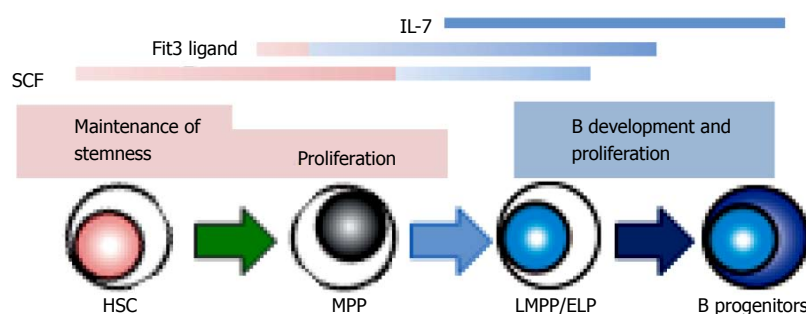


Figure 3 The role of signaling molecules in B lymphopoiesis. Several cytokines, such as stem cell factor (SCF), Flt3 ligand and IL-7, show the various effects depending on the developmental stages. There are several species differences in the role of cytokines between human and mouse. IL-7 is required for adult mouse B lymphopoiesis, but not for that of human. Recent studies indicate that Flt3 signaling plays a crucial role in lymphoid, but not in HSC or myeloid development in mouse, while human Flt3 ligand affects the survival of hematopoietic stem/progenitor cells as well as B cell differentiation. HSC: Hematopoietic stem cells; MPP: Multipotent progenitors; LMPP/ELP: Lymphoid-primed multipotent/early lymphoid progenitors.

lowers differentiation with the consequent expression of B cell-specific genes^[10,11]. Moreover, recent studies have shown that the expression of IL-7Ra denotes the transition from LMPP to CLP, and Flt3L regulates the survival and proliferation of MPP/LMPP with commitment to B lineage fate^[42,59,60]. The combination of crucial cytokines changes during ontogeny. Studies using IL-7 knockout mice showed that only adult but not fetal or neonatal B development is inhibited^[61]. Thymic stromal lymphopoietin (TSLP) regulates IL-7-independent fetal B lymphopoiesis. SCF and chemokines recognized by the CXCR4 receptor also affect the differentiation^[62].

In contrast, IL-7 is not required for human B cell development^[63-66]. Crucial transcription factors including E2A, EBF and Pax5 are expressed during the differentiation from HSC to B lineage progenitors before acquisition of CD19, in the same manner as mouse B lymphopoiesis^[67]. The importance of SCF and CXCL12 for B cell development has been recognized^[68-70]. In human, Flt3L is critical to cell survival and proliferation of HSPC as well as to B lymphopoiesis^[68,69]. Several groups reported that human HSPC could develop B lineage cells independently of IL-7 stimulation, and IL-7 induces little increase of B production in co-cultures^[64]. Moreover, patients with disruption of the human IL-7 receptor spared B lymphopoiesis while development of T and NK cells was severely impaired^[65,66]. Some groups questioned about the interpretation because in these studies fetal materials or murine stromal cells might influence the consequences^[71,72]. However, we found that addition of neutralizing antibody to IL-7 or TSLP has no effect in stromal cell-free cultures we established^[28,29]. In our study, hMSC conditioned medium could support human B lineage generation, indicating the existence of unknown stromal cell-derived factors facilitating B lymphopoiesis. Interestingly, we and others reported that G-CSF promotes human B production from HSC *in vitro*^[23,28]. G-CSF is originally cloned as a glycoprotein which stimulates the production of granulocytes, and now is known the important role in HSC proliferation and mobilization, and bone resorption. For now, nothing has been reported about the influences on early B lymphopoiesis *in vivo* while clinical studies showed a higher proportion of Th2 cells present in peripheral blood cell grafts from G-CSF-stimulated donors and T cell hyporesponsiveness in association with increase of Th2-inducing dendritic cell^[73,74].

There are several possibilities about the mechanism how G-CSF affects B lymphocyte generation *in vitro*. It might have direct effects on cultured cells. Another speculation is that HSC or progenitors with the specific stage or lineage stimulated by G-CSF might regulate B generation indirectly. In B cell cultures, short-term expansion of myeloid cells is observed before emerging B lineage cells^[27,28].

Collectively, the essential key of human B lymphopoiesis is still remained unknown. The recent study with the depth of single-cell mass cytometry and an algorithm analysis of human BM showed the exclusive activation of STAT5, which phosphorylation is known to be induced by IL-7, in early B progenitors^[75]. Using novel technologies, the precise biology could be unveiled in the near future.

ROLE OF MICROENVIRONMENT

In 1978, Schofield proposed the hypothesis that a specialized niche in BM preserves the reconstituting and differentiating ability of HSC, but could not prove that^[76]. It is believed that bone marrow contains specialized niches for differentiation of specific lineage progenitors^[77-79]. With the great advances of gene-modified mice generation and imaging techniques, the anatomical location and cellular components of HSC niches have been elucidated since 2000s, although our understanding is still incomplete and novel analysis tools are needed^[80,81]. In parallel, the roles of molecular and environmental factors in the niches have been extensively studied. Niches make specialized environments, consisting of soluble or surface-bound signaling factors, cell-cell contacts, extracellular matrix (ECM) proteins, and local mechanical environments such as the concentration of oxygen and calcium.

Cellular components

In marrow, there are many types of non-hematopoietic cells including mesenchymal stem/progenitor cells, osteoblastic lineage cells, adipocytes, endothelial cells, reticular cells, pericytes, fibroblasts and nerve cells^[80,81]. The effects of several molecular regulators produced by niche cells, such as chemokines like CXCL12, cytokines (SCF, thrombopoietin, angiopoietin-2, and angiopoietin-like 3), Wnt, Notch, TGF- β and hedgehog signaling, and ECM proteins (osteopontin, decorin, and tenascin C) have been

reported. Based on the concept of HSC niche, the cellular components are supposed to neighbor with HSC, and more importantly, the influence on HSC maintenance should be direct.

Several immunofluorescence imaging studies showed that HSC is consistently located adjacent to the sinusoidal vasculature^[39,82]. In perivascular niches, mesenchymal stem/progenitors which express Nestin, leptin receptor, or fibroblast activation protein (FAP), CXCL12-abundant reticular cells, and endothelial cells are co-localized with HSC and secrete HSC supporting factors like SCF or CXCL12^[39,83-86]. The sympathetic neurons, arteries, macrophages such as osteoclasts and regulatory T cells in the niches affect the frequency, function and localization of HSC^[87-91]. Surrounded by these cells and molecular components, HSC can maintain the capacity of self-renew and multipotent differentiation.

On the other hand, whether the osteoblastic lineage cells at the endosteal surface of the bone, described first as the place where HSC reside, could be the niche is under debate^[82,92]. Although osteoblasts may not to be adjacent to HSC, they do have the distinct influences on HSPC through the production of CXCL12 and SCF, and expression of adhesion molecules. It is known that HSPC frequently move out from their own niche^[93,94]. Thirty percent of IL-7Rα⁺ B progenitors are co-localized with bone-lining cells, and acute depletion of them are observed when osteoblastic cells are conditionally deleted^[95]. Interestingly, the deletion of CXCL12 from osteoblasts depletes early lymphoid progenitors, but not HSC or myeloid progenitors^[95,96]. These findings suggest that osteoblasts could be the niches for B lymphopoiesis in endosteal area (Figure 4).

Anatomical location

As well as the identification of cellular components of niches, the anatomical localization of HSC in BM has been the subject of intense researches. The initial studies indicated that HSC might reside in the endosteum, adjacent cortical bones with osteoblasts^[92]. With the great advance in immunostaining methods and understanding HSC characteristics, however, others showed that most of accurate HSC localize adjacent to sinusoid vessels while less than 20% to bone-lining cells^[82,84]. It is consistent that HSC are found in the trabecular regions at metaphysis.

Inside marrow, HSC is mobile when HSC divides or starts to differentiate. Interestingly, it is known that HSC periodically leave and reenter the niches for circulation with circadian oscillation and in response to infection or G-CSF stimulation^[87,91]. *In vivo* time-lapse imaging makes it possible to observe HSC motility and localization of activating HSPC. Another unanswered question is skeletal localization. In human adult, the sternum is active hematopoietic site while long bones are occupied by adipocytes with aging. The three-dimensional, whole-mount confocal immunofluorescence imaging techniques showed the same is true in mice^[89].

Niches in human

In clinical settings, hematopoietic stem cell transplantation offers patients with refractory hematological diseases a curative treatment option. Several types of stem cell sources, CB, BM and G-CSF mobilized peripheral blood are used for the therapy, although differences among sources are still remained unclear^[97]. After transplantation, HSC migrate, localize in niches and start to proliferate and reconstitute all lineage bloods in the recipient BM damaged by the conditioning. A full understanding of the whole process is critical for choosing the adequate strategy of donor sources, conditioning and immunosuppressive therapy before or after transplantation.

Several types of mesenchymal stem/progenitors, osteoblast, and endothelial cells in human have been reported the supportive effects on HSC maintenance or specific lineage differentiation^[27,98,99]. Imaging analysis using bone biopsy specimens to evaluate the actual distance between HSPC and niche component showed that human CD45⁺ CD34⁺ CD38⁻ HSC localize in the trabecular area similar to mice HSC, while CD34⁺ CD38⁺ HPC are dispersed evenly in BM^[99]. HSC in the trabecular area own better HSC functions compared to those in long bone area. There is no information about the niches for human B lymphopoiesis.

SIGNALING MOLECULES

Signaling molecule families like TGF-β, Wnt, Notch and hedgehog are highly conserved in mammals, and control proliferation or cell fate determination during embryonic development and adult homeostasis. In hematopoiesis, the specific ligand-receptor interactions regulate the maintenance of HSC stemness and differentiation through direct and indirect effects via the affected micro-environments.

TGF-β superfamily

The TGF-β superfamily is composed of more than 20 members, including three TGF-βs, bone morphogenetic proteins (BMP), growth and differentiation factors (GDF), Activins, and Nodal. TGF-β signaling regulates HSC quiescence, and is reduced in aged HSC^[88,100]. The activation is restricted although many cells can produce TGF-β ligands and express the receptors. For HSC maintenance, the latent type of ligand is produced from the HSC microenvironment and activated by the nonmyelinating Schwann cells ensheathing sympathetic nerves in contact with HSC^[88]. We and others reported the effects of TGF-β signaling for mouse and human B lymphopoiesis^[27,101-103]. We showed that both Activin A and TGF-β1 inhibit generation of B cells from CB CD34⁺ cells in cultures. The receptors are expressed by not only CD34⁺ HSPC but also CD34⁺ CD10⁺ cells, and we observed the same effects of the signaling when the inhibitor was added at the later periods of the co-cultures. These findings indicate TGF-β superfamily might affect early B lymphocyte progenitors. Transition into IgM⁺ immature

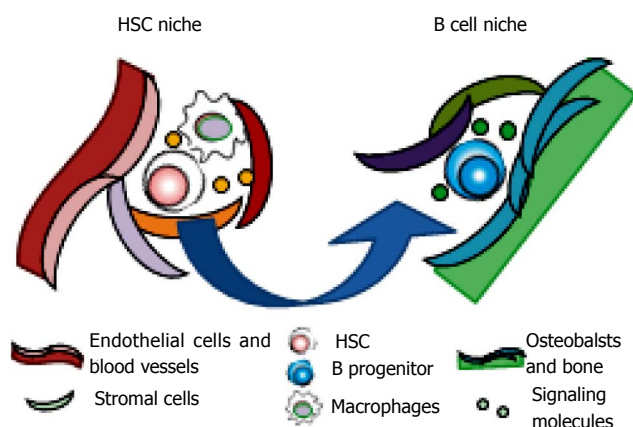


Figure 4 Motility of hematopoietic stem and progenitor cells in bone marrow. Hematopoietic stem and progenitor cells reside in their own specialized niches where they could preserve the reconstituting and/or differentiating ability. The cellular components and anatomical localization make specialized environments, consisting of soluble and surface-bound signaling molecules, cell-cell contacts, extracellular matrix proteins, and local mechanical environments. The niches for stem cell maintenance and differentiation are distinct. It is believed that hematopoietic stem cells (HSC) self-renew in their niches adjacent to the sinusoidal vasculature with mesenchymal progenitors, endothelial cells, sympathetic neurons, arteries, and macrophages such as osteoclasts. Once the differentiating daughter cell is generated after asymmetrical division of HSC, it moves to the favorable space for undergoing specific lineage commitment. For B lymphopoiesis, progenitors are co-localized with bone-lining osteoblasts in endosteal area.

B-cells was not influenced by the TGF- β superfamily in our culture systems.

Wnt family

Wnt is a large family of glycoproteins. Canonical pathway used by Wnt3a has been most studied in hematopoiesis^[104]. After Wnt3a binds Frizzled receptor, this canonical signaling stabilizes intracellular β -catenin by inhibition of GSK-3 β , and then β -catenin translocates to the nucleus and interacts with transcription factors. The role of Wnt for HSC maintenance has been a debatable issue. Constitutively active β -catenin blocks their differentiation, but induces exhaustion by shifting HSC cell-cycling status, although some of conditional deletion of β -catenin mouse have no abnormality in hematopoiesis^[105,106]. However, now it is known that these discrepant results from studies using gain or loss of functions reflect the sensitivity to the dosage^[107]. Wnt5a associated with noncanonical pathway also regulates HSC maintenance and differentiation^[108-110]. Recent studies showed that noncanonical signaling is balanced with canonical signaling under inflammatory and aging condition^[109].

Our and other groups reported the inhibitory effects on B lymphopoiesis^[108,110,111]. Wnt3a, using canonical pathway, inhibits B and pDC but not cDC development, and Wnt5a promotes B lymphopoiesis *in vitro*. The observations about canonical Wnt signaling can translate from mouse to human^[111]. It is known that Wnt ligands and receptors are expressed in both of hematopoietic tissues and the niche cells, and Wnt3a regulates mesenchymal lineage differentiation^[111,112]. While hematopoietic

cells themselves are Wnt responsive, we showed that the regulation of niches by Wnt3a mediates the effects. Specifically, Wnt3a strongly induces the production of ECM protein, decorin, which inhibits B lymphopoiesis and retains the HSC phenotype, from stromal cells. Decorin is a small leucine-rich proteoglycan secreted by MSC, and regulates TGF- β signaling, although the detailed mechanisms have not been elucidated^[111,113]. Collectively, the findings suggest that Wnt signaling is important for maintaining not only hematopoiesis but also the niches.

Notch family and hedgehog family

The ligands of Notch signaling are membrane bound proteins, and the function depends on the type of ligand, such as Delta-like and Jagged, and responsive receptors, Notch 1-3. Notch is essential for early T lymphopoiesis, and B lymphopoiesis is suppressed by the interactions between Delta-like and Notch1 to avoid B cell generation in thymus^[114]. The precise role in adult HSC at physiological levels is still controversial. Loss of the function in HSC did not show any influences for reconstitution and differentiation in mice, while *in vitro* expansion of HSC is promoted by the signaling^[115,116]. The same is true in human^[117]. The two recent studies published in 2013 emphasized the importance of Notch signaling in the interaction between human HSC and the microenvironment. Human CD146⁺ perivascular cells maintain stemness of HSC via Notch activation^[98]. Bhatia and colleagues showed that in the trabecular bone area where HSC can hold the regenerative and self-renewing capacity, 3-fold greater of proportion of mesenchymal cells express Jagged-1 compared to those in long bone area^[99]. More recently, it is reported that Notch signaling in HSC stimulated after the activating mutation of β -catenin in mouse osteoblasts induces the leukemogenesis^[118]. The mutation induces Jagged-1 expression in osteoblast leading the Notch activation in HSC, and the inhibition of Notch signaling prevents the onset of leukemia. According to this study, 38% of patients with acute myeloid leukemia or myelodysplastic syndromes showed increased β -catenin in osteoblasts and increased Notch signaling in hematopoietic cells. The cooperation between Wnt and Notch is also reported in HSC maintenance^[119]. Further study about the role of Notch signaling is warranted.

Although hedgehog signaling is also important for the development, stem cell maintenance, and tumorigenesis in various organs, the detailed effects on hematopoiesis have remained unclear. In mice, hedgehog signaling in HSC is not required for hematopoiesis although several studies showed the effects on cell-cycle and differentiation of HSC^[120,121]. The activation of hedgehog signaling in stromal cells promotes B lymphopoiesis and HSC expansion^[122]. Both of cell-extrinsic and cell-autonomous effects might be critical.

CONCLUSION

B lineage commitment starts at the early stage of HSC in

asynchronous ways. The fate decision and development are affected by the microenvironmental factors including cellular niche components and signaling molecules. In this review, we described the common and different features in early B lymphopoiesis between human and mouse. The surface phenotypes on human HSC and B progenitors and requirement of cytokines are distinct while many effects of signaling molecules are consistent with mice.

It is known that immune system can be harmed by malignant disease, chronic inflammation and normal aging. Many studies concerning impairments in cellular and humoral immunity have focused on regulation of mature lymphocyte function. Recent studies, however, revealed that the earliest stage of B lymphopoiesis plays an important role in the immune decline. Understanding the precise mechanism in human and mouse BM, and the assessment of species variations with novel technologies would make the potential applications to cancer immunotherapy and the discovery of novel treatment for autoimmune diseases possible.

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Fetal stem cell transplantation: Past, present, and future

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Core tip: Based on the history of fetal stem cell transplantation since 1928, this article discusses strategies for transplantation, with a focus on donor cells, cell processing, and the therapeutic cell niche, in addition to ethical issues associated with fetal origin. We described the stream line to current clinical trials using fetal and embryonic stem cells based on Clinical. Trials. gov. Finally, we discussed the perspective of fetal stem cell transplantation.

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Abstract

Since 1928, human fetal tissues and stem cells have been used worldwide to treat various conditions. Although the transplantation of the fetal midbrain substantia nigra and dopaminergic neurons in patients suffering from Parkinson's disease is particularly noteworthy, the history of other types of grafts, such as those of the fetal liver, thymus, and pancreas, should be addressed as there are many lessons to be learnt for future stem cell transplantation. This report describes previous practices and complications that led to current clinical trials of isolated fetal stem cells and embryonic stem (ES) cells. Moreover, strategies for transplantation are considered, with a particular focus on donor cells, cell processing, and the therapeutic cell niche, in addition to ethical issues associated with fetal origin. With the advent of autologous induced pluripotent stem cells and ES cells, clinical dependence on fetal transplantation is expected to gradually decline due to lasting ethical controversies, despite landmark achievements.

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Key words: Fetal tissue; Fetal stem cells; Fetus, Embryonic stem cells; Transplantation; Clinical trials

INTRODUCTION

In 1988, an article reported the hopeful results of a clinical trial in which the fetal mesencephalic substantia nigra was transplanted in patients with Parkinson's disease (PD)^[1]. In the preceding year, 1987, a Chinese team had reported similar findings of fetal tissue transplantation conducted in August 1985^[2]. Following the publication of these reports, similar neural tissue transplantation procedures became widespread. Most notably, a double-blind, sham surgery controlled study of transplantation of fetal dopaminergic neurons in PD patients was reported in 2001^[3], which provided convincing data regarding the efficacy of fetal tissue transplantation for treating this condition. Since then, fetal tissue transplantation has advanced to include the clinical development of isolated fetal cells, particularly neural stem cells in business entities.

Although many review articles have focused on the application of neural tissue and/or cells in fetal tissue transplantation^[4-14], the clinical use of fetal cells is not new or simply confined to the field of neurological field. The rationale of fetal tissue transplantation lies in the potential for fetal cell proliferation and differentiation,

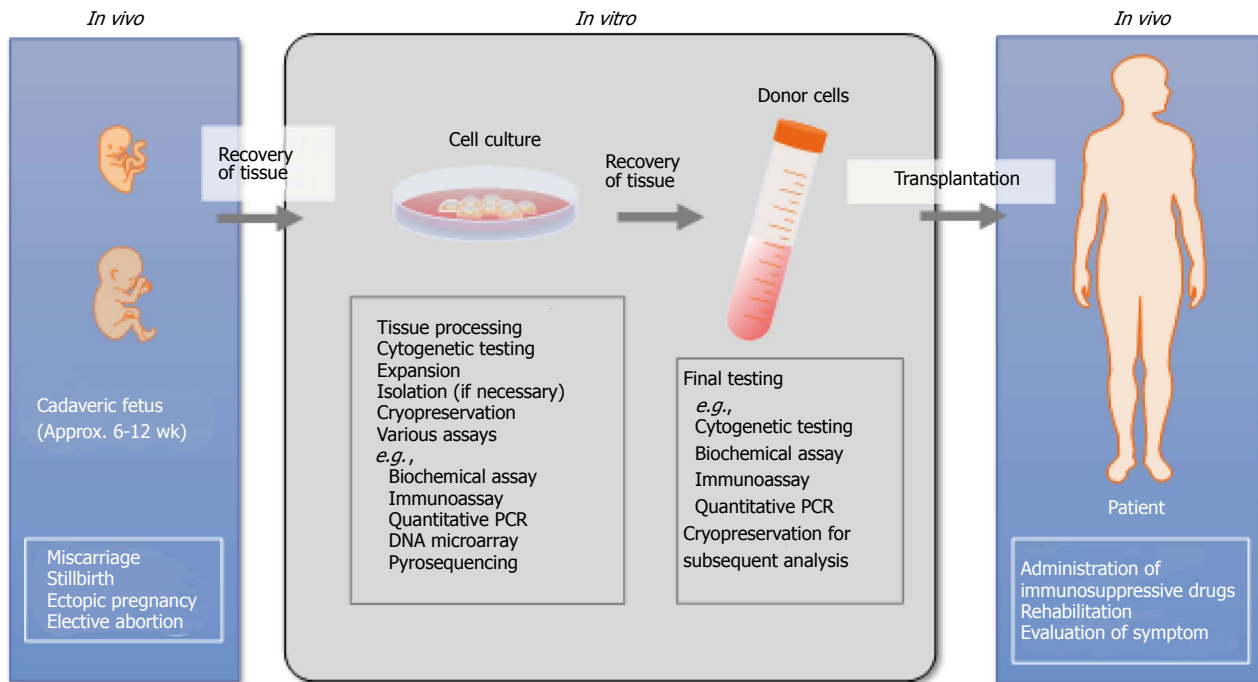


Figure 1 Fetal tissue transplantation procedures. Fetal tissue can be obtained from cadaveric fetuses for medical and non-medical reasons in obstetrics and gynecology hospitals. Procured fetal tissue, which was donated with consent for research, is processed *in vitro*, confirming cell function without contamination and genetic abnormality. After careful examination, donor cells are used for grafts primarily in the form of a cell suspension, which is usually intravenously or intraperitoneally injected or, otherwise, transplanted into predefined implant sites during surgery. Although fetal tissue cells are less likely to be rejected by transplant recipients, immunosuppressive drugs are administered in some cases. PCR: Polymerase chain reaction.

and fetal grafts may be integrated into the host without inducing immune rejection. These features of fetal tissue are well known, as is the established clinical use of transplants derived from cadaveric fetuses in the history of transplantation therapy. For example, early as 1928, a form of fetal tissue transplantation in Italy was documented in a medical journal as a treatment for diabetes mellitus^[15]. Subsequently, the indications for fetal tissue transplantation expanded to other subjects with therapeutic efficacy in conditions other than diabetes. Since the early 1960's, a tremendous number of fetal liver and thymus transplantations have been performed worldwide to treat immunodeficiency and hematological disorders.

In order to gain new perspectives on future clinical application of stem cells, it is worth considering the history of fetal tissue transplantation, taking into account an overview of current fetal stem cell research. In this report, the authors examine the history of fetal tissue transplantation, as well as many associated complications including procuring and processing fetal tissue, selecting appropriate diseases and subjects, developing new transplantation strategies, assessing graft survival and integrity *in vivo*, providing long-term monitoring of patients treated with fetal grafts for adverse events. Moreover, important ramifications of ES cell research are addressed and transplantation strategies are considered from the viewpoint of donor cells, cell processing and the therapeutic cell niche, in addition to ethical concerns. Finally, the authors provide future perspectives on fetal stem cell transplantation.

In this article, the authors offer a discussion of the progression from previous applications of fetal tissue transplantation to current uses of stem cell transplantation. In humans, the product of conception after implantation in the uterine wall through the eighth week of development is referred to as the embryo. From the ninth week to birth, the embryo is called a fetus. The authors largely follow this nomenclature.

FETAL TISSUE TRANSPLANTATION PROCEDURES

Fetal tissue contains a sufficient number of stem cells and progenitor cells for development, making it valuable for some treatments. Namely, fetal tissue cells are easier to culture and proliferate more readily than comparable adult tissue cells^[16-24], with the exception of pancreatic cells^[25,26]. Fetal tissue cells are also less likely to be rejected by transplant recipients, as these cells are less antigenic, expressing HLA-G for immune tolerance during pregnancy^[27]. This fact and the findings of animal experiments suggested a reduced need for an exact tissue match, which is frequently difficult to obtain^[28]. Collectively, the features of fetal tissue cells facilitate engraftment *in vivo* and may provide beneficial effects against diseases difficult to treat.

Fetal tissue can be obtained from cadaveric fetuses following spontaneous abortion, stillbirth, or surgery due to ectopic pregnancy in obstetrics and gynecology hospitals (Figure 1). In addition, such tissue may be derived

from elective abortions. The obtained fetal tissue is ordinarily processed and used for grafts in the form of a cell suspension, which is usually intravenously or intraperitoneally injected or, otherwise, transplanted into predefined implant sites during surgery.

PREVIOUS FETAL TISSUE TRANSPLANTATION PROCEDURES

Early attempts

A bibliographic survey revealed the use of fetal pancreatic transplantation to treat insulin-dependent diabetes mellitus, as well as an attempt to treat human cancer in Italy as early as 1928^[15]. The applied tissues were acquired from three human fetuses. Prior to this period, a diabetic dog experiment was conducted in Canada in 1921, the result of which suggested that injections of insulin, a hormone secreted from the pancreas may be used to treat diabetic patients. The following year, a clinical trial involving a 14-year-old boy with diabetes was performed; the boy recovered from his condition following insulin injections^[29]. This therapeutic achievement was awarded the Nobel Prize in Physiology or Medicine in 1923 and provided a background for the development of fetal pancreatic transplantation in Italy, as the fetal transplants may be used to circumvent the need of repeated insulin injections while offering the potential for curative therapy for diabetes. Nonetheless, this attempt eventually failed, due to a lack of treatment. Meanwhile, the first fetal pancreatic transplantation in the United States was carried out in 1939^[30]. In the clinical setting, pancreatic tissue removed from an aborted fetus was transplanted into a diabetic patient twice, albeit in vain. Subsequently, in 1959, two United States physicians reported the transplantation of fetal tissue derived from six stillborn fetuses into their diabetic mothers^[30]. However, only a transitory reduction in the need for insulin was observed in one case. Although fetal tissues are less likely to be rejected due to their reduced antigenicity, allotransplantation remained difficult until the availability of immunosuppressive drugs, such as azathioprine, in the early 1960's.

In contrast, fetal tissue was frequently used in biomedical research at that time. For instance, fetal kidney cell cultures were applied to produce large quantities of viruses, leading to the development of the polio vaccine, which was awarded the Nobel Prize in Physiology or Medicine in 1954. The application of fetal tissue cultures also contributed to the development of the rubella vaccine.

1960's to mid-1980's

The first bone marrow transplantation to treat fatal leukemia was reported by United States researchers in 1957^[31]. However, the results of marrow transplantation achieved in six patients, after first destroying their marrow with radiation, was disappointing; none of the patients survived beyond 100 d. It was not until the late 1970's when the marrow transplantation consistently resulted in success-

ful outcomes due to tissue matching, thus controlling both infectious complications and graft-*vs*-host disease (GvHD). These experiences in marrow transplantation simultaneously facilitated the development of fetal tissue transplantation, which ultimately became a frequently used therapeutic option in cases where no histocompatible donor was available for marrow transplantation.

In adult humans, hematopoiesis normally occurs in the bone marrow; however, a succession of organs sustains blood cell production during human embryogenesis^[32]. The process of hematopoiesis is initiated in the yolk sac during the third week of development, then subsequently relayed to the liver, thymus, and bone marrow at the 11th week, at which time stabilization of definitive post-natal hematopoiesis begins. Most elective abortions are performed during the first trimester. In this era, clinical availability of fetal liver and thymus tissue has encouraged researchers to performed transplantation to treat hematological disorders and cases of severe immunodeficiency.

In 1958, it was reported that a devastated immune system in rodents was restored by inoculating fetal hematopoietic tissue following lethal total body X-irradiation^[33]. In 1961, a United Kingdom group reported the results of transplantation of fresh or stored fetal liver cells ($1-20 \times 10^9$ /case, gestational age unknown) *via* intravenous injection to treat aplastic anemia, stating that remission was achieved in two of 14 patients (18 mo to 55 years of age)^[34]. Similar findings were subsequently reported from China^[35,36], Hungary^[37], India^[38-41], Italy^[42-44], and United States^[45,46].

In 1975, a United States group reported successful fetal liver transplantation in a male infant (3 mo of age) with adenosine-deaminase (ADA) deficiency, which causes severe combined immunodeficiency (SCID)^[47]. In that case, an 8.5-wk-old embryo was obtained, with permission from a mother undergoing termination of pregnancy and sterilization with hysterectomy. A suspension containing 2.5×10^8 liver cells was injected into the recipient intraperitoneally, who developed immunocompetent T and B cells in an orderly manner until one year after the procedure, when he died of fatal nephrotic disease. Soon after that case, a United States group reported the results of transplantation of fresh fetal liver cells (obtained from 8-, 9-, and 10-wk-old fetuses) in two infants with SCID in 1976^[48]. Although no functional immunological improvements were achieved in the first infant, both clinical and functional immunological improvements were noted in the other patient, who was monitored for 19 mo after transplantation. In that case, the engraftment of fetal cells, as confirmed by chimerism in the recipient's lymphocytes, reversed the patient's immunodeficiency. Similar treatment of ADA-SCID was also reported by a Japanese group in 1985^[49]. In addition, according to a case report published in 1985, a patient with X-linked SCID whose parents and siblings were not suitable HLA-compatible bone marrow donors underwent, embryonic liver cells were transplanted intravenously in 3 stages ($6 \times 10^6 - 9 \times 10^7$)^[50]. Although the procedure resulted in T-cell recon-

stitution in addition to the initiation of immune globulin production, the child died at five months of age due to respiratory failure. In another SCID case reported by a French group in 1979, an infant who received two separate grafts of both hepatic and thymus cells recovered from the same fetus exhibited a partially restored immune system^[51].

Fetal liver transplantation has also been attempted to treat leukemia. In 1982, an Italian group reported the use of fetal liver transplantation in two patients with acute leukemia following the administration of a conditioning regimen consisting of cyclophosphamide and total body irradiation^[52]. Although each patient achieved remission with a hematopoietic recovery, the survival time after transplantation was only 153 and 30 d, respectively. A similar transplantation procedure was subsequently conducted to treat acute myeloid leukemia in India^[53]. In 1986, a Chinese group reported the results of fetal liver transplantation in 10 patients with malignant tumors^[54]. The authors prepared fetal liver cells using 3.5-6-mo-old fetuses and observed 1.8×10^8 - 4×10^{12} fetal liver cells in a fetus over five mo of age, in which most of the cells were are CFU-Cs (granulocyte progenitor cells). These findings suggest that fetal liver transplantation improves the peripheral blood profile and stimulates the production of bone marrow.

In February 1986, a symposium on fetal liver transplantation was held in New-Delhi, India^[55]. A relevant review article critically analyzed progress in the field at that time and reported that over 300 individuals had received fetal liver transplants for a spectrum of disorders, including immunodeficiency, aplastic anemia, leukemia and genetic conditions. Additionally, in a review article published in 1987, a United States researcher, Gale, examined the results of fetal liver transplantation in patients with hematological disorders^[46]. With respect to aplastic anemia, 122 two patients received transplants, with engraftment reported in four patients and GvHD in no cases. Although complete and partial responses were reported in half of the patients, the majority displayed no evidence of engraftment. Meanwhile, 39 patients with leukemia received transplants; transient engraftment was reported in 40% of cases, and two patients developed GvHD. In that report, the survival was extended to more than two years. The relatively high rate of engraftment also suggested the efficacy of pretransplant immune suppression. Therefore, the risk of GvHD appears to be low, despite complete HLA-mismatching.

Regarding thymus transplantation, two cases were reported in 1968, in which fetal thymus tissue was transplanted into neonates suffering from DiGeorge syndrome, which is characterized by the absence or incomplete development of the thymus with varying degrees of T-cell immunodeficiency^[56]. In addition, August *et al*^[50] reported the case of a 21-mo-old male with DiGeorge syndrome who underwent transplantation of thymus fragments derived from a 16-wk-old female fetus. In that case, abnormalities in the patient's lymphocyte function

were promptly ameliorated. Cleveland *et al*^[57] also reported the implantation of three thymus fragments derived from a 13-wk fetus into a 7-mo-old male infant. Although no XX cells were identified in the host, the infant's immunological data and ability to resist infection suggested that his immunological function was reconstituted by the fetal transplants^[57]. Another article reported that the combined transplantation of the fetal thymus and liver resulted in effective immunological reconstitution in a presumed case of DiGeorge syndrome^[58]. Two similar thymus transplantation procedures were performed in Japan^[59,60].

During this period, various cases of fetal tissue transplantation were reported in medical journals. However, the clinical results and patient survival rates were largely dismal. At that time, most fetal tissue transplantations were conducted based on previous experience with bone marrow transplantation in which irradiation-based or chemical conditioning is performed prior to transplantation in order to facilitate post-transplantation engraftment following the administration of immunosuppressive drugs. However, cellular characteristics of fresh or preserved fetal tissue were insufficient in most cases, with total cell count usually being the only parameter reported, while the cell functions was not thoroughly assessed. Moreover, in general, precautions measures to prevent infectious diseases were not taken. For example, fetal tissue donors were not carefully screened, and testing of fetal tissue prior to transplantation was largely insufficient. Despite clinical success in some cases, the use of fetal liver and/or thymus transplantation should have been based on sufficient data from preclinical research using disease model animals, as is common in current stem cell research.

Mid 1980's to early 2000's

Around the mid-1980's, the application of fetal neural transplantation to treat neurological diseases began to receive significant attention. In this era, clinical trials using fetal cerebral tissue were conducted worldwide primarily in patients with Parkinson's disease (PD), a progressive disorder of the central nervous system that affects movement. PD is characterized by the death of dopaminergic neurons, the substantia nigra in the brain for unknown reasons. Langston *et al*^[61] identified a chemical, MPTP (1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine), that selectively damages cells in the substantia nigra, resulting in the development of marked parkinsonism in monkeys and humans, and the injection of MPTP can be used to create an animal model of PD. Preclinical research using such animals has demonstrated that transplanting the fetal substantia nigra significantly improves motion symptoms^[62-65]. Although L-Dopa therapy has been applied to PD since the 1960's, this medication induce troublesome side effects, such hypotension and a variety of abnormal involuntary movements^[66]. Therefore, the transplantation of fetal neural tissue, including dopaminergic neurons, is thought to be an alternative treatment for PD.

In addition to preclinical research using animal dis-

ease models, fetal neural tissue transplantation was performed based on preclinical data, including the impact of cryopreservation^[67], and screening for infection and cytogenetic abnormalities^[68]. Regarding the *in vivo* survival of fetal tissues and cells, Freeman *et al.*^[69] reported the implantation of human mesencephalic dopaminergic neurons in a rat model and suggested that the upper age limit should be postconception (PC) day 56 for suspension grafts and PC day 65 for solid implants.

In September 1986, a Mexican group conducted a renowned clinical trial in which the fetal mesencephalic substantia nigra procured from a 13-wk-old fetus of spontaneous abortion, was transplanted in the caudate nucleus in two PD patients. The cases were subsequently reported in 1988^[1], and the results of monitoring at three months showed a dramatic improvement in symptoms; in particular both rigidity and dyskinesia disappeared^[70]. In the preceding year, 1987, however, a Chinese team had already reported the transplantation of similar fetal tissue in a PD patient in August 1985, the first clinical trial in which brain tissue was transplanted from one human being to another^[2]. In that case, a suspension containing substantia nigra fragments was implanted into the striatal caudate nucleus to which a collateral projection extends from the substantia nigra. The case involved a 54-year-old male patient whose HLA status was determined prior to transplantation, although the fetal HLA status was not tested. The transplanted tissue was obtained from a 5-mo-old fetus, as the authors considered the clinical use of the substantia nigra derived from fetuses of 4.5-5.5 mo of age to be appropriate based on the stage of tissue development at that age. However, this presumption was inaccurate compared to the evidence (in embryos up to nine weeks of age) provided by Freeman *et al.*^[69]. However, the Chinese team reported a reduction in limb tremors and rigidity on the third day after the surgery, with satisfactory control of parkinsonism confirmed after eight months of diagnostic monitoring. Moreover, a United Kingdom group published a case report of fetal tissue transplantation for PD in 1988^[71]. The authors stated that two patients (a 60-year-old female and 41-year-old male) with early and late parkinsonism, respectively, showed immediate improvements in motion symptoms following the administration of a mesencephalic cell suspension (fetal age unknown). These cases, with the exception of the China case, made worldwide headlines, commanding considerable attention from patients and their families. However, all three cases lacked comprehensive, long-term results, including the findings of behavioral, biochemical, psychological, physiological, and motor assessments.

Subsequently, a Swedish group demonstrated that deep brain transplantation of fetal brain tissue could be used to restore local dopamine production and relieve symptoms^[72]. According to their report published in 1990, mesencephalic dopamine neurons derived from embryos of eight to nine weeks of gestation exhibited survival in the recipient. The grafts, which were implanted unilaterally into the putamen *via* stereotactic surgery,

restored dopamine synthesis and storage in the grafted area, as assessed on positron emission tomography with 6-L[18F]fluorodopa. These neurochemical changes resulted in a significant reduction in severe rigidity and bradykinesia, with marked diminution of fluctuations in the patient's condition under optimal medication. Following this achievement, long-term (up to 46 mo) stable improvements and graft integrity were reported in various cases^[73-76]. Stable integration and the persistence of fetal grafts have also been confirmed on functional imaging as well as postmortem analyses^[77-79]. Such clinical results have encouraged many researchers worldwide to apply this therapeutic approach as a treatment for PD. Namely, fetal brain tissue transplantation, which began in China^[2,80], has been attempted in Canada^[81], Cuba^[82], the Czech Republic^[83], France^[84], Mexico^[1], Poland^[85], Slovakia^[86], Spain^[87,88], United Kingdom^[71,89], United States^[2,3,73,77,90,91], and USSR (currently Russia)^[92]. Moreover, the Network of European CNS Transplantation and Restoration (NECTAR) was founded in 1990 to bring together European groups who share the common goal of protecting, repairing and restoring the central nervous system damage resulting from degenerative diseases and/or injury^[93].

Fetal tissue transplantation for PD has also been conducted using fetal adrenal medullary tissue^[1,80] other than the substantia nigra, and several clinical trials have assessed the efficacy of fetal neural transplantation for neurological conditions other than PD. For instance, patients suffering from Huntington's disease (HD) have been evaluated in the United Kingdom^[94]. In the report, cell suspensions of fetal ganglionic eminence were transplanted unilaterally into the striatum in four patients with early to moderate HD, all of whom received immunotherapy with cyclosporin A, azathioprine, and prednisolone for at least six months postoperatively. During the six month post-transplantation period, the only adverse events related to the procedure were associated with the immunotherapy regimen. Magnetic resonance imaging demonstrated the presence of tissue at the implantation site, although no signs of tissue overgrowth were detected. The United Kingdom team concluded that the unilateral transplantation of fetal striatal tissue in patients with HD is safe and feasible. Meanwhile, an Indian group issued a report in which human fetal neuroretinal cells were transplanted in patients with advanced retinitis pigmentosa^[95]. The results of a long-term phase I safety study (12-40 mo) prompted the initiation of phase II trials.

Notably, in 2001, a United States group reported the results of a double-blind, sham surgery controlled-study of transplantation of fetal dopamine neurons in PD patients^[5]. The neural tissues were recovered from 7- to 8-wk-olds embryos, and the tissue cell culture, in which dopamine production was monitored according to homovanillic acid concentration in the medium, was transplanted up to four weeks after recovery. Consequently, a reduction in motor symptoms was observed in the patients 60 years of age or younger, but not in the older

patients. This study provided the first direct evidence that fetal grafts can be used to improve the condition of some PD patients, separate from the placebo effect. Another United States group reported the results of a similar double-blind controlled trial in which, approximately half of the patients treated with solid mesencephalic grafts derived from 6- to 9 wk-old embryos developed dyskinesia, with no significant overall treatment effect^[96]. Moreover, postmortem analyses revealed the subjects who displayed significant improvements had at least 100000 dopaminergic neurons per sides with organotypic reinnervation of the striatum^[97,98]. In these cases, four 6- to 9-wk-old fetuses were required to obtain the requisite number of cells for a graft. Therefore, some research groups have introduced a temporal moratorium on such procedures since 2003 owing to the uncertainty and difficulty in conducting clinical trials^[99].

ETHICAL ISSUES AND POLITICAL RESPONSES

As mentioned above, the results of fetal brain tissue transplantation for PD have received significant attention, making worldwide headlines in the news media since the late 1980's. Such advancements have simultaneously raised profound ethical concerns and objections against the medical use of cadaveric fetal tissue, which is frequently derived from cases of elective abortion. This report briefly addresses this issue and associated political responses. The ethical debate in the United States, which involves anti-abortion movement, led to a moratorium on federal funding (1987-1992) of fetal tissue transplantation research^[100-103]. There are five issues related to fetal tissue transplantation. First, females may be advised or persuaded to undergo induced abortion on the grounds that it may help others by donating fetal tissue. Second, the widespread use of fetal tissue transplantations may result in an increase in the number of abortions. Third, the successful use of fetal tissue may make such procedures more socially acceptable. Fourth, the abortion procedure may be changed based on medical needs. Most notably, the question as to whether rightful informed consent for the use of fetal tissue can be obtained in cases of induced abortion is the most controversial issue. Ideally, the decision to undergo induced abortion should be completely separate from the consent to fetal tissue donation (*i.e.*, the "principle of separation"^[104]).

In the United Kingdom, the Department of Health and Social Security issued a report by their advisory group (the Peel report) regarding the use of fetuses and fetal material for research in 1972. The Department subsequently reviewed their guidelines on the research and use of fetuses and fetal material in the Polkinghorne report in 1989^[105]. The main issue in that report was the consistent application of the "principle of separation"^[104]. In contrast, the British Medical Association (BMA) dissented from the Polkinghorne report in their guidelines on the use of fetal tissue^[106], and United Kingdom sociol-

ogists expressed concerns about the fetal tissue economy from the abortion clinic to the stem cell laboratory^[107, 108]. In 1994, NECTAR considered the ethical issues and published guidelines for the use of human embryonic or fetal tissue for research and clinical use^[109]. In Japan, discussions in the Ministry eventually resulted in the development of guidelines on clinical research using human stem cells, in which the clinical use of fetal cells is intentionally excluded due to potential, profound ethical issues^[110].

Thus, fetal tissue transplantation has raised ethical controversy worldwide. The "principle of separation" suggests fetal tissue can be procured if informed consent is separately obtained from females who underwent spontaneous abortion and still birth in Europe and the United States. It is also suggested that fetal tissues may be obtained if the induced abortion is conducted for a clear medical reason (*e.g.*, ectopic pregnancy). However, if the informed consent is obtained from females who will undergo induced abortion only for a social reason, some would query the validity of the informed consent^[107]. The difficulty in the 'principle of separation' in some cases is likely to lead to the exclusion of fetal cells in stem cell transplantation, as in Japan if future results of fetal tissue transplantation are overhyped.

CHANGES WITHIN THE LAST DECADE

Since 2000, fetal cell transplantation has advanced to the clinical development of isolated fetal stem cells. As mentioned above, there are hundreds of investigator-initiated clinical trials of fetal transplantation in the academic setting. In addition, several companies have developed or are developing fetal stem cell products *via* the use intracerebral or spinal transplantation^[111].

A wide variety of conditions have been assessed using fetal stem cell transplantation. Recently, evaluated conditions can be categorized into six groups: neurological diseases, central nervous system (CNS) injury, heart failure^[112], diabetes^[113], skin wounds^[114], and osteogenesis imperfecta^[115]. Neurological diseases include amyotrophic lateral sclerosis (ALS)^[116-118], cerebral palsy (CP)^[119,120], cerebral atrophy^[121], Huntington's Disease^[122,123], and PD^[124-126]. With respect to CNS injury, spinal cord injury (SCI)^[127] and traumatic brain injury^[128] have been recent topics in the setting of fetal cell transplantation. Some of these reports are described below.

Olfactory ensheathing cells (OECs) are radial glia with a variety of functions. These cells phagocytose axonal debris and dead cells in the olfactory system^[129]. OECs are also known to secrete many neurotrophic factors. A Chinese group, Chen *et al.*^[120] conducted a randomized controlled clinical trial among 33 patients in order to confirm the feasibility of OEC transplantation for treating CP in children and adolescents. In that report, OECs were isolated from aborted human fetal olfactory bulbs, cultured and propagated for two to three weeks and then characterized using immunostaining with Abs against p75. OECs derived from one to two fetuses, representing

two million cells, were transplanted in each patient, and HLA-DR-matching analyses ensured histocompatibility between the donors and recipients. The trial ultimately demonstrated that fetal OEC transplantation is effective for obtaining functional improvements in children and adolescents with CP, without obvious side effects. Another Chinese group, Wu *et al.*^[127] followed patients with complete chronic SCI for an average of 14 mo after OEC transplantation. Consequently, both sensation and spasticity improved moderately, whereas the recovery in locomotion recovery was minimal. In contrast, Piepers and den Berg asserted that there are no benefits from experimental treatment with fetal OECs in patients with ALS^[118]. The authors carried out a prospective study of seven patients who underwent fetal OEC treatment in China^[130], following the subjects for four months to one year after treatment, and found no objective improvements, while the outcome measurements gradually declined in all patients. Two patients experienced severe side effects. Therefore, although careful examination is needed, fetal OEC transplantation is likely to be effective against trauma-induced neurological conditions, but not ALS or the selective degeneration of motor neurons. These findings highlight the significance of selecting appropriate diseases and conditions for each type of stem cell transplantation.

Regarding fetal neural progenitor cells (NPCs) and neural stem cells (NSCs), a Chinese group, Luan *et al.*^[119] performed fetal NPC transplantation in 45 patients with severe CP by injecting NPCs derived from aborted fetal tissue into the lateral ventricle. The NPCs were isolated from aborted human fetal forebrain tissue and likewise propagated. The cells used for transplantation were characterized as nestin-positive and microbe-free with normal karyotype, viability of over 95%, and endotoxin level below 2 EU/mL. After one year, the developmental level for each functional sphere (gross motor, fine motor, and cognition) was significantly higher in the treatment group than in the control group, with no delayed complications. Therefore, both fetal NPC and OEC transplantation appear to be efficacious against CP^[119,120]. A United States group, Grass *et al.*^[117], consequently reported the results of a phase I trial of the intraspinal injection of fetal NSCs in patients with ALS. This study was a first-in-human clinical trial with the goal of assessing the safety and tolerability of introducing stem cells into the spinal cord, in association with the administration of immunosuppressants. Twelve patients received either five unilateral or five bilateral (10 total) injections into the lumbar spinal cord at a dose of 100000 cells per injection. Clinical assessments ranging from six to 18 mo after transplantation demonstrated no evidence of acceleration of disease progression due to the intervention; therefore, the goal of the clinical trial was attained. Hence, ALS may be treated with fetal NSC transplantation, but not fetal OEC transplantation^[117,118].

In addition to the above bibliographic survey, relevant trials were searched on ClinicalTrials.gov in order

to provide an overview of recent clinical trials of fetal transplants (Table 1). Consequently, 11 trials were identified, most of which (7/11) were sponsored by business entities. In addition, fetal neural stem cells were used in most trials (8/11), focusing on ischemic stroke, SCI, age-related macular degeneration, and neurological disorders, including ALS and Pelizaeus-Merzbacher disease (an inherited dysmyelination disorder). Meanwhile, fetal mesencephalic tissue or dopamine neuronal precursor cells were used for transplantation in PD patients in two trials and fetal liver cells were used in one trial. Most of these studies (7/11) were sponsored by private companies, including Stem Cell, Inc. (California, United States), Neuralstem Inc. (Maryland, United States), and ReNeuron Ltd. (United Kingdom). Stem Cell Inc. has developed a neural stem cell product for use in Batten's disease (neuronal ceroid lipofuscinosis) and obtained approval for a new investigational drug (IND) from the FDA, although a phase I trial was terminated due to difficulties in recruiting an adequate number of patients. Instead, the company opted to focus on thoracic SCI, age-related macular degeneration, congenital Pelizaeus-Merzbacher disease for clinical development. Other companies are currently developing neural stem cell products to treat stable ischemic stroke (ReNeuron) as well as ALS and chronic SCI (Neuralstem Inc.).

Among the above companies, Stem Cell, Inc. is the most active developer of fetal neural stem cells. For example, it has generated unique mAbs and isolated neural stem cells derived from fetal brain tissue using cell sorters. The company has identified and enriched CD133⁺ CD24^{-/lo} population cells using their unique mAbs against CD133 and CD24. The transplantation of CD133⁺ sorted/expanded neurosphere cells into the lateral ventricle in newborn NOD-SCID mouse brains has been shown to result in specific engraftment in numerous sites, according to the levels of brain markers. The researchers therefore concluded that human central nervous system (CNS) stem cells can be clonally isolated^[153]. Using CNS stem cells, the company is currently developing stem cell products for use in patients with SCI, macular degeneration, and Pelizaeus-Merzbacher disease. In most cases, de novo neurogenesis is not the goal, but rather the treatment of enzyme deficiencies, as well as remyelination, or the modulation of endogenous repair *via* neoangiogenesis and/or neuroprotection^[131-134]. Moreover, the company has isolated fetal liver progenitor cells and developed a unique co-culture system with endothelial cells in a three-dimensional matrix^[135]. These liver cells are studied for the future application of transplantation therapy and drug discovery assay systems.

Recent fetal stem cell transplantation procedures have used isolated and well-characterized fetal tissue cells designed in a sufficiently rational manner. Clinical trial results also allow researchers to be optimistic about the future of fetal stem cell transplantation. Nevertheless, uncertainties abound in the clinical settings. Amariglio *et al.*^[136] reported an adverse event following NSC trans-

Table 1 Ongoing clinical trials of fetal stem cell transplantation

Clinical Trials.gov	Start (yr)	Sponsor	Status	Title	Interventions	Cell source
NCT 01013194	2007	The Mediterranean Institute	Unknown	Human fetal liver cell Transplantation in chronic liver failure	Human fetal liver cell transplantation	Fetal liver cells derived from fetuses between the 16 th and 26 th week of gestation
NCT 01151124	2010	ReNeuron Limited.	Active, not recruiting	Pilot Investigation of Stem Cells in Stroke	Surgical delivery of a neural stem cell line to the damaged area of the brain	CTX0E03 neural stem cells ¹
NCT 01321333	2011	StemCells, Inc.	Active, not recruiting	Study of HuCNS-SC in patients with thoracic spinal cord injury	Intramedullary spinal cord transplantation of human CNS stem cells	HuCNS-SC cells (Human Central Nervous System Stem Cells) ²
NCT 01348451	2009	Neuralstem Inc.	Active, not recruiting	Human neural stem cell Transplantation for the Treatment of amyotrophic lateral sclerosis	Surgical implantation of human neural stem cells	Human spinal cord derived neural stem cells ³
NCT 01391637	2011	StemCells, Inc.	Active, not recruiting	Long-term follow-up study of human stem cells transplanted in subjects with pelizaeus-merzbacher disease	HuCNS-SC transplantation	HuCNS-SC cells (Human Central Nervous System Stem Cells) ²
NCT 01632527	2012	StemCells, Inc.	Recruiting	Study of HuCNS-SC in age-related macular degeneration	Transplanting HuCNS-SC cells directly into the subretinal space of one eye.	HuCNS-SC cells (Human Central Nervous System Stem Cells) ²
NCT 01640067	2011	Azienda Ospedaliera Santa Maria	Recruiting	Human neural stem cell Transplantation in Amyotrophic Lateral Sclerosis	Surgical microinjection of human neural stem cells	Human foetal neural stem cells suspension
NCT 01730716	2013	Neuralstem Inc.	Enrolling by invitation	Dose escalation and safety study of neural stem cell transplantation for the treatment of amyotrophic lateral sclerosis	Human spinal cord stem cell implantation	Human spinal cord derived neural stem cells ³
NCT 01772810	2014	Neuralstem Inc.	Not yet recruiting	Safety study of human spinal cord-derived neural stem cell transplantation for the treatment of Chronic SCI	Human Spinal Cord-derived Neural Stem Cell Transplantation	Human spinal cord derived neural stem cells ³
NCT 01860794	2013	Bundang CHA Hospital	Recruiting	Evaluation of safety and tolerability of fetal mesencephalic dopamine neuronal precursor cells for Parkinson's disease	Transplantation of fetal mesencephalic dopamine neuronal precursor cells	Fetal mesencephalic dopamine neuronal precursor cells
NCT01898390	2012	University of Cambridge	Enrolling by invitation	TRANSEURO open label transplant study in Parkinson's disease	Neural allo-transplantation with fetal ventral mesencephalic tissue	Fetal ventral mesencephalic tissue

The survey was conducted in ClinicalTrials.gov using key words “fetal + transplantation, or fetus + transplantation”. The status is on June 24, 2014. The description of the table is based on the database. See also the details by entering the identifier No. into the database website. Additional investigation confirmed that the neural stem cells¹ are derived from first trimester human fetal cortical cells, the central nervous system stem cells² from fetal brain tissue, and the spinal cord derived neural stem cells³ from a single eight-week-old fetus.

plantation in a boy with ataxia telangiectasia treated with the intracerebellar and intrathecal injection of human fetal NSCs. Four years after the first injection, the patient was diagnosed with a multifocal brain tumor, a biopsy of which showed a glioneuronal neoplasm. In addition, molecular and cytogenetic studies demonstrated the tumor to be of nonhost origin and microsatellite and HLA analyses revealed that the tumor was derived from at least two donors. This is the first report of a human brain tumor complicating the outcome of NSC therapy. These findings suggest that neuronal stem/progenitor cells may induce gliomagenesis. Therefore, considerable caution is required when implementing NSC transplantation, although clinical trials of NSCs are proceeding worldwide.

RAMIFICATIONS OF EMBRYONIC STEM CELL RESEARCH

The results of previous fetal neural transplantation therapy for PD have indicated that the use of more biologically defined and clinically reliable sources of dopaminergic neurons is required in future clinical trials. For this reason, other stem cell sources are often investigated in parallel with clinical trials of fetal stem cell transplantation.

Pluripotent ES cells are established from preimplantation, not implantation, embryos. ES cells possess self-renewal properties and almost infinitely proliferate in petri dishes. In addition, under appropriate differentiation protocols, ES cells exhibiting pluripotency can be differ-

entiated into any lineages of the ectoderm, mesoderm, or endoderm. Therefore, ES cells can be used to obtain the number of cells required for transplantation therapy for various diseases.

Two reports regarding the establishment of mouse ES cell lines were published in 1981^[137,138]. The first derivation of human ES cell lines was based on knowledge obtained *via* the establishment of non-human primate ES cells, first attained in 1995^[139,140]. It took a considerable amount of time to transition from mouse to human ES cells due to differences in molecular and cellular mechanisms between mice and humans that hampered the technical establishment of the culture method. For instance, human ES cells, unlike their mouse counterparts, do not appear to require leukemia inhibitor factor (LIF) for propagation or the maintenance of pluripotency^[140,141]. Instead, fibroblast growth factor (FGF) signaling has a central role in the self-renewal of human ES cells. It has been previously demonstrated that basic FGF (bFGF) stimulates the clonal growth of human ES cells on fibroblasts in the presence of a commercially available serum replacement^[142]. In addition, while the expression of many of markers is similar in mouse and human ES cells, significant differences are noted in the expression levels of vimentin, β -III tubulin, alpha-fetoprotein, eomesodermin, HEB, ARNT, FoxD3, and the LIF receptor complex LIFR/IL6ST (gp130)^[143]. Furthermore, focused microarray analyses have identified significant differences in cell cycle and apoptosis regulation as well as cytokine expression^[143].

Human ES cells which were first reported in 1998 were established from surplus *in vitro* fertilization (IVF) embryos, a byproduct of assisted reproduction treatment. The creation of embryos for research purposes, which is associated with ethical issues and requires rigorous reviews in many countries even if legally permitted^[144], was not conducted to establish the ES cells. Nonetheless, an ethical debate ensued, as some regard preimplantation embryos to constitute the beginning of human life. Meanwhile, in 2009, the United States FDA approved an IND applied by the Geron Corporation (California, United States)^[111]. The biologics of human ES cell-derived cells was developed in the first clinical trial after the company verified that there were no problems with the cell product regarding the formation of micro-cysts in animal transplants. The approved phase I study was conducted to assess the safety of transplantation of human ES cell-derived oligodendrocyte precursor cells in patients with thoracic spinal cord injury. In that study, the subjects with functionally complete spinal cord injury at the T3 to T10 spinal segments underwent grafting of oligodendrocyte progenitors into the spinal cord at the site of injury under conditions of immunosuppression. Although Geron terminated the study for financial reasons in 2011, another company plans to restart the trial^[145].

Current clinical trials of ES cells (Table 2) include at least eight trials of ES cell-derived cells underway in France, South Korea, United Kingdom, and the United

States. Again, most of these studies are being sponsored by business entities (6/8). Namely, Advanced Cell Technology (ACT), Inc. (Massachusetts, United States) is currently developing ES cell-derived retinal pigment epithelium cells to treat conditions such as age-related macular degeneration and macular dystrophy using an orphan drug status to accelerate clinical trials. In addition, CHA Bio and Diostech (South Korea) is advancing two pipelines similar to that of ACT using the cell product developed by ACT. Pfizer is also currently developing a similar pipeline to that of ACT and CHA Bio and Diostech; however, Pfizer is using a different cell product. Hence, macular generation is the primary condition currently receiving attention with respect to the development of ES cells. The remaining two trials are being sponsored by French and United States universities. UCLA is attempting to initiate a clinical trial in which ACT's cell product applied to treat macular regeneration, while Assistance Publique - Hôpitaux de Paris is recruiting patients to develop a treatment for ischemic heart disease using ES cell-derived CD15⁺ Isl-1⁺ progenitors. All of these trials are open-label, not blind, studies. More recently, the use of autologous ES cells, which reduces the possibility of immune rejection, has recently become realistic based on somatic cell nuclear transfer^[146,147]. Clinical success rates of transplantation using autologous ES cell-derived cells would be expected to increase, although there is a potential ethical issue when procuring oocytes from females.

Another type of pluripotent stem cell, embryonic germ (EG) cells, can be established from cultured human primordial germ cells (PGCs) derived from early embryos. The first establishment of human EG cells from 5-to 9-wk-old embryos obtained as a result of the therapeutic termination of pregnancy, was reported in 1998^[148], followed by other reports^[149]. However, knowledge of human PGCs and EG cells is insufficient, as these cells are difficult to study in the gonadal ridge during the fifth and sixth week of development, with further PGCs often being detected in the gut mesentery, most likely during transit^[149]. To our knowledge, there have been no clinical trials of human EG cells.

FUTURE DIRECTIONS OF STEM CELL TRANSPLANTATION

In the 20th century, clinical issues abounded in the field of fetal tissue transplantation and many lessons were learned from such practices. After reflecting on the history of fetal tissue cell transplantation, this report will now consider the future direction of stem cell transplantation based on issues related to donor cells, cell processing, and therapeutic cell niche.

Donor cells

Earlier fetal tissue cell transplantation procedures required careful screening of maternal donors and testing of fetal tissues in order to prevent infectious diseases as well as match histocompatibility; however, such analyses

Table 2 Ongoing clinical trials of embryonic stem cell-derived cell transplantation

Clinical Trials.gov	Start (yr)	Sponsor	Status	Title	Condition	Intervention	Remarks
NCT01344993	2011	Advanced cell technology	Recruiting	Safety and tolerability of transplantation of MA09-hRPE cells in patients with advanced dry age related macular degeneration	Advanced dry age related macular degeneration	Sub-retinal transplantation of MA09-hRPE	A Phase I / II, open-label, multi-center, prospective study in United States. MA09-hRPE cells are human embryonic stem cell derived retinal Pigmented epithelial cells.
NCT01345006	2011	Advanced cell technology	Recruiting	Transplantation of MA09-hRPE cells in patients with stargardt's macular dystrophy	Stargardt's macular dystrophy	Sub-retinal transplantation of MA09-hRPE	A Phase I / II, open-label, multi-center, prospective study in United States
NCT01469832	2011	Advanced cell technology	Recruiting	Safety and tolerability of transplantation of hESC-RPE cells in patients with stargardt's macular dystrophy	Stargardt's macular dystrophy; fundus flavimaculatus; juvenile macular dystrophy	Sub-retinal transplantation of MA09-hRPE	A Phase I / II, open-label, multi-center, prospective study in the United States
NCT01625559	2012	CHA Bio and diostech	Recruiting	Safety and tolerability of MA09-hRPE cells in patients with stargardt's macular dystrophy	Stargardt's macular dystrophy	Sub-retinal transplantation of MA09-hRPE	A Phase I, open-label, prospective study in Korea
NCT01674829	2012	CHA Bio and diostech	Recruiting	Safety and tolerability of transplantation of MA09-hRPE cells in patients with advanced dry age-related macular degeneration (AMD)	Dry age Related macular degeneration	Sub-retinal transplantation of MA09-hRPE	A Phase I / II a, open-label, single-center, prospective study in Korea
NCT01691261	2014	Pfizer	Not yet recruiting	Implantation of human embryonic stem cell derived retinal pigment epithelium in subjects with acute wet age related macular degeneration and recent rapid vision decline	Age related macular degeneration	Implantation of human embryonic stem cell derived retinal pigment epithelium	Phase 1, open-label, safety and feasibility study in United Kingdom. PF-05206388 is human embryonic stem cell derived retinal pigment epithelium living tissue equivalent.
NCT02057900	2013	Assistance publique - hôpitaux de Paris	Recruiting	Transplantation of human embryonic stem cell-derived progenitors in severe heart failure (ESCORT)	Ischemic heart disease	Human embryonic stem cell-derived CD15+ Isl-1+ progenitors	Phase 1, open-label, feasibility and safety study in France
NCT02122159	2014	University of California, Los Angeles	Not yet recruiting	Research with retinal cells derived from embryonic stem cells for myopic macular degeneration	Myopic macular degeneration	MA09-hRPE cellular therapy	A Phase I / II, Open-label, prospective study to determine the safety and tolerability in United States

The survey was conducted in ClinicalTrials.gov using key words "Embryonic + Stem + Cells". The status of clinical trials listed is confirmed on June 19, 2014. The description of the table is based on the database. See the details by entering the identifier No. into the database website.

were often not conducted sufficiently. In addition, mouse transplantation experiments showed that the immunogenicity of first-trimester human fetal pancreatic grafts (6- and 9-wk-old embryos) is less than that of older, second-trimester human fetal pancreatic grafts^[28]. This reduced immunogenicity is insufficient to completely circumvent the need for immunosuppressive conditioning in the recipient^[150]. Such precautions are now common sense for assuring safety in present-day stem cell transplantation.

The authors emphasize the need for sufficient implementation of cytogenetic testing, such as karyotyping and CGH arrays, in order to attain the therapeutic goal (Figure 1). Fetal tissue can be obtained from cadaveric fetuses following spontaneous abortion, stillbirth, or surgery due to ectopic pregnancy, in addition to elective abortion. Among these types of cells, fetal tissues de-

rived from spontaneous abortion and stillbirth are more likely to induce adverse events after transplantation, and frequent chromosomal or genetic causes of spontaneous abortion and stillbirth are likely to affect the pre- and post-transplantation behavior of donor cells. In addition, genetic changes may occur during cell culture. Therefore, cytogenetic testing is required to confirm the therapeutic validity of stem cells for transplantation. From this viewpoint, fetal tissue derived from cases of elective abortion or ectopic pregnancy is more likely to be an appropriate source for transplantation. However, the use of such cells remains still ethically, and socially controversial, primarily mainly due to the difficulty in consistently applying the "principle of separation" in cases of elective abortion^[100-103,107,108]. For these reasons, the procuring of the required amount of fetal tissue for transplantation is

challenging.

In contrast, adult tissue stem or progenitor cells, or terminally differentiated cells derived from non-fetal, adult tissues are more likely to be candidates for transplantation. In addition, the clinical use of human pluripotent stem cells recently became realistic (Table 2). As mentioned above, ES cells have been established from a more ethical source, surplus IVF embryos^[144]. Compared with adult tissue stem cells, ES cells proliferate more readily *in vitro*, and the directed differentiation of human ES cells can be used to produce a desired lineage, with some types of differentiated cells currently being applied as grafts in clinical trials (Table 2). Furthermore, a far more ethical source, induced pluripotent stem (iPS) cells, which are established from reprogramming the patient's own somatic cells *via* ectopic expression of defined factors, is now available^[151]. Human iPS cells can be likewise differentiated and used for autologous transplantation. Recently, the Japanese Ministry of Health, Labour, and Welfare approved a clinical research application for the use of iPS cell-derived retinal pigment epithelium cells in patients with age-related macular degeneration^[152]. Therefore, with the exception of fetal stem cells, a variety of human pluripotent stem cells are available for study in clinical trials.

Cell processing

A few weeks of culture has frequently been applied to expand fetal cells prior to transplantation^[3,119,120,153]. Close monitoring during cell culture is needed to assess whether the culture changes the cell population and/or function. If a change in cell population is detected, the population intended for use in transplantation must be isolated *via* methods such as a cell sorting^[154], as the presence of a remaining unintentional cell population in the culture may cause side effects. Notably, the effects of intermingled serotonergic neurons in part explain the onset of graft-induced dyskinesia in the setting of fetal neural transplantation^[155]. Such caution should also be applied to cell cultures resulting from the directed differentiation of pluripotent stem cells. In addition, culture additives, such as serum replacement and bFGF, must be carefully tested to avoid contamination with viruses or other microorganisms as well as potential epigenetic effects. Therefore, cell processing requires sufficient optimization in preclinical research.

Again, cell-processing also requires cytogenetic testing to confirm that the absence of karyotype or genetic changes during cell culture. Regarding application of human pluripotent stem cells, there remain still technical obstacles. For example, human ES cells and iPS cells exhibit a progressive tendency to acquire genetic changes during prolonged culture^[156]. In addition, it is necessary to take precautions against genetic instability (in the nucleus and mitochondria) of iPS cells, which may occur regardless of the reprogramming method used^[157]. However, future advances in stem cell research would overcome such obstacles.

Therapeutic cell niche

The selection of appropriate diseases and symptoms largely constitutes successful transplantation therapy, subsequently requiring the systematic consideration of autonomous or non-autonomous cell pathology, the localization of the affected tissue, and the assessment of progressive *vs* chronic disease.

Although only cell transplantation is considered to be efficacious in the setting of autonomous pathology, non-autonomous conditions are more likely to require extrinsic cues (cytokines, growth factors, inflammatory mediators, *etc.*) for proper use in stem cell transplantation. Although the therapeutic intervention requires only NSCs in the two identified pipelines developed for a CNS injury, including SCI (Table 1), the application of extrinsic cues may facilitate graft integration at the site of implantation, thus maximizing the therapeutic efficacy. Hepatocyte growth factor (HGF), a mitogen for mature hepatocytes and mediator of the inflammatory responses to tissue injury, was recently highlighted as a potent neurotrophic factor in the CNS. In addition, the intrathecal administration of human HGF in non-human primates has been demonstrated to have therapeutic efficacy in cases of SCI^[158]. Therefore, combined treatment with HGF and NSCs may improve the outcomes of therapy for SCI.

The localization of affected tissue defines the required number of cells and transplantation methodology. A survey of current clinical trials indicated that macular degeneration is a major subject of current studies using ES cell-derived cells (Table 2). For instance, four cohorts, ranging from 50000 to 200000 MA09-RPE cells, were designed in the NCT01344993 trial. These numbers are relatively small, as the cells are confined to application at the affected site in patients with retinal disease. With respect to fetal neuronal transplantation for PD, significant motion improvements require the integration of at least 100000 dopaminergic neurons into the striatum^[97,98]. However, graft-induced dyskinesia may occur in the setting of cell transplantation in the striatum^[3,96]. The development of a new transplantation procedure to construct dopamine projections from the substantia nigra to the striatum may eliminate the occurrence of dyskinesia.

Presumed pathological changes must be sufficiently considered in patients undergoing stem cell transplantation for progressive diseases. Notably, fetal neural tissue transplantation for in cases of PD has been reported to be efficacious in young and earlier-phase patients, but not old or later-phase patients^[3]. This finding implies that the efficacy of cell transplantation depends on the condition of the recipient. Such indications are represented by a key concept, the therapeutic cell niche, the local environment surrounding the cell graft that makes the graft functional *in vivo*. The therapeutic cell niche may vary based on symptoms depending on the disease.

Currently, researchers are able to differentiate stem cells into the desired lineage *in vitro* to obtain highly specified, isolated differentiated cells. Many pipelines are

sponsored by business entities (Tables 1 and 2). However, current stem cell transplantation procedures may lack firm evidence regarding the therapeutic cell niche *in vivo*. Therefore, it is necessary to provide proof of the therapeutic concept in disease model animals and subsequently confirm the safety and efficacy of the treatment in clinical trials, consistently paying attention to the therapeutic cell niche. Otherwise, similar side effects to the adverse events caused by NSC transplantation^[136] may occur in clinical trials. It is thus vital to continue to take a cautious approach to designing stem cell transplantation protocols for various conditions.

CONCLUSION

This report considered perspectives on fetal stem cell transplantation. To date, hundreds of clinical trials using various types of fetal transplants have been performed worldwide. Although success has been observed in some cases, most cases of fetal tissue or cell transplantation have been hastily implemented, and research groups must share their knowledge and experience. Meanwhile, research communities have learned many important lessons through these experiences and continue to improve transplantation strategies, leading to clinical trials of isolated fetal stem cells and ES cell-derived cells (Tables 1 and 2).

Although there remain still ethical and social issues with respect to the clinical use of fetal tissue, ongoing clinical trials of fetal transplants should proceed as fetal transplantation may be currently the sole benchmark for other types of stem cell transplantation. Indeed, the decade-long moratorium on cell transplantation for PD was recently lifted^[99], and European, United States and Japanese research groups recently formed the Parkinson's Disease Global Force to assess fetal transplant protocols for ES and iPS cell-derived dopaminergic neurons. In this process, essential issues, including those associated with the therapeutic cell niche, donor cells, and cell processing, should be sufficiently considered in order to develop more successful transplantation therapies.

Finally, clinical dependence on fetal transplantation, despite its landmark achievements, is expected to gradually fade in the setting of stem cell research owing to lasting ethical controversies and the advent of autologous iPS cells and ES cells.

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WJSC 6th Anniversary Special Issues (1): Hematopoietic stem cell transplantation

Allogeneic hematopoietic cell transplant for acute myeloid leukemia: Current state in 2013 and future directions

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AML. Besides the conventional sibling and unrelated donor allografts, we review the available data and recent advances for alternative donor sources such as haploidentical grafts and umbilical cord blood. We also discuss conditioning regimens, including reduced intensity conditioning which has broadened the applicability of allo-HCT. Finally we explore recent advances and future possibilities and directions of allo-HCT in AML. Practical therapeutic recommendations have been made where possible based on available data and expert opinion.

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Key words: Acute myeloid leukemia; Allogeneic hematopoietic cell transplantation; Reduced intensity conditioning; Myeloablative conditioning; Haploidentical; Umbilical cord blood

Abstract

Acute myeloid leukemia (AML) represents a heterogeneous group of high-grade myeloid neoplasms of the elderly with variable outcomes. Though remission-induction is an important first step in the management of AML, additional treatment strategies are essential to ensure long-term disease-free survival. Recent pivotal advances in understanding the genetics and molecular biology of AML have allowed for a risk-adapted approach in its management based on relapse-risk. Allogeneic hematopoietic cell transplantation (allo-HCT) represents an effective therapeutic strategy in AML providing the possibility of cure with potent graft-versus-leukemia reactions, with a demonstrable survival advantage in younger patients with intermediate- or poor-risk cytogenetics. Herein we review the published data regarding the role of allo-HCT in adults with AML. We searched MEDLINE/PubMed and EMBASE/Ovid. In addition, we searched reference lists of relevant articles, conference proceedings and ongoing trial databases. We discuss the role of allo-HCT in AML patients stratified by cytogenetic- and molecular-risk in first complete remission, as well as allo-HCT as an option in relapsed/refractory

Core tip: Acute myeloid leukemia (AML) represents a heterogeneous group of high-grade myeloid neoplasms of the elderly with variable outcomes. We discuss the role of allo-hematopoietic cell transplantation (HCT) in AML patients stratified by cytogenetic- and molecular-risk in first complete remission, as well as allo-HCT as an option in relapsed/refractory AML.

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INTRODUCTION

Acute myeloid leukemia (AML) comprises a group of high-grade clonal neoplasms of the myeloid progenitor cells. With a median age of 66 years, AML is a disease

of the older age group with an annual incidence of 4.4 per 100000. It is estimated that approximately 15000 new cases of AML will be diagnosed in the United States in 2013^[1]. While the goal of initial therapy in AML is attaining complete remission (CR), without additional post-remission therapy disease relapse is inevitable in vast majority of the cases^[2]. In the past two decades little has changed in AML induction chemotherapy regimens, but our improved understanding of the disease biology in identifying high-risk groups with modern cytogenetics and molecular testing have led to better risk-stratification that facilitates customization of post-remission therapy based on the relapse-risk^[3-5]. While allogeneic hematopoietic cell transplantation (allo-HCT) has been long considered a potentially curative therapy for AML^[6], advances in human leukocyte antigen (HLA)-matching, supportive care, optimal pre-transplant conditioning and advent of alternative donor allografting have broadened the availability and improved transplant outcomes^[7]. Herein we review the role of allo-HCT in adults with AML in first complete remission (CR1), discuss the allograft options in advanced AML (beyond CR1), and review the current state of reduced-intensity and alternative donor allo-HCT in the management of AML.

PROGNOSTIC FACTORS IN AML

Traditionally used prognostic factors in AML include age, leukocyte count at diagnosis, performance status, extra-medullary involvement, antecedent hematologic disorders and initial response to therapy. Cytogenetics by metaphase and interphase analysis are one of the most powerful prognostic factors in AML, providing us the ability to risk-stratify patients at diagnosis. Acute promyelocytic leukemia t(15;17) and core binding factor (CBF) leukemia t(8;21) and inv(16)/t(16;16) are favorable-risk AML, largely retaining their good prognosis even with additional cytogenetic abnormalities^[8-10]. Chromosomal abnormalities conferring poor outcomes include abnormalities of chromosome 3q (abn1 3q), deletions of 5q (-5q), monosomies of chromosome 5 or 7 (-5/-7), and complex karyotype. Large cooperative group studies have confirmed the impact of cytogenetics on survival rates, reporting 55%-65% and 5%-14% 5-year overall survival (OS) for patients with favorable- and poor-risk cytogenetics, respectively^[8,11,12]. Grimawade *et al.*^[10] reported outcomes in 5876 patients treated on Medical Research Council (MRC) trials and identified abn1 3q (excluding t(3;5)(q25;q34)), inv(3)(q21;q26)/t(3;3)(q21;q26), add5q/-5q, -5, -7, add(7q)/-7q, t(6;11)(q27;q23), t(10;11)(p11;q23), other t(11q23) (excluding t(9;11)(p21;q23) and t(11;19)(q23;p13)), t(9;22)(q34;q11), -17, abn1(17p) and complex karyotype as poor risk cytogenetic aberrations. Presence of monosomal karyotype (defined as 2 or more autosomal monosomies or combination of 1 monosomy with structural abnormalities) is associated with very poor prognosis with 4-year OS < 5%^[13,14]. Similarly, the presence of subclones within the poor risk cytogenetic category (*i.e.*, clonal heterogeneity) may confer poorer

Table 1 The European LeukemiaNet Standardized Reporting System for risk stratification of acute myeloid leukemia based on cytogenetics and molecular testing¹

Risk category	Cytogenetic abnormalities	Molecular abnormalities
Favorable risk	t(15;17) inv(16)/t(16;16) ² t(8;21) ²	CN-AML with biallelic CEBPA mutation CN-AML with NPM1 mutated but FLT3-ITD negative
Intermediate risk	CN-AML t(9;11) All others abnormalities not classified as favorable or adverse risk	CN-AML with: NPM1 mutated/FLT3-ITD positive NPM1 wild type/FLT3-ITD negative t(8;21)/inv (16) with c-KIT mutation
Adverse risk	inv (3)/t(3;3) t(6;9) t(v;11)/MLL rearranged -5/-5q -7 Monosomal karyotype Abnormal 17p Complex cytogenetics	CN-AML with FLT3-ITD positive

¹Table modified from Mrózek *et al.*^[24]; ²The good prognosis of inv(16) and t(8;21) is maintained even with additional cytogenetic abnormalities. The presence of concomitant c-KIT mutation may increase relapse risk in t(8;21) and to lesser extent inv(16). CN-AML: Cytogenetically normal acute myeloid leukemia; CEBPA: CCAAT enhancer binding protein alpha; FLT3-ITD: FMS-like tyrosine kinase 3 gene-internal tandem duplication; MLL: Mixed lineage leukemia; NPM: Nucleophosmin.

outcomes^[15]. Recently, Middeke *et al.*^[16] found the presence of abn1(17p) and -5/-5q, within complex and monosomal karyotype AML characterized ultra high-risk disease.

Work done in the last decade has further enhanced our ability to stratify cytogenetically normal AML (CN-AML) based on presence of molecular aberrations into poor-risk [*e.g.*, FMS-like tyrosine kinase 3 gene-internal tandem duplication (FLT3-ITD), mixed-lineage leukemia gene-partial tandem duplication (MLL-PTD), overexpression of Wilms' tumor gene 1 (WT1), brain and acute leukemia, cytoplasmic gene (BAALC), ETS-related gene (ERG), KIT-gene and ecotropic viral integration site 1 gene (EVI1)] and good-risk [nucleophosmin (NPM1), isocitrate dehydrogenase (IDH 1/2) and CCAAT enhancer binding protein alpha (CEBPA)] categories^[15,17-23]. Integrating conventional cytogenetics and the commonly utilized molecular testing markers (FLT3-ITD, CEBPA and NPM1), the European LeukemiaNet validated the effect of prognostic factors on remission rates, disease-free survival (DFS) and OS (Table 1)^[24,25]. The improved understanding of the molecular basis of AML and its ramifications on patient outcomes has important relevance in clinical decision making, heralding the era of "individualized" post-remission therapy (Figure 1).

CONSOLIDATION WITH ALLOGENEIC HCT IN CR1

Remission induction reduces the leukemic burden

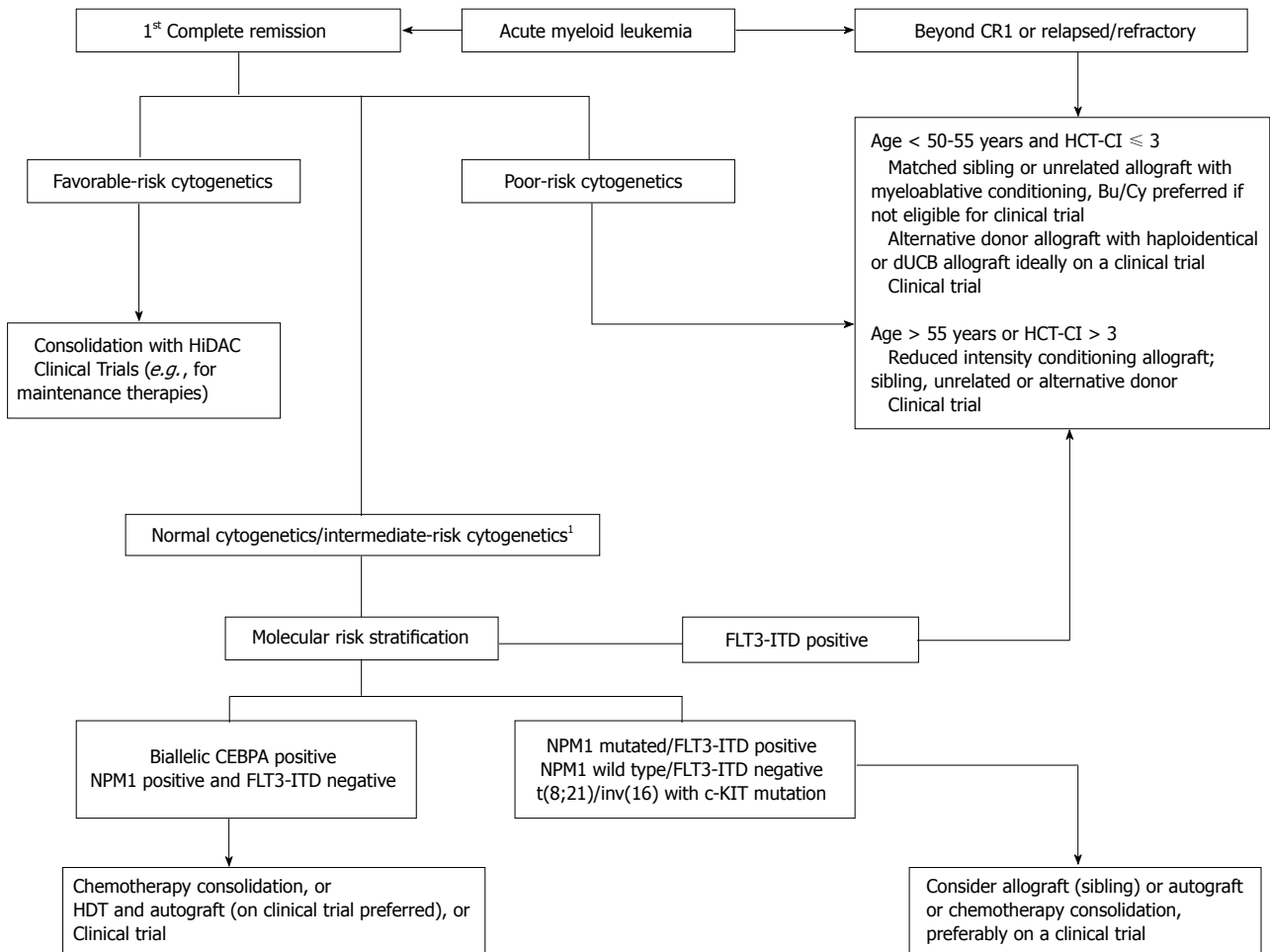


Figure 1 Clinically useful algorithm for optimal consolidation for acute myeloid leukemia patients based on cytogenetic and molecular genetic aberrations, based on available data and practice preference. ¹Allogeneic HCT may be considered in medically fit AML patients with intermediate risk/normal cytogenetics in CR1. Bu/Cy: Busulfan/cyclophosphamide; CEBPA: CCAAT enhancer binding protein alpha; CR: Complete remission; dUCB: Double umbilical cord blood; FLT3-ITD: FMS-like tyrosine kinase 3 gene-internal tandem duplication; HCT-CI: Hematopoietic cell transplantation-comorbidity index; HDT: High dose therapy; HiDAC: High dose cytarabine; NPM: Nucleophosmin.

roughly from 1×10^{12} cells to approximately 1×10^9 cells, if the patient achieves a morphologic CR. Hence additional consolidative therapy is necessary to eradicate a sizeable leukemic clone in patients in morphologic CR to achieve long-term DFS. Generally using chemotherapy-based consolidation approaches alone, the relapse rates in intermediate- and poor-risk cytogenetic groups remain unacceptably high^[26] and represent an area where alternative consolidation approaches are warranted. Allogeneic HCT for patients in CR, not only provides a “tumor-free” graft, but more importantly the donor effector T-cells recognize and mount an effective immune response against the leukemia cells [*i.e.*, the graft-versus leukemia (GVL) effect], to provide patients with durable disease control. While the potent GVL effects of allogeneic HCT provide the most effective post-remission therapy for AML patients in CR1, the associated morbidity and mortality warrants careful selection of high-risk patients, likely to benefit the most from this approach, and sparing the toxicity in lower-risk cohorts.

SIBLING DONOR ALLOGENEIC HCT IN CR1

Prospective single institution studies comparing allo-HCT with consolidation chemotherapy (CC) in the 1980s and early 1990s showed lower relapse rates and improved DFS with allo-HCT for AML patients in CR1, but none conclusively demonstrated an OS advantage^[27,28]. Subsequently, six cooperative group trials (Table 2) have examined the role of allo-HCT in AML in CR1^[28-33]. Those with HLA-matched siblings were offered allo-HCT (“genetic randomization”) while the others were randomized to autologous transplantation or CC on an intention-to-treat analysis. In the European Organization for Research and Treatment of Cancer (EORTC)-Gruppo Italiano Malattie Ematologiche Maligne Ddell’Adulto (GIMEMA) trial^[29], superior 4 year DFS was noted with allo-HCT (55%) and autologous HCT (48%) compared to CC (30%). However, no OS improvement was seen with either transplant modality^[34]. In the Groupe Ouest-

Table 2 Cooperative group trial of allogeneic hematopoietic cell transplantation for acute myeloid leukemia in first complete remission

Cooperative group	Relapse rate			Disease free survival			Overall survival		
	Allo	Auto	CC	Allo	Auto	CC	Allo	Auto	CC
EORTC/GIMEMA AML-8	24% ¹	41%	57%	55% ¹	48% ¹	30%	59%	56%	46%
GOELAM	37%	45%	55%	49%	48%	43%	55%	52%	58%
ECOG/CALGB/SWOG	29% ¹	48%	61%	43%	34%	34%	46%	43%	52% ¹
EORTC/GIMEMA AML-10	30% ¹	52%	-	52% ¹	42%	-	58%	50%	-
UK MRC AML-10 ^{2,3}	36% ¹	52%		50% ¹	42%		55%	42%	
HOVON-SAKK ³	32% ¹	59%		48% ¹	37%		54%	46%	

¹Represents statistically significant and favorable outcome with the treatment modality; ²The 4-year relapse rate, disease free survival and overall survival shown in all studies, except the UK-MRC AML-10 which reported 7-year outcomes; ³All studies designed to compare outcomes between allograft *vs* autograft *vs* consolidation chemotherapy except the UK MRC AML-10 and HOVON-SAKK trial which did not differentiate between autograft and chemotherapy. Allo: Allogeneic transplantation; auto: Autologous transplantation; CC: Consolidation chemotherapy.

Est Leucémies Aigues Myeloblastiques study, the relapse rates following allo-HCT were unusually high (37% at 4 years) and likely explain the lack of therapeutic benefit with allografting in this study^[30]. The MRC reported improved DFS but not OS in the MRC AML-10 patients randomized to allo-HCT^[31]. Similarly the US intergroup trial showed that the higher treatment related mortality (TRM) in patients randomized to allo-HCT arm negated the benefits of lower relapse rates in this group, resulting in no net OS advantage with transplantation in CR1 over chemotherapy alone^[28]. Although provocative, the data from these cooperative group trials failed to provide any concrete guideline for selecting the optimal post-remission strategy for individual patients with a matched sibling donor available in CR1.

Impact of cytogenetic and molecular markers on allo-HCT in CR1

Integrating information regarding cytogenetic-risk categories in the outcome analysis of aforementioned cooperative group trials was the next logical step. Reanalysis of the EORTC/GIMEMA AML-10 trial by cytogenetic-risk stratification showed superior DFS (43% *vs* 18%) and OS (50% *vs* 29%) with allo-HCT compared to autografting in patients with poor-risk cytogenetics^[32]. However allo-HCT did not benefit patients with good-risk [t(8;21), inv(16)] or intermediate-risk (normal or -Y) cytogenetics. Similar cytogenetic-risk stratification of the US intergroup trial showed a 5-year OS of 44%, 13% and 15% with allo-HCT, autologous-HCT and CC respectively, in patients with poor-risk cytogenetics^[12]. No improvement in OS was observed in patients with good or intermediate-risk disease. Unlike the prior studies, the Dutch-Belgian Haemato-Oncology Co-operative Group (HOVON) and Swiss Group for Clinical Cancer Research (SAKK) trial demonstrated superior DFS with allo-HCT for both intermediate and poor cytogenetic-risk patients^[33]. It may be noted that risk stratification in the HOVON-SAKK trial included additional variables. Patients with intermediate-risk cytogenetics requiring two induction cycles to achieve CR1 were classified as poor-risk, only t(8;21) AML patients with a white blood cell count of $< 20 \times 10^9/L$ were considered favorable and patients with

unknown cytogenetics ($n = 89$) were considered intermediate-risk group. Two separate meta-analyses conducted by the HOVON-SAKK group and Koreth *et al*^[35] have confirmed survival benefit with allo-HCT in patients with intermediate- and poor-risk cytogenetics in CR1. Allogeneic HCT in CR1 also appears to improved DFS and OS in AML with monosomal karyotype, compared to other consolidation strategies.

Recognition of the prognostic value of additional molecular markers is facilitating further risk stratification of the heterogeneous group of patients with CN-AML. The German-Austrian Acute Myeloid Leukemia Study Group showed that transplantation might have an important role in a molecular subset of patients with CN-AML. Patients with normal cytogenetics were randomized based on availability of an HLA-identical sibling donor for allo-HCT in CR1 *vs* chemotherapy alone. No benefit of allogeneic transplantation was seen in patients whose leukemia was *NPM1* mutated without *FLT3*-ITD. Conversely, patients with the *FLT3*-ITD mutation or the genotype consisting of wild-type *NPM1* and *CEBPA* without *FLT3*-ITD, benefited from an allogeneic transplant performed during CR1^[36]. In double mutant *CEBPA* allo-HCT or autografting in CR1 improved DFS without impacting OS compared to CC^[37].

Matched sibling allo-HCT in medically fit AML patients, with poor- and intermediate-risk (at least in the *FLT3*-ITD+ or *NPM1*-/*CEBPA*-/*FLT3*-ITD- subgroups) cytogenetics, who are able to achieve CR1 should be considered a standard option.

UNRELATED DONOR ALLOGENEIC HCT IN CR1

The strength of evidence presented above supports allo-HCT from a sibling donor in intermediate-/poor-risk AML in CR1. Unfortunately only approximately 25%-30% of AML patients have an HLA-identical sibling. No randomized trials have looked at unrelated donor (URD) allo-HCT for AML in CR1. Yakoub-Agha *et al*^[38] reported similar outcomes with respect to acute graft-versus-host disease (GVHD), TRM, and OS in patients with standard-

risk hematologic malignancies who received HLA-A, -B, -C, -DRB1, and -DQ (10/10) allele-matched allografts from either sibling or unrelated donors. Although randomized, prospective trials of URD transplantation for AML in CR1 are lacking, a number of retrospective studies provide evidence in support of the approach. Sierra *et al*^[39] reported outcomes of URD transplantation in 161 AML patients at various stages of disease including 16 patients with poor-cytogenetic risk AML in CR1 with a 5-year DFS of 50%. The corresponding DFS for those undergoing allo-HCT in CR2, relapse, or primary induction failure were 28%, 7%, and 19%, respectively. Bashir *et al*^[40] reported a 3-year OS and TRM of 78% and 15% respectively in a cohort of 44 patients (59% poor risk cytogenetics) who underwent URD allo-HCT in CR1. In a Center for International Blood and Marrow Transplant Registry (CIBMTR) analysis of 476 patients undergoing URD allo-HCT; adjusted 3-year OS, and DFS, in CR1 were 44%, and 43% respectively^[41]. Interestingly, Tallman *et al*^[42] found no difference in survival by cytogenetic-risk stratification for AML patients undergoing URD allo-HCT in CR1. However, the reported 5-year DFS of 30% in cytogenetically poor-risk AML likely represents a better outcome than with other non-HCT treatment strategies^[43]. The presence of complex cytogenetics (> 3), however likely represent a high-risk group with poorer outcomes even with allo-HCT in CR^[44].

European Group for Blood and Marrow Transplantation (EBMT) recently reported outcomes of 206 CN-AML patients in CR1 undergoing HLA-identical sibling or matched URD allo-HCT with reference to their *FLT3-ITD* status (present: $n = 120$, 58%; absent: $n = 86$, 42%)^[45]. *FLT3-ITD*-positive patients, compared with *FLT3-ITD*-negative patients had higher 2-year relapse incidence (30% *vs* 16%, $P = 0.006$) and lower DFS (58% *vs* 71%, $P = 0.04$). More importantly, more than half of the patients harboring this mutation who received matched sibling or URD allo-HCT were alive and leukemia free at 2 years. URD allo-HCT in CR1 however may be associated with a higher TRM as noted in a registry study that reported trends of outcomes over the last two decades, underlining the need to carefully select patients for URD allo-HCT. For poor-risk cytogenetics and *FLT3-ITD*+ CN AML patients in CR1 lacking an HLA-matched sibling donor, it is certainly reasonable to consider matched URD allo-HCT.

OPTIMAL CONDITIONING REGIMENS

Myeloablative conditioning regimens (MAC) utilizing chemotherapy and/or total body irradiation (TBI) have been the basis of most of the studies discussed thus far. The two most commonly utilized MAC regimens are busulfan/cyclophosphamide (Bu/Cy) and cyclophosphamide/TBI (CY/TBI). Although prior studies showed inferior DFS and OS with Bu/Cy conditioning^[46,47], a large meta-analysis did not show any difference between the two regimens with regards to survival and relapse^[48]. It has been widely noted that the erratic bioavailability

of oral busulfan was the likely cause inferior outcomes. Recent EBMT data comparing intravenous Bu/Cy to CY/TBI in AML found increased incidence of GVHD with TBI conditioning, and a trend towards improved TRM with Bu/Cy but no difference in DFS at 2-year^[49]. A larger CIBMTR analysis clearly showed better DFS (RR = 0.70, 95%CI: 0.55-0.88, $P = 0.003$) and OS (RR = 0.68, 95%CI: 0.52-0.88, $P = 0.003$) in AML patients receiving IV, but not oral busulfan compared to TBI^[50]. Similar observations (lower TRM with Bu/Cy and better OS compared to TBI-based regimens) were made in a prospective cohort study of CIBMTR^[50,51]. Collectively these data suggest that in the era of pharmacokinetically driven adjustment of intravenous busulfan dosing, in younger (< 50 -55 year) AML patients Bu/Cy should be considered the preferred MAC regimen for allo-HCT.

The use MAC is limited to medically fit, younger AML patients. The observed lower TRM rates using the so-called non-myeloablative (NMA) or reduced-intensity conditioning (RIC) regimens have broadened the applicability of allo-HCT to elderly patients or younger patients with comorbidities. Unlike MAC regimens; the NMA/RIC allo-HCT relies more heavily on the GVL effects to eradicate disease in the recipient. The decision to use NMA or RIC regimens for AML patients undergoing allo-HCT is not always clearly delineated, and significant variations exist in the selection criteria used by transplant centers across the globe. Sorror *et al*^[52] evaluated the impact of a priori medical comorbidities on transplant outcomes by using the HCT-Comorbidity Index (HCT-CI), and reported significantly higher TRM rates and inferior OS in patients with an HCT-CI score of ≥ 3 . While not validated in prospective clinical trials, it is increasingly becoming common practice to offer RIC allo-HCT to AML patients of advanced age (generally > 50 -55 years), and/or HCT-CI > 3 (regardless of age), or with a prior history of autologous transplantation or less optimal performance status^[53,54].

The acute leukemia working party of the EBMT compared transplantation outcomes for 315 RIC and 407 MAC recipients^[55]. While the incidence of grade II-IV acute GVHD (22% *vs* 31%) and 2-year TRM (18% *vs* 36%) significantly favored the RIC group, more patients with RIC allograft experienced disease relapse compared to MAC regimens (41% *vs* 24%). The DFS and OS did not differ between the two groups. Another report noted grade II-IV acute GVHD rates and 2-year relapse rates of 40% and 39% respectively in 122 AML patients who received a RIC regimen with 2-year DFS of 44%^[56]. A Spanish prospective, multicenter trial of patients with poor-risk AML/myelodysplastic syndrome reported 4-year DFS and OS rates of 43% and 45% with RIC and showed that development of chronic GVHD was strongly associated with reduced risk of relapse and improved OS and DFS, providing proof of concept for clinically relevant GVL effects with RIC allotransplantation^[57].

RIC in AML has generally shown lower TRM with comparable OS and DFS to MAC regimens, but follow up is relatively short thus limiting conclusions. The ongoing

ing prospective randomized BMT-CTN 0901 clinical trial (NCT01339910) comparing RIC regimens against MAC in AML/myelodysplastic syndrome will hopefully clarify the optimal conditioning intensity in AML. The advent of RIC allo-HCT has indeed extended the feasibility and applicability of allogeneic transplantation to include those with advanced age and multiple co-morbidities, thus offering them possibly a better chance for long term DFS.

ALTERNATIVE DONOR TRANSPLANTATION

Umbilical cord blood transplantation

For those high-risk patients who do not have an HLA-identical sibling or unrelated donor available, alternative donor sources may be necessary. Umbilical cord blood transplantation (UCBT) is an attractive alternative donor option due to its rapid and easy availability^[58-62]. UCBT is associated with lower GVHD rates for the degree of HLA-disparity. In a direct comparison of outcomes in adults with hematological malignancies, Laughlin *et al*^[61] reported no difference in TRM or relapse rates between UCBT and mismatched URD bone marrow transplantation, although outcomes were inferior to matched bone marrow allografts. Similarly, Rocha *et al*^[59] in a study that included patients with acute leukemia who received UCB or matched URD marrow ($n = 582$) grafts showed no difference in TRM, relapse rate, DFS, and OS between the two groups.

The low cell dose available from individual cord blood units has been the major limitation against the widespread use of UCBT in adults with AML or other hematologic malignancies. However work done by the group in University of Minnesota has firmly established the feasibility of combining two cord blood units, in the so-called double UCBT (dUCBT), to overcome dose limitation of a single cord unit for adult patients^[63]. A large multicenter collaborative effort comparing dUCBT, matched-sibling allo-HCT, matched URD allo-HCT and mismatched URD allo-HCT showed similar 5-year DFS with all 4 modalities. dUCBT was associated with lower relapse rates but higher TRM^[64]. The preliminary results of Societe Française De Greffe De Moelle Osseuse Et Therapie Cellulaire and Eurocord's multicenter phase II trial for UCBT in patients with AML were presented in abstract form^[65]. At 1 year the rates of OS, DFS, relapse and TRM for the 65 AML patients on the study were 60%, 52%, 30% and 18%, respectively. The wider acceptance of UCBT has markedly extended the application of allogeneic transplantation, particularly to minority populations who are underrepresented in current volunteer donor databases.

Haploidentical transplantation

Almost all AML patients without an HLA-identical donor will find a haploidentical related (parents, sibling or children) donor. Enthusiasm for this modality was subdued early on due to the increased risks of GVHD, TRM, graft

rejection and opportunistic infections. However, renewed interest in haploidentical transplants has been noted with T-cell depleted as well as unmanipulated allografts with novel strategies for GVHD prevention^[66,67]. The Perugia group reported DFS of 30%-45% in AML with rigorous *ex-vivo* T-cell depletion and intense myeloablative conditioning^[68-70]. Although such transplantation has been demonstrated as feasible, it is associated with slow immune reconstitution and high rates of TRM, in smaller centers.

Recently, an alternative approach to haploidentical allo-HCT was developed with the addition of post-transplant cyclophosphamide to prevent GVHD and graft rejection in the setting of a marrow allograft after reduced intensity conditioning^[71,72]. This approach has demonstrated promising results, including acceptable rates of TRM and severe GVHD in single- and multi-institution studies. Variations including myeloablative conditioning and use of peripheral blood grafts with post transplant cyclophosphamide treatment are being studied in prospective trials^[73]. Limited retrospective data suggest comparable outcomes of matched sibling HCT, URD all-HCT and haploidentical transplantation utilizing post-transplant cyclophosphamide administration, in patients with hematological malignancies^[74]. Bone Marrow Transplantation-Clinical Trials Network's (BMT-CTN) two parallel multicenter phase II trials (BMT-CTN 0603 and BMT-CTN 0604) showed comparable 1-year OS and progression-free survival with RIC dUCBT (54% and 46%, respectively) and haploidentical bone marrow transplantation (62% and 48%, respectively) in hematological malignancies^[75]. These trials have paved the way for the ongoing BMT-CTN 1101 trial (NCT01745913) randomizing patients with hematological malignancies to either haploidentical transplantation or dUCBT. This study will hopefully guide us further in choosing the optimal alternative donor source.

Continued research is needed to better define preferred conditioning regimens, methods and degree of T-cell depletion, reduce high relapse rates with haploidentical transplantation and improved delayed immune-reconstitution inherent to all alternative donor HCT. Recently, allelic polymorphism in donor natural killer-cell immunoglobulin like receptor (*KIR*) gene has been shown to impact allograft outcome and may play important role in donor selection, including alternative sources^[76]. In centers with available expertise, alternative donor allo-HCT for carefully selected high- or intermediate-risk AML patients in CR, or those beyond CR1 is reasonable, however enrollment of such patients on any available protocols is preferred.

ALLOGENEIC-HCT FOR AML BEYOND CR1

Second complete remission (CR2)

Relapsed AML patients, who are able to achieve a second CR (CR2), typically do not enjoyed sustained responses

with chemotherapy alone. A retrospective matched-pair analysis that compared the outcomes of autologous HCT versus HLA-identical sibling allo-HCT in AML CR2 ($n = 288$) showed that while allograft recipients had higher TRM it was offset by a much lower relapse rate leading to better OS (39% *vs* 30%) at 4-years^[77]. Burnett *et al*^[78] reported outcomes of 1271 patients aged 16-49 years who entered the MRC AML10, AML12, and AML15 trials and did not receive a transplant in CR1 and then subsequently relapsed. Fifty-five percent of patients who relapsed entered CR2. Sixty-seven percent of remitters received an allotransplant that delivered superior OS compared with patients who did not receive a HCT (42% *vs* 16%). A more-stringent assessment of a transplant by using delayed-entry (Mantel-Byar) analysis confirmed the benefit of transplant overall and within intermediate- and poor-risk groups but not the favorable-risk subgroup. Allo-HCT is the preferred option for most medically fit patients with AML in CR2, including carefully planned alternative donor allografts. For those unable to undergo an allograft (due to comorbidities, personal preference, *etc.*) are best treated in the context of a clinical trial when available.

Beyond CR2

Allogeneic HCT offers the best prospect of long term DFS for patients with relapsed/refractory AML beyond CR2^[79,80]. Sierra *et al*^[39] reported 5-year DFS of 50%, 28%, 27% and 7% with allo-HCT in CR1, CR2, beyond CR2 and in untreated relapse respectively. The corresponding relapse rates were 19%, 23%, 25% and 44%, respectively. A history of prior autologous transplantation adversely affects the success of a subsequent allo-HCT^[79].

The first relapse of AML poses a management dilemma regarding whether to proceed directly with allo-HCT or to administer salvage chemotherapy to attain remission. Retrospective data indicate 3-year DFS rates of approximately 30% for patients transplanted in untreated first relapse^[81,82]. Salvage chemotherapy generally induces subsequent CR in approximately 30% of relapsed AML patients^[83]. Considering that only 35%-45% of these patients may achieve long-term DFS with allo-HCT (approximately 15% of all relapsing patients), theoretically allografting in untreated relapse may cure more patients than additional chemotherapy. However, in clinical practice the logistics of HLA-typing, identifying and evaluating potential donors, and stem cell collection generally necessitate administration of chemotherapy for disease control before transplantation. Moreover, relapse/refractory patients may not be prime candidates for myeloablative conditioning regimes that are likely required for optimal disease control to facilitate graft-versus-leukemia effect. This fact also highlights the importance of initiating the donor search in AML patients at the time of diagnosis^[84,85].

Primary refractory AML

Allo-HCT likely represents the only curative option for patients with primary refractory AML^[83]. Retrospec-

tive analyses have shown long-term survival in a subset of patients receiving allo-HCT for primary refractory AML^[86-89]. Despite the relatively high TRM (30%-50%), the reported 3-year OS and DFS of approximately 20%-30% are encouraging for this otherwise poor prognosis group. CIBMTR reported outcomes of 1673 AML patients undergoing allo-HCT with refractory/active disease^[90]. Five adverse pre-transplantation variables significantly influenced survival: first CR duration < 6 mo, circulating blasts, non-HLA-identical sibling donor, Karnofsky score < 90, and poor-risk cytogenetics. Patients who had 0 adverse factors had 42% OS at 3 years, whereas OS was 6% for a score ≥ 3 . These important results highlight that allo-HCT can salvage a highly select subgroup of AML patients, who are not able to achieve a CR before transplantation. Based on promising phase I / II data, the use of novel clofarabine and busulfan conditioning is being explored in this population (NCT01457885)^[91].

FUTURE DIRECTIONS

Great strides have been made in the field of AML and allo-HCT resulting in a steady increase in the number of allogeneic transplantation done for AML. Risk stratification of AML based on conventional cytogenetics and now molecular profiling has been instrumental in identifying higher-risk groups who may benefit from early allo-HCT. Studies looking at whole-genome and whole-exome sequencing have been reported^[92] and this information will be vital not only in prognostication but is likely to lead to discovery of novel therapeutic targets. The cytogenetic and molecular signature of AML has become expansive and its clinical application ought to be carefully interpreted. The identification of higher-risk cytogenetic groups, novel molecular stratifications incorporating coinciding aberrations and the presence of clonal heterogeneity in poor-risk AML may allow us to better predict relapse risk, recommend allo-HCT and other strategies to improve disease control and survival in an individualized fashion. The presence of minimal residual disease (MRD) is another area of active interest that may help identify those subsets of AML with the highest risk of early relapse and thus may benefit from early interventions such as allo-HCT. This may be especially important in good-risk and intermediate-risk group AML^[93]. Similarly evidence of persistent MRD post allo-HCT is a marker of poor outcomes. Such AML patients with evidence of MRD post allografting could be enrolled in trials designed to eradicate persistent low level disease (*e.g.*, by rapid taper of immune suppression, planned/escalated donor-lymphocyte infusions, low-dose chemotherapies, or novel targeted agents, *etc.*).

Allogeneic HCT itself has indeed undergone tremendous advancement in the last 2 decades. High-resolution allele level HLA-typing, improvements in supportive care, use of alternative donor allograft and RIC has widely broadened the use of allo-HCT in AML. The newest concept of adoptive cellular therapy is the so-

called “microtransplantation” where HLA-mismatched peripheral blood stem cells are infused into the recipient after consolidative chemotherapy with cytarabine, the hypothesis being that the alloreactive HLA-mismatched cells would not engraft, but during their transitory period will destroy AML clone without causing GVHD^[94]. Concerted efforts are needed to devise strategies to prevent relapse post allo-HCT using novel maintenance or consolidation strategies (*e.g.*, FLT3 inhibitors post allo-HCT in FLT3⁺ patients, hypomethylating agent administration to eradicate minimal residual disease). Rigorous research efforts in the development of novel preparative regimens able to provide better early disease control and limiting TRM are need. In this regard total marrow irradiation programs and/or immune-radioisotope-based conditioning appear promising. Additional avenues include using propylene glycol free melphalan (to limited renal toxicity), and pharmacokinetically dose busulfan (to limited organ damage, and prevent underdosing) as safer conditioning drugs. Immunological strategies to modulate patient or donor’s immune system, so that they mount response against tumor specific antigens are ongoing. Various antigens (Wilms Tumor gene, NOTCH, PR1, *etc.*) are being tested to develop vaccine to achieve a lasting immune response in the setting of relapsed leukemia or MRD after transplant. Newer mobilization regimens (*e.g.*, plerixafor for sibling donor mobilization) and more effective methods to prevent GVHD^[95-101] as well as increased availability of alternative-donor approaches, are ongoing and will add to our ability to cure patients with AML in the coming years.

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Haploidentical vs cord blood transplantation for adults with acute myelogenous leukemia

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Abstract

Hematopoietic cell transplantation is established as a curative treatment for patients with acute myelogenous leukemia. Haploidentical family donor and umbilical cord blood (UCB) are alternative sources of stem cells for patients lacking a matched sibling or unrelated donor. The early challenges of transplant complications related to poor engraftment and graft-vs-host disease have been overcome with new strategies such as using 2 units and increased cell dose in UCB and T-cell depletion and post transplantation cyclophosphamide in haploidentical transplantation. The outcomes of alternative transplantation for acute leukemia were compared to other traditional graft sources. For patients lacking a matched sibling or unrelated donor, either strategy is a suitable option. The choice should rely mostly on the urgency of the transplantation and the available cell dose as well as the expertise available at the transplant center. This manuscript reviews the options of alternative donor transplantation and highlights recent advances in each of these promising transplantation options.

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Key words: Umbilical cord blood transplantation; Hap-

loidentical transplantation; Leukemia

Core tip: Allogeneic hematopoietic cell transplantation is a curative treatment for patients with acute leukemia. Many patients lack a suitable matched donor and require another stem cell source. The choice between cord blood and mismatched relative is challenging as there is no direct comparison between the two transplantation modalities. This manuscript highlights the studies and current innovative approaches with either modality with an emphasis on the recent studies aiming at decreasing complications, enhancing engraftment and speeding immune recovery.

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INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) is a potential curative treatment for patient with leukemia. The preferable donor is a fully matched sibling; however, two thirds of patients needing transplant lack this donor option^[1]. In the absence of sibling donors, most centers choose a matched unrelated volunteer donor as the next option. Report from the National Marrow Donor Program's registry indicates an 8/8 HLA-matched adult unrelated donor is available for 51% of Whites, 30% of Hispanics, 20% of Asians and 17% of African-Americans^[2]. Hence, a 30% of all patients requiring HCT lack a suitable matched donor. The high relapse risk of many leukemia patients lacking a matched donor has led to the use of alternative sources of stem cells such as unrelated donor umbilical cord blood (UCB) and haploidentical

family donors.

First attempts with alternative HCT carried a high risk of mortality, engraftment complications and graft *vs* host disease^[3]. Progress in recent years has significantly improved the outcomes post alternative donor HCT. The improved outcomes are mostly credited to better donor selection, vigorous T-cell depletion in haploidentical transplantation, use of post infusion cyclophosphamide in haploidentical setting and use of 2 units in adult UCB transplantation (DUCBT) and introduction of more suitable conditioning regimens.

This article will review the recent advances in alternative donor HCT for acute leukemia in adults, describe the outcomes of HCT using these alternative donor sources and discuss ongoing studies in alternative HCT.

UMBILICAL CORD BLOOD TRANSPLANTATION

UCB offers several benefits over unrelated adult donors^[1,4]. UCB is safe for the donor as it is collected from the placenta during delivery. UCB units are readily available with less risk of transmission of infections, in particular cytomegalovirus (CMV), since most units are CMV negative. It permits a higher HLA disparity between donor and recipient when compared to MUD or related donors^[5,6]. Finally, UCB HCT may carry less risk of chronic GVHD compared to other cell sources^[7-10]. The main limitations associated with UCB are related to the small number of progenitor cells in each unit and the lack of access to donor lymphocytes for donor lymphocyte infusion (DLI), if needed. UCB constitutes a significant proportion of unrelated donor transplantations in children (40%) compared to only 10% in adults.

Laughlin *et al*^[3] reported on 68 patients [15 with acute lymphoblastic leukemia (ALL), 19 acute myeloid leukemia (AML) and 17 chronic leukemia] who received myeloablative UCB transplantation^[3]. Engraftment was better for patients with a nucleated cell dose $\geq 2.4 \times 10^7$ /kg. Median time to engraftment was 27 d. Five patients experienced primary graft failure. CD34+ cell dose ($\geq 1.2 \times 10^5$ /kg) was associated with a higher event free survival (EFS). EFS was not influenced by HLA matching (3-6/6) or patient age. This study established the safety of UCB transplantation in adults despite limited cell content and a more HLA mismatch than what has been reported in pediatrics^[11]. The COBLT study prospectively evaluated the outcomes of UCB transplantation^[12]. This study evaluated 34 adult subjects [AML = 19, ALL = 9, CML = 3, myelodysplastic syndrome (MDS) = 1]. Patients had a myeloablative conditioning (MA) with total body irradiation (TBI) plus cyclophosphamide and busulfan or melphalan with 4-6/6 HLA matched UCB units. The required cell dose was $> 1 \times 10^7$ nucleated cells/kg. Overall, 34 % had primary graft failure and 6 mo survival was only 30%. The reasons for higher mortality and complications with initial studies of UCB were due to patient selection and long duration from diagnosis to transplan-

tation. However, these reports established the importance of cell dose for successful UCB HCT and set the background for future studies of strategies to limit complications (*e.g.*, double umbilical cord blood transplantation, *ex vivo* expansion).

Double umbilical cord blood transplantation

The use of two UCB units was started at the University of Minnesota to overcome the cell dose limitation of single UCB units^[3,13,14]. DUCBT has yielded better engraftment, lower mortality and improved disease free survival comparable to other hematopoietic cell sources^[15].

A recent report from Minnesota group assessed 536 patients who received HCT with HLA MRD ($n = 204$), HLA allele matched or 1 antigen mismatched unrelated donor (MUD = 152, MMUD = 52) or HCT using 4-6/6 HLA matched two UCB units ($n = 128$) after myeloablative conditioning^[15]. Disease free survival (DFS) was similar for the different graft sources (UCB 51%, MUD 48%, MRD 33%, and MMUD 38%). UCB recipients had a lower relapse risk but a higher TRM. Another study from Minnesota suggested that using double UCB units carries a lower risk of relapse and a higher risk of acute GVHD when compared to single unit UCB transplantation^[16,17].

BMT-CTN 0501 is a myeloablative study that randomizes 1 *vs* 2 UCB grafts for children with leukemia. BMT-CTN 0604 study addressed RIC regimen in the DUCBT setting. Longer follow up from both studies will help improve our understanding of the use of DUCBT.

UCB outcomes

UCB has been compared to other donor sources in the myeloablative setting (Table 1). Transplant outcomes post UCB used to be inferior but recent series show similar outcomes for UCB when compared to other graft sources. The differences in outcomes between prior and current studies is related to many reason, the most important being an increase in the minimum acceptable cell dose in the cord unit to proceed with transplantation^[18].

Laughlin *et al*^[5] compared outcomes of 450 patients receiving 5-6/6 HLA matched unrelated donor transplants to 150 patients receiving 4-6/6 UCB transplants through the CIBMTR registry. The median time to neutrophil engraftment was delayed with UCB (27 d) compared to 18 d among 6/6 and 20 d among 5/6 HLA-matched unrelated bone marrow. Acute GVHD and relapse rates were similar between UCB and 6/6 MUD. UCB had higher TRM and poorer LFS. MUD had a better overall survival at 3 years (33% *vs* 23%) compared to UCB HCT. When UCB was compared to 5/6 MMUD, UCB was shown to have a lower risk of acute GVHD, but a similar risk of TRM, relapse, and LFS. Rocha *et al*^[6] on the other hand; reported that UCB had a lower risk of GVHD and similar rates of relapse, TRM and LFS. Both authors suggested UCB as a reasonable stem cell source in the absence of 6/6 MUD.

Single unit UCB transplantation outcomes were compared to MUD peripheral blood stem cells (PBSC) and

Table 1 Hematopoietic cell transplantation after myeloablative conditioning in adult patients comparing umbilical cord blood and other donor sources

Year	Graft type	Number of patients	Median age	ANC > 500/ μ L (median, d)	aGVHD II-IV (%)	Extensive cGVHD (%)	100 d TRM (%)	Relapse rate (%)	Survival (%)
2004 ^[5]	UCB	150	16-60	27	41	51	63	17 (3 yr)	26 (3 yr)
	MUD BM	367	16-60	20	48	35	46	23	35
	MMUD BM	83	16-60	18	51	40	65	14	20
2004 ^[6]	UCB	98	25	26	26	30	44	23 (2 yr)	36 (2 yr)
	MUD BM	584	32	19	39	46	38	13	42
2007 ^[19]	UCB	100	38	22	60	23	8	17 (3 yr)	NA
	MRD (BM and PB)	71	40	17	55	30	4	26	
2008 ^[10]	UCB	148	29	NA	NA	NA	41	26 (2 yr)	35 (2 yr)
	MUD PB	518	35	NA	NA	NA	27	30	45
	MMUD PB	210	NA	NA	NA	NA	42	24	36
	MUD BM	243	29	NA	NA	NA	26	28	48
	MMUD BM	111	NA	NA	NA	NA	37	26	38
2009 ^[62]	UCB AML	173	38	NA	32	8	32 (2 yr)	31 (2 yr)	43 (2 yr)
	MUD BM	311	38	NA	35	20	22	24	60
	UCB ALL	114	34	NA	28	10	24	31	49
	MUD BM	222	32	NA	42	17	25	24	57
2010 ^[15]	MRD	204	40	NA	65	47	24 (5 yr)	43 (5 yr)	NA
	MUD	152	31	NA	80	43	14	37	NA
	MMUD	52	31	NA	85	48	27	35	NA
	DUCB	128	25	NA	60	28	34	15	NA

ANC: Absolute neutrophil count; aGVHD: Acute graft versus host disease; cGVHD: Chronic graft versus host disease; TRM: Treatment related mortality; UCB: Umbilical cord blood; MUD: Matched unrelated donor; BM: Bone marrow; MMUD: Mismatched unrelated donor; NA: Not available; MRD: Matched related donor; PB: Peripheral blood stem cells; AML: Acute myeloid leukemia; ALL: Acute lymphoblastic leukemia; DUCB: Double umbilical cord blood.

bone marrow in a multiregistry study^[10]. Graft sources included 4-6/6 HLA matched single unit UCB ($n = 165$), 8/8 HLA matched PBSC ($n = 632$), 8/8 HLA matched bone marrow ($n = 332$), 7/8 HLA matched PBSC ($n = 256$) and 7/8 HLA matched bone marrow ($n = 140$). Endpoints included hematopoietic recovery, TRM, LFS and GVHD. Both acute Grade II-IV and chronic GVHD were lower in UCB than in PBSC MUD, while only chronic was lower in UCB than in 8/8 matched bone marrow patients. TRM was higher after UCB than after 8/8 allele matched PBSC (HR 1.62, $P = 0.003$) or bone marrow transplantation (HR = 1.69, $P = 0.003$). Overall, LFS was comparable between UCB and 7-8/8 allele matched unrelated donor.

A recent report from Minnesota and Fred Hutchinson group in Seattle showed that myeloablative DUCBT has comparable leukemia free survival as matched and 1 antigen mismatched unrelated donor.

UCB has also been compared to related donor transplantation. Takahashi *et al*^[19] reported on 171 adults who received single unit UCB ($n = 100$), 5-6/6 HLA matched related donor bone marrow transplant ($n = 55$) or 5-6/6 HLA matched related donor PBSC HCT ($n = 16$). UCB recipients had a delayed hematologic recovery and a lower incidence of grade III-IV acute and extensive chronic GVHD. Both UCB and related donor transplantation had similar relapse, TRM and DFS.

In summary, there is enough evidence to suggest UCB as an acceptable source of stem cells for patients requiring myeloablative HCT but lack a suitable matched donor.

UCB Transplantation after reduced-intensity conditioning

Older patients with AML requiring allogeneic HCT are at increased risk of complications with myeloablative conditioning. Studies with RIC UCB had variable TRM and this variability could be related to different study populations^[20-22]. Overall, most studies have reported OS and DFS that is similar to HCT using other stem cell sources.

A Minnesota study evaluated older patients after UCB transplantation and compared their outcomes to matched related donors, MUD and Mismatched URD. The TRM was higher (35% *vs* 27%), LFS was lower (28% *vs* 35%) and overall survival was lower (30% *vs* 43%) among UCB recipients when compared to MUD transplantation^[23]. This study and other reports establish the efficacy of UCB after RIC for patients who are not eligible for myeloablative conditioning.

Other factors in selecting cord blood units

The selection of cord blood units has been traditionally based on low resolution typing of HLA-A, B and high resolution at DRB1 and on the total nucleated cell dose. Recent studies have evaluated the importance of high resolution HLA typing, HLA-C match and KIR ligand status. Eapen *et al*^[24] found that patients who had units matched at HLA-A, B, DRB1 and HLA-C had better 3 year TRM (9%) and 3 year OS (57%) than patients who were matched at HLA-A, B, DRB1 but with mismatch at HLA-C (TRM 26%; OS 51%) and better outcomes than those with a mismatch on HLA-C with additional mismatch at HLA-A, B, DRB1 (TRM 31%, OS 37%)^[24].

Allele level typing was recently analyzed through a combined CIBMTR and Eurocord registry databases. The investigators showed that the frequency of neutrophil recovery was lower for recipients of mismatches at 3 or more alleles. Nonrelapse mortality was higher with units mismatched at 1 to 5 alleles compared with matched units. Overall mortality was not different except for those that received units mismatched at 5 alleles^[25]. The author concluded that cord blood transplantation with ≥ 3 allele level mismatches should be avoided.

When a fetus is exposed to non-inherited maternal antigen (NIMA) in utero, fetal T regulator cells are induced to that haplotype. It was hypothesized that recipients who are matched to donor NIMA may have lower mortality post transplantation. 5 year TRM was lower and OS was better among NIMA matched UCBT compared to NIMA mismatched UCBT (TRM 18% *vs* 32%, $P = 0.05$; OS 55% *vs* 38%, $P = 0.04$)^[26]. It was suggested that NIMA matching can be considered in a patient with multiple UCB units harboring adequate cell dose.

The role of Donor killer cell immunoglobulin-like receptor (KIR) ligand incompatibility has shown variable conclusions. A study from Eurocord showed that patients receiving UCB units mismatched at KIR-ligand had lower relapse and better leukemia-free survival^[27]. The results were significant for patients with AML, where recipients of KIR-ligand mismatched in the GVH vector had a better LFS (73% *vs* 38%, $P = 0.004$) and incidence of relapse (5% *vs* 36%, $P = 0.005$). This finding was not reproduced in a recent analysis by the Japan society for HCT^[28] or by an earlier study from Minneapolis in the myeloablative setting^[29]. In the same analysis, Minnesota group found that KIR ligand mismatch is associated with increased grade III-IV acute GVHD and increased risk of death in the reduced intensity setting.

Recent advances in UCB transplantation

Recent work in UCBT is aimed at achieving faster neutrophil engraftment and minimizing early TRM. Direct injection of stem cells into the marrow cavity was hypothesized to reduce systemic “wasting” of such cells. In one unit UCBT, intra-bone marrow injection was associated with lower risk of acute graft *vs* host disease with a sustained engraftment^[30]. These results were not reproducible in the DUCBT setting where one of the two units was injected directly into the bone marrow^[31].

New methods to enhance engraftment focus on *ex vivo* expansion and co-infusion of purified committed hematopoietic progenitors. One trial evaluated the effects of co-infusion of highly purified “of the shelf” CD34+ progenitors from healthy volunteers. The aim of this strategy was to assess if the additional CD34+ cells will help enhance neutrophil recovery without leading to long term engraftment. *Ex vivo* expansion is also receiving more support. One expansion method include cocultures of UCB derived CD34⁺CD38⁻ precursors with immobilized Notch I ligand^[32]. A study by de Lima *et al*^[33] reported on 31 patients who received *ex vivo* expanded

UCB with cocultures from mesenchymal stem cells. Time to engraftment was significantly improved at 15 d compared to 24 d for patients with unmanipulated cord infusion^[34]. The role of *ex-vivo* expansion in UCB transplantation is still an ongoing process.

Engraftment can also be improved by increasing stem cell homing. One such method include the use of complement fragment 3a and diprotein A^[34,35] that increase homing through stromal cell-derived factor 1 (SDF1). A recent study through the University of Minnesota established safety of infusing C3a primed units but failed to show effect on engraftment^[36].

HAPLOIDENTICAL FAMILY DONOR TRANSPLANTATION

Haploidentical transplantation has gained significant interest in the last few years with the introduction of new GVHD strategies such as T cell depletion with high CD34+ doses to overcome risk of graft failure^[37,38], and high dose cyclophosphamide post transplantation. Haploidentical donors are usually defined as having ≥ 2 HLA antigen mismatches at HLA-A, -B and -DRB1 loci. Some studies of haploidentical transplantation included family donors with one HLA antigen mismatch^[39]. There are several platforms for performing haploidentical transplantation including *ex vivo* T cell depletion prior to infusion, post infusion depletion with drugs such as cyclophosphamide and unmanipulated infusion with vigorous GVHD prophylaxis. With the choice of multiple available donors, selection can be based on factors such as sex, age, cytomegalovirus status (CMV) and killer immunoglobulin receptor (KIR) incompatibility. One advantage over UCB, is the availability of haploidentical donors for more cells if needed.

OUTCOMES OF HAPLOIDENTICAL TRANSPLANTATION IN ACUTE LEUKEMIA

Ex vivo T-cell depleted haploidentical transplantation

The Perugia group evaluated 104 adult leukemia patients who were conditioned with TBI, fludarabine, thiopeta and antithymocyte globulin (ATG)^[37]. Grafts were T-cell depleted using CD34+ immunoselection and no post-transplantation GVHD prophylaxis was used. Ninety-one percent of the patients engrafted, and for the seven patients who failed to engraft, engraftment was successful after a second transplant in six cases. Acute GVHD developed in 8% of patients (2% grade III-IV) and five patients developed chronic GVHD. 16/67 AML patients and 10/37 ALL patients relapsed. The event free survival for patients who were transplanted in complete remission was 48% for AML and 46% for ALL. Table 2 Summarizes studies that compared haploidentical transplantation to other donor sources.

Table 2 Haploidentical hematopoietic cell transplantation compared to transplantation from other graft sources

Year	Number of patients	Neutrophil engraftment (median d)	aGVHD II-IV (%)	cGVHD (%)	100 d NRM (%)	Relapse (2 yr)	Survival (%)
2002 ^[63]	MUD BM 81	16	42	57	23	25	58 (2 yr OS)
	MMUD BM 58	15	33	51	45	26	34
	Haplo 48	14	46	50	42	42	21
2005 ^[53]	Haplo-ALL 74	NA	8	NA	49	38	13 (2 yr LFS)
	UCB-ALL 91		26		41	23	36
	Haplo-AML 151		12		58	18	24
	UCB-AML 91		26		24	24	30
2009 ^[48]	Haplo 56	54/56 (13)	27	23	13	22	68 (2 yr LFS)
	MRD 51	48/51 (12)	14	31	8	17	76

aGVHD: Acute graft versus host disease; cGVHD: Chronic graft versus host disease; NRM: Non-relapse mortality; AML: Acute myelogenous leukemia; ALL: Acute lymphoblastic leukemia; NA: Not available; HCT: Hematopoietic cell transplant; MUD: Matched unrelated donor; BM: Bone marrow; MMUD: Mismatched unrelated donor; Haplo: Haploidentical family donor; OS: Overall survival; LFS: Leukemia free survival; UCB: Umbilical cord blood; MRD: Matched related donor.

Another T-cell depleted study evaluated 173 AML patients and 93 ALL patients who received a haploidentical transplantation^[40]. Patients received high dose of CD34⁺ cell with a median of 10×10^6 CD34⁺ cells/kg and 11.6×10^6 CD34⁺ cells/kg in AML and ALL patients, respectively. All patients received myeloablative conditioning containing TBI (74% AML and 92% ALL patients received TBI). Transplant related mortality was 66% for AML and 44% for ALL patients. Relapse incidence was 32% in AML and 49% in ALL patients. Among these patients with advanced disease, LFS was only 1% and 7% for AML and ALL, respectively. However, among patients transplanted in complete remission, the outcomes were more encouraging. Ninety-one percent of recipients engrafted with median time to engraftment of 12 d. The incidence of Grade II-IV GVHD was 5% and 18% among AML and ALL patients, respectively. In the AML group, recipients with a parent or sibling donor had lower TRM than other relatives (35% *vs* 65%, $P = 0.03$). The most common cause of TRM was infections, particularly viral infections such as adenovirus and CMV. Among these patients transplanted in remission, leukemia free survival at 2 years was 29% in AML and 23 % in ALL recipients. This multicenter study showed that infusion of high doses of immunoselected CD34⁺ cells without post-transplant immunosuppression can yield rapid and sustained engraftment and a low risk of GVHD.

A more selective T cell depletion can be performed by the Clini-MACS system. This system removes the α/β T cells and B cells, and keeps γ/λ T cells, natural killer and other cells. Locatelli *et al*^[41] reported on this method at the annual European BMT meeting where patients received myeloablative conditioning regimen of TBI, thiotepa, fludarabine and ATG followed by infusion of TCR α/β /CD19 T cell depleted grafts. This approach yielded sustained engraftment, faster immune reconstitution and low incidence of GVHD.

T-cell replete haploidentical transplantation

Di Bartolomeo *et al*^[42] studied the outcome of unmanipulated, G-CSF primed bone marrow haploidentical HCT for patients with high risk hematologic malignancies^[42].

The most common conditioning regimen used was thiotepa, busulfan and fludarabine in the myeloablative setting with GVHD prophylaxis comprised of 5 drugs: antithymocyte globulin, cyclosporine, methotrexate, mycophenolate mofetil and basiliximab. The 100 d incidence of grade III-IV acute GVHD was 5%, 1 year cumulative incidence of TRM was 36% and 3 year OS was 54% for standard risk patients^[42]. This study showed the feasibility of haploidentical transplantation without ex vivo T cell depletion by using a vigorous pre- and posttransplantation pharmacologic GVHD prophylaxis.

A group from china published results of unmanipulated G-CSF primed marrow haploidentical HCT followed by intensive immunosuppression. The incidence of grade III-IV acute GVHD was 13.4% and the 3 year LFS was 70.7% and 55.9% in standard and high risk AML^[43]. Another group from china published on the use of mismatched peripheral stem cells without conditioning regimen but post chemotherapy with cytarabine and mitoxantrone and showed an improvement of complete remission rate (80% *vs* 42.8%; $P = 0.06$) when compared to chemotherapy alone^[44].

Cyclophosphamide post haploidentical transplantation

A new Platform for RIC haploidentical transplantation was pioneered by John Hopkins university using high-dose post transplantation cyclophosphamide. Cyclophosphamide induced immune tolerance was first studied by Berenbaum *et al*^[45] who showed that mice treated with cyclophosphamide had a prolonged survival of mismatched skin graft if given up to the fourth day post grafting. The ability of post-transplant cyclophosphamide to prolong engraftment post a major histocompatibility mismatched skin graft, several immunologists became interested in developing durable chimerism before solid organ transplantation using post-transplant cyclophosphamide^[46]. These earlier studies established the fact that post-transplant cyclophosphamide kills T cells that undergo antigen driven proliferation and hence facilitates decrease risk of GVHD post transplantation.

Earlier phase II clinical studies with high dose cyclophosphamide were published in 2008 where cyclophos-

phamide 100 mg/kg given was administered over days +3 and +4 post RIC haploidentical marrow transplantation. The conditioning regimen included fludarabine, cyclophosphamide and TBI. Tacrolimus and mycophenolate were used for GVHD prophylaxis. Neutrophil engraftment was achieved at day 15 with very acceptable acute GVHD rates (grade II-IV GVHD was 35%). Relapse rate was 40%-50% at 1 year with DFS of 34%^[47]. Overall and EFS at two years were 36% and 26% respectively. A multicenter trial sponsored through the CIBMTR (CTN0603) using haploidentical BMT for high risk hematologic malignancies was run in parallel with another phase II trial (CTN 0604) using DUCBT. The probability of 1 year overall and PFS were 54% and 46% after DUCBT and 62% and 48% after haploidentical transplantation^[48].

Post-transplant cyclophosphamide was also applied in the myeloablative setting with peripheral blood cell source in the haploidentical setting. A study by the group in Philadelphia used a high dose TBI based conditioning with cytoxan 120 mg/kg given on days -3 and -2 followed by CD34 selected peripheral blood stem cells^[49]. The cumulative incidence of NRM was 22%, grade III-IV acute GVHD 7% and the 3 year survival was 27% for patients with active disease at the time of transplant. Other studies with myeloablative haploidentical transplantation using peripheral blood stem cell and post-transplant cyclophosphamide showed similar results of low incidence of acute GVHD and a 1 year of EFS in the range of 50%-60%^[50,51].

The use of peripheral blood as a source of stem cells in the nonablative haploidentical setting with post-transplant cyclophosphamide will allow wider applicability of this approach^[52].

Haploidentical transplantation vs UCB transplantation

The outcomes of 407 adult leukemia patients (AML = 242; ALL = 165) after UCB or haploidentical HCT were compared by the eurocord group^[53]. Compared to haploidentical HCT, recipients of UCB HCT had delayed neutrophil recovery, higher incidence of acute GVHD and similar incidence of relapse, LFS and TRM. A similar analysis among children with ALL showed that UCB HCT had higher rate of graft failure (23% *vs* 11%, $P = 0.07$). Both UCB and haploidentical HCT had similar TRM and DFS but more relapses were seen in the haploidentical group (RR = 1.7, $P = 0.01$)^[54]. These studies show that either UCB or haploidentical HCT is an acceptable option for both adult and children with leukemia in the absence of a fully matched sibling or unrelated donor.

A multicenter trial by the Clinical Trials network (BMT-CTN) is comparing the two stem sources in the reduced intensity setting for patients with acute leukemia. This study will hopefully help find some answers on the selective role of each of these procedures among leukemia patients.

Future strategies in haploidentical HCT

T-cell depletion has become the cornerstone of haplo-

identical transplantation. This usually leads to profound immunodeficiency lasting for 4-6 mo. Adoptive transfer of memory T lymphocytes helps protect against infections in the first months after transplantation. Infusion of virus-specific cell lines (CMV, Epstein-Barr virus, adenovirus and aspergillus) had inconsistent results in preventing and treating infections^[55,56]. Other strategies to hasten the post transplantation immune reconstitution without triggering GVHD have included infusion of donor T cells after engineering with a suicide gene^[57], photodynamic purging^[58], and the use of anti-CD25 monoclonal antibody to remove alloreactive cells^[59]. The Perugia group studied the infusion of haploidentical donor derived regulatory T cells followed by CD34 cells and donor mature T cells in the setting of T cell depleted haploidentical HSCT^[60]. With this approach, Perugia group was able to achieve a very low incidence of acute GVHD and a faster immune reconstitution.

More single centers are showing that usage of peripheral stem cell in the haploidentical RIC setting yields equivalent results to bone marrow infusion.

CONCLUSION

Patients with high risk acute leukemia requiring allogeneic HCT and lacking a fully matched related or unrelated donor have alternative options of stem cell sources. Either haploidentical or UCB is an acceptable option in this situation. The choice of best alternative donor is center dependent and several algorithms have been published to address donor selection^[40,61]. As studies continue to improve on engraftment rates in UCB, GVHD and relapse rates in haploidentical HCT, the order of donor choices will likely change with time.

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Identification and targeting leukemia stem cells: The path to the cure for acute myeloid leukemia

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HSC. In this review, we will first provide a historical overview of the discovery of LSC, followed by a summary of identification and separation of LSC by either cell surface markers or functional assays. Next, the review will focus on the current, various strategies for eradicating LSC. Finally, we will highlight future directions and challenges ahead of our ultimate goal for the cure of AML by targeting LSC.

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Key words: Acute myeloid leukemia; Leukemia stem cell; Immunotherapy; Cancer stem cell; Cell therapy

Core tip: Acute Myeloid Leukemia (AML) remains an incurable disease in most of cases. Leukemia stem cells (LSC) are a subpopulation of leukemic cells responsible for the continued proliferation and propagation of bulk leukemic cells. Growing evidence support the notion that LSCs are the root source of disease relapse and treatment resistance. Here we review the literature on historical overview of the discovery of LSC, identification and separation of LSC and strategies of targeting LSC as a potential cure for AML.

Abstract

Accumulating evidence support the notion that acute myeloid leukemia (AML) is organized in a hierarchical system, originating from a special proportion of leukemia stem cells (LSC). Similar to their normal counterpart, hematopoietic stem cells (HSC), LSC possess self-renewal capacity and are responsible for the continued growth and proliferation of the bulk of leukemia cells in the blood and bone marrow. It is believed that LSC are also the root cause for the treatment failure and relapse of AML because LSC are often resistant to chemotherapy. In the past decade, we have made significant advancement in identification and understanding the molecular biology of LSC, but it remains a daunting task to specifically targeting LSC, while sparing normal

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INTRODUCTION

Acute myeloid leukemia (AML) remains a hefty challenge for hematologists and oncologists. There are approximate 18800 new cases diagnosed with AML each year in United States alone, but estimated death cases is as high as 10000, ranking AML as the 6th highest cancer-related

death in male population (Cancer Facts and Figures 2014, American Cancer Society). AML is a group of morphologically, genetically and epigenetically heterogeneous disorders characterized by the accumulation of differentiation-arrested abnormal hematopoietic progenitor cells in the bone marrow and blood. The complexity of AML is further complicated by the existence of a spectrum of functionally diverse leukemic and preleukemic clones. Recent strides in massively parallel sequencing technology and powerful bioinformatic tools enable us to gain a deep and panoramic insight of AML genome and epigenome at unprecedented level. Elegant studies tracking clonal evolution from diagnosis to relapse revealed the greater clonal heterogeneity in AML than we previously estimated^[1-3]. Some clones either founding clone (major clone) or subclones (minor clone) at diagnosis, can survive chemotherapy. These survival clones may gain a small number of cooperating mutations, eventually leading to a relapse^[1-3]. For example, a subclone within the founding clones containing somatic mutations in some well-characterized pivot genes such as *DNMT3A*, *FLT3*, *NPM1*, *etc.*, can develop into dominant clone after acquiring additional mutations in *ETV6* and *MYO18B*. The mutations in these pivot genes are recurrent in AML^[1].

From the identification of chromosomal translocation in the 1970s, leukemia has been a prime and pioneering paradigm for the breakthrough discoveries in cancer genetics and the development of novel therapeutics^[4]. For example, the demonstration of the presence of leukemia stem cells (LSC) has preceded the discovery of the first cancer stem cells (CSC) in solid tumor (breast cancer) by almost 10 years^[5]. LSC, or leukemia initiating cells (LIC), are a subpopulation of cells that acquire self-renewal function and sustain the disease. AML LSC is the not only the first identified CSC, but also the best characterized CSC. It has become increasingly apparent that AML LSCs are generally insensitive to the conventional chemotherapy. They reside in the bone marrow micro-environment and are poised to propagate, leading to the treatment failure and relapse. This suggests that the LSC subpopulation is the culprit for the poor outcome of AML patients and selectively targeting LSC will be a important strategy towards curing AML.

IDENTIFICATION OF LSC-CELL SURFACE MARKERS IN COMBINATION WITH FUNCTION ASSAYS

CD34+CD38-: the beginning of LSC hunting

Pioneer studies from John Dick's group in 1990s firmly established the AML LSC model, that AML is a hierarchical disease which is initiated and sustained by a rare subset of LSC. Only the subset of immature CD34+CD38- leukemia cells is capable of not only initiating leukemia in sublethally irradiated immunodeficiency mice, but also transplantable in second and third generation mice. In contrast, the fraction of more mature CD34+CD38+

leukemia blasts failed to imitate disease under the same condition. The estimated frequency of LSC in the CD34+CD38- cells is one in one million, thus LSC represent a very rare of unique population of leukemia cells sharing the similar cell surface marker as normal immature hematopoietic cells. Importantly, several clinically observatory studies demonstrated that high frequency of CD34+CD38- cells, but not total CD34+ cells, amongst blast cells at diagnosis correlates with poor survival in both adult and pediatric AML patients^[6,7]. More recently, gene expression profiles generated from this rare subset of CD34+CD38- cells support their clinical impact that high expression of LSC signature predicts worse outcome^[8-11].

However, recently findings derived from newly generated NOD/ShiLtSz-*scid*/IL2Rγ^{null} (NSG) and NOD/ShiJic-*scid*/IL2Rγ^{null} (NOG) mice, the most immunodeficient strains, cast new light on the origin of LSC. These two strains of mice don't express the IL-2 receptor common gamma chain, which allow more efficient engraftments of human hematopoietic cells than SCID or NOD/SCID mice in previous studies. Using these more immunosuppressive mice as hosts, CD34+CD38+ cells from some primary AML can induce transplantable disease, indicating CD34+CD38+ cells have LSC activity too^[12,13]. Works from Bonnet's laboratory unveiled the possibly confounding factor that the anti-CD38 antibody used for separation of primary AML cells has significant inhibitory effect on engraftment of leukemia cells^[13]. Taken together, these studies suggest LSC might co-exist in CD34+CD38- and CD34+CD38+ subpopulation.

Cell surface markers differentially expressed between LSC and normal HSC

Because LSC and HSC sharing similar CD34+CD38- surface immunophenotype, the search of cell surface markers unique to LSC (ideal circumstances) or at least differentially expressed has attracted intensive enthusiasm in hematology and oncology field. Such makers will provide excellent therapeutic windows for specifically targeting LSC, while sparing normal HSC. Such therapies are expected to be much tolerable for AML patients.

CD90

CD90, also known as Thy-1, is a small glycosylphosphatidylinositol (GPI)-anchored protein (25-37 kDa) regulating multiple signaling cascades which control cellular survival, proliferation, adhesion and response to cytokines^[14]. One of the early studies reported that the majority of AML blasts did not express CD90 and CD34+CD90- cells were capable of maintaining the disease *in vitro* and *in vivo* as demonstrated by production of leukemic clonogenic cells (CFU) and engraftments in nonobese diabetic severe combined immune deficient (NOD/SCID) mice, respectively^[15]. However, independent study to validate CD90 as a possible LSC marker is scarce in the literature. In contrast, CD90 expression was detected

at high frequency of a group of high-risk AML, such as secondary AML (40%) and elderly > 60 years AML (24%) patients^[16]. Univariate analysis revealed that CD90 expression was an independent prognostic factor for a shorter survival^[16]. This finding appears to contradict to the proposal of CD34+CD90- fraction is the source of LSCs because it is generally believed that abundant level of LSC markers is associated with poor survival. Interestingly, CD90 has been identified as marker of cancer stem cell (CSC) of hepatocellular carcinoma^[17], esophageal cancer^[18] and high-grade gliomas^[19].

CD96

CD96 (also known as TACTILE), a type I membrane protein, belongs to the immunoglobulin superfamily. CD96 plays a role in the antigen presentation of immune response the adhesive interactions of activated T and NK cells. CD96 is expressed on the majority of CD34+CD38- AML cells and vice versa^[20]. In contrast, CD96 is weakly expressed in cells in the normal HSC-enriched population [Lin(-)CD34(+)-CD38(-)CD90(+)]. Significant level of engraftment is only achieved in mice implanted with CD96+ AML cells, but not CD96- AML cells^[20]. From a therapeutic point view, this LSC marker offers a few new avenues for treatment of AML disease. Firstly, CD96 specific monoclonal antibody can be used to selectively eradicate AML-LSCs before autologous stem cell transplantation^[21]. Secondly, Fc-engineered mini-antibodies directed against CD96 shows enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) activity of affinity and the highest cytolytic potential^[22].

CD123

CD123 is also known as interleukin 3 receptor, alpha (IL-3R α). IL3R is a heterodimeric cytokine receptor comprised of the alpha unit and beta unit, which is activated by the ligand binding and necessary of IL-3 activity^[23]. IL-3 is one of the prominent cytokines that controls proliferation, growth and differentiation of hematopoietic cells^[24]. Compared to all other cell surface antigens as potential LSC markers, the studies on CD123 have been investigated into much more details and targeting CD123 is now in clinical trials^[23].

Jordan and colleagues^[25] first reported that CD123 was aberrantly expressed on CD34+CD38- cells from AML patients, but not detectable on CD34+CD38- cells from healthy controls. Moreover, purified CD34+CD123+ cells from AML patients were capable of establishing and propagating leukemia disease in NOD/SCID mice^[25]. This result functionally validated CD123 as a LSC marker. A following-up study from the same group further revealed that NF κ B activity was constitutively activated in the CD123+ LSCs, but not CD123+ normal HSC, providing a molecular difference between these two cell entities^[26]. Higher level of spontaneous signal transducer and activator of transcription 5 (STAT5) activity is an-

other factor contributing to the proliferative advantage and resistance to apoptosis of AML blasts with elevated CD123^[27]. It is well documented that enhanced STAT pathway activity confers drug resistance in AML^[28], possibly through two distinct mechanisms: upregulation of anti-apoptotic survivin (*BIRC5*), Bcl-xL (*BCL2L1*) genes and ATP-binding cassette (*ABC*) family genes, which encode multidrug-resistance (MDR) transport proteins.

The utility of CD123 as a LSC marker has been convincingly confirmed by many other studies^[29,30]. A flow cytometric analysis of CD123 expression of diagnostic blasts from 111 *de novo* AML patients younger than 65 years old shows the presence of more than 1% population of CD34(+)-CD38(low/-)-CD123(+) cells adversely affected the disease-free-survival and over-all survival^[30]. Notably, not only the percentage of CD123+ cells, but also the expression level of CD123+ predicts clinical outcome. Patients whose AML blasts have higher CD123 expression have a lower complete remission (CR) rate and shorter survival duration than those showing normal CD123 expression level^[27]. In AML arising from Fanconi anemia (FA) background, only CD123+ cells achieve significant level of engraftment and cause leukemia in a "humanized" FA xenotransplant model^[29].

Other studies have depicted what other molecules are co-expressed with CD123 in AML-LSCs. High CD123 AML cells often exhibit elevated level of receptor tyrosine kinases (RTKs) such as FLT3 (Fms-Related Tyrosine Kinase 3), c-Kit^[31], N-cadherin and Tie2 (Tunica Interna Endothelial Cell Kinase)^[32]. Both FLT3 and c-Kit are important RTKs for the survival of hematopoietic stem/progenitor cells. N-Cadherin and Tie2 play a pivotal role in regulation of interaction between LSCs and their niche in the bone marrow microenvironment. These findings reinforce the role of CD123 as a LSC marker because these co-expression molecules provide CD123+ cells survival advantages and sanctuary in their niche environments.

Antibody therapy specifically targeting CD123 has been advanced to clinical development over a short 5-year period since the first report of *in vivo* preclinical study^[33]. Anti-CD123 monoclonal antibody 7G3 has been shown to completely inhibit bone marrow engraftment by ex vivo treatment and partially impede bone marrow engraftment in a pre-established disease model in mice. CSL360, a recombinant chimeric IgG1 mAb derived from 7G3, was evaluated in phase I clinical trial against AML. The preliminary results showed that anti-CD123 mAb therapy with CSL360 is safe and tolerable and biological effects have been observed (ClinicalTrials.gov Identifier: NCT00401739). A humanized, affinity-matured version of anti-CD123 antibody, CSL362, was developed through engineering the Fc-domain for increased affinity for human CD16 (Fc γ RIIIa) on (natural killer) NK cells. CSL362 exhibits greater ADCC against both bulks of AML blasts and CD34+CD38-CD123+ LSCs^[34]. Currently, CSL362 is under phase I clinical trials in patients with CD123+ AML in complete remis-

sion (CR) or CR with incomplete platelet recovery at high risk for early relapse (Clinical Trials.gov identifier: NCT01632852). Novel molecules targeting both CD123 and CD33 have been shown to have stronger anti-AML effect than mono-targeting agents *in vitro*^[35]. It will be interesting to test these dual-targeting or triple-targeting molecules in animal studies or even in human clinical trials against LSC.

Adoptive T cell therapy is an alternatively attractive approach for the treatment of cancer utilizing chimeric antigen receptors (CARs)^[36]. The third generation of CARs consist of an extracellular antigen-binding domain and three or more intracellular signaling domains^[36]. CD123 chimeric antigen receptor (CAR) redirected T cells/cytokine-induced killer (CIK) cells show robust activity against CD123+ cell lines, primary AML cells and mouse xenograft models transplanted with patient AML cells^[37-39]. One important advantage of this approach lies on the observation that relapsed or refractory AML cells which often are chemotherapy-resistant are still vulnerable to CD123 CAR T cell therapy^[37]. However, depletion of normal human myelopoiesis caused by CD123 CAR T cells as a potential side effect should be taken account when planning a clinical trial^[38].

Taken together, novel immunotherapy approaches such as improved variants of anti-CD123 monoclonal Ab and CD123 CAR T cell therapy hold great promising for AML treatment.

CD47

CD47 (also known as Integrin-associated protein, IAP) is one of the unique member of the Ig superfamily, consisting of a V-type Ig-like extracellular domain at its N-terminus, five hydrophobic membrane-spanning segments and a variably spliced (3-36 amino acids) cytoplasmic tail at its C-terminus^[40]. CD47 is a receptor for the C-terminal cell binding domain of thrombospondin-1 (TSP-1) and a ligand for the extracellular region of signal-regulatory protein alpha (SIRPα)^[41]. CD47 is ubiquitously expressed on human cells and involved in many fundamental cellular processes including immune and angiogenic responses^[40].

Majeti and co-workers first discovered higher expression of CD47 on AML LSC compared to their normal counterparts, HSC and multipotent progenitor cells (MPP), by flow cytometer and microarray gene expression analysis^[42,43]. The association between increased CD47 expression with worse outcome has been validated in 3 independent, large clinical cohorts with total 664 AML patients. Moreover, increased CD47 expression remains a prognostic factor for poor event-free survival and over-all survival in multivariable analysis considering age, FLT3-ITD status^[42]. SIRPα serves as inhibitory receptor expressed on phagocytic cells such as macrophages and dendritic cells. It was previously reported CD47 expressed on red blood cells (RBC) as a marker of self and interaction of CD47 and SIRPα on phagocytic

cells delivered a “do not eat me” message, limiting clearance of circulating RBC by the means of phagocytosis^[44]. Similarly, upregulation of CD47 on AML LSCs prevents themselves from the attack of phagocytic cells through the interaction of CD47 with its inhibitory ligand SIRPα. This conclusion is supported by several lines of evidence. Firstly, human AML cell line with low endogenous CD47 level fails to engraft in immunodeficient mice, while ectopic expression of mouse CD47 in this cell line improves engraftment^[45]. In an inducible and controlled expression of CD47 *in vitro* and *in vivo* models, it has shown that the level of CD47 expression negatively correlates the percentage of phagocytosis by the macrophages^[45]. Secondly, transgenic mice expressing SIRPα variants with differential ability to bind human CD47 demonstrates that the engraftment of AML LSCs depends on the interaction of CD47 with SIRPα and AML LSCs are eliminated by macrophage-mediated phagocytosis in the absence of SIRPα signaling. In addition, pharmacological disruption of CD47-SIRPα binding by SIRPα-Fc fusion protein augments phagocytosis of AML cells by both mouse and human macrophages and damages engraftment of CD34+CD38- AML LSCs in mice^[46]. Thirdly, AML patients with high SIRPα mRNA expression on AML blasts have poor survival and inhibition of SIRPα signaling lead to reduced cell proliferation and enhanced apoptosis of AML cells^[47]. Based on the aforementioned evidence generated from *in vitro* experiments, *in vivo* mouse model and clinical data, we believe elevation of CD47 expression in AML LSCs appears to enable them to evade host immune surveillance.

A few anti-CD47 monoclonal antibodies have been tested *in vitro* and animal models. Two antibodies that block CD47/SIRPα interaction induce phagocytosis of AML cells *in vitro* and *in vivo* and eradicate LSCs in xenograft mouse and isogenic mouse leukemia models, while an anti-CD47 antibody that does not disrupt CD47 binding to SIRPα fails to promote phagocytosis of AML cells^[42]. The other promising strategy to target this interaction is to use soluble SIRPα-Fc fusion proteins to neutralize CD47^[46]. Treatment of SIRPα-Fc fusion proteins leads to activate macrophages mediated phagocytosis, resulting in potent anti-AML effect and clearance of LSCs^[46].

Taken together, these evidences indicate that delivering a “do not eat” signal to phagocytic cells is a prime consequence of CD47/SIRPα interaction, which suppresses phagocytosis. Disruption of this interaction would successfully initiate innate immune response to eliminate LSCs through macrophage phagocytosis.

CD44

CD44 belongs to a family of transmembrane glycoproteins that act primarily as a receptor for hyaluronan acid (HA), but it also binds to other receptors including osteopontin, collagens, matrix metalloproteinases (MMPs), *etc.*^[48]. Hyaluronan is one of the major components of

the extracellular matrix^[49]. The major function of CD44 is to regulate cell-cell adhesion and cell-matrix interaction through binding to HA and other receptors^[49]. Specifically, the roles of CD44 in haematopoiesis include cell migration, proliferation, differentiation, survival and bone marrow homing of hematopoietic stem/progenitor cells^[50].

It has been long recognized that CD44 is expressed in normal and leukemic CD34+ early hematopoietic cells and empowers them to seek intramedullary or extramedullary sanctuary^[51]. It has been postulated that such protective ability resulted from CD44 interaction with various cellular receptors and matrix components allows small numbers of leukemic cells to survive from the attack of cytotoxic chemotherapy^[52].

Detection of CD44 and coexpression of CD123 (abovementioned) on CD34+CD38- AML cells indicates the CD44 is a potential candidate of LSC marker^[53]. Jin *et al.*^[54] first comprehensively characterized CD44 as a critical regulator of AML LSCs in a few mouse models. Treatment with H90, a monoclonal antibody targeting CD44, significantly prolonged survival of NOD/SCID mice transplanted with CD34+CD38- AML LSCs and reduced the number of LSCs in mouse bone marrow as compared to control IgG treatment. Furthermore, in a secondary transplantation experiment, leukemic cells obtained from H90 treated mice (primary mice) failed to engraft into the secondary receipt mice. However, in the parallel experiment, leukemic cells harvested from primary mice treated with control IgG initiated robust engraftment in the secondary receipt mice^[54]. The power of eliminating LSCs by anti-CD44 monoclonal antibody treatment could be explained by three different mechanisms by which targeting CD44 induces leukemic cell differentiation^[54-58], inhibits cell cycle progression and cell proliferation^[58,59] and impedes LSCs homing to bone marrow niches^[54]. Collectively, these data conclusively demonstrate that CD44 is functional important for LSCs.

CD32 OR CD25

CD32 is a member of a family of immunoglobulin Fc receptors, expressed on macrophages, neutrophils and nature killer cells^[60]. CD32 binds to the Fc region of immunoglobulins gamma (Igγ) and executes phagocytosis and clearing of immune complexes^[60]. CD25 is also known as interleukin 2 (IL2) receptor alpha (IL2RA)^[61]. IL-2 cytokine regulates cell proliferation, differentiation, survival and apoptosis^[62].

CD32 and CD25 were discovered to be overexpressed on quiescent and chemotherapy-resistant human AML LSCs by microarray study of LSCs *vs* normal HSCs. Normal CD34+CD38-CD133+ HSCs are negative for CD32 or CD25 expression^[63]. In xenotransplantation experiments with sorted human AML cells injected into immunodeficient mice, CD32+CD34+CD38- or CD25+CD34+CD38- cells were capable of engraftment and inducing AML. On the contrary, no engraft-

ment was detected in mice inoculated with CD32-CD34+CD38- or CD25-CD34+CD38- cells^[63]. The CD32+CD34+CD38- or CD25+CD34+CD38- cells not only survived after treating the mice with cytosine arabinoside (Ara-C), but also initiated *in vivo* AML when injected into the secondary receipt mice in a serial transplantation model^[63].

CLL-1

C-type lectin-like molecule-1 (CLL-1) is a member of type II transmembrane receptor family containing C-type lectin/C-type lectin-like domain (CTL/CTLD). CLL-1 was initially identified as a novel surface marker of AML cells through phage display technology combined with flow cytometry^[64]. Further studies revealed that CLL-1 was expressed on CD34+CD38- cells in 87% of AML patients, but was not expressed in normal HSCs^[65]. Successful engraftment was observed in all 3 NOD/SCID mice transplanted with CD34+CLL-1+ AML cells^[65]. The same group also reported that side population (SP) cells isolated from AML samples which were highly enriched for LSCs also expressed CLL-1^[66].

A series of monoclonal antibodies against CLL-1 was developed and two lead antibodies were chosen based on their high affinity and potent cytotoxic activity^[67]. These antibodies induced dose-dependent complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity against AML cell lines, primary AML patient cells and xenograft mice implanted with HL-60 AML cells. However, the possibility of targeting LSCs was not assessed in this study^[67]. It would be of interesting to further evaluate the impact of these anti-CLL-1 antibodies on LSCs in animal experiments. Nanomicelles decorated with CLL1-targeting peptides can specifically binds to CD34+CLL-1+ primary AML cells and delivered chemodrug daunorubicin directly to target cells^[68]. Importantly, these nanomicelles did not bind to normal CD34+ cells, so it was not expected to harm normal hematopoiesis^[68]. The challenge of *in vivo* delivery of nanomicelles remains a concern.

TIM-3

T cell immunoglobulin-3 (TIM-3) belongs to the mucin domain-containing molecule (Tim) superfamily and is a member of the T cell Ig^[69]. TIM-3 is expressed on CD4+ Th1, CD8+ T cytotoxic 1 (Tc1) cells, monocytes/macrophages, dendritic cells and mast cells^[70]. TIM-3 plays an important role in T cell response and regulation of innate immunity^[69,70].

TIM-3 was found to be expressed on CD34+CD38- fraction of AML cells except FAB M3 subtype (acute promyelocytic leukemia, APL) but absent on normal CD34+CD38- HSCs through comparative analysis of transcriptome of these two populations^[71]. TIM-3 expression was significantly higher in a distinct subtype of AML with core binding factor (CBF) translocation or

CEBP α mutation^[72]. This association was a bit puzzling because AML patients with CBF and CEBP α abnormalities often have favourable prognosis^[73]. Reconstitution of AML in immunodeficient mice was established only when TIM-3+ AML cells were transplanted, but not TIM-3- AML cells. Treatment of mice injected with human primary AML cells with an anti-TIM-3 monoclonal antibody, ATIK2a, effectively blocked reconstitution of AML. Importantly, human CD45+ AML cells harvested from the primary recipient mice treated with ATIK2a lose the ability to initiate AML retransplanted into secondary recipient mice^[71]. Normal HSCs were not damaged by ATIK2a treatment because normal HSCs appear to reside in TIM-3- population^[71,72]. These data suggest that TIM-3 could serve as a useful marker to distinguish LSCs from HSCs and monoclonal antibody against TIM-3 holds promise to eradicate LSCs.

Aldehyde dehydrogenase

Aldehyde dehydrogenase (*ALDH*) gene superfamily consists of 19 functional genes and three pseudogenes. ALDH oxidise a wide range of endogenous and exogenous aldehyde substrates, thus detoxifying large portion of adverse aldehydes to the cells. ALDH is highly expressed in primitive stem cells from several tissue origins, including bone marrow and intestine^[74]. HSCs have high level of ALDH activity^[75] and can be distinguished using a fluorescent aldehyde, dansyl aminoacetaldehyde (DAAA) in conjunction with FACS analysis^[74,76].

Since LSCs share some functional similarity with HSCs, researchers soon started to investigate the role of ALDH in AML LSCs. In total, 3 distinct patterns of ALDH activity were documented. In the first pattern, the subpopulation of AML cells with high ALDH activity was rare, which was similar to the pattern seen in normal core blood. In the second pattern, the frequency of cells with ALDH activity was more frequent and their side scatter profiles were higher than normal stem/progenitor cells. No fraction of cells with high ALDH activity was present in the third pattern^[77]. Xenograft transplantation experiments demonstrated that ALDH+ cells were enriched for LSCs and engrafted better than ALDH- cells^[77,78]. From a clinical point of view, higher ALDH activity is associated with dismal prognosis, drug resistance and relapse^[78-80].

SMALL MOLECULE INHIBITORS

TARGETING LSCS

Parthenolide and analogs

Dimethylamino-parthenolide (DMAPT), modified analog of parthenolide (PTL) which is a major active component of herbal medicine Feverfew, possesses improved pharmacologic properties and is orally bioavailable^[81,82]. DMAPT and PTL preferentially kill AML leukemia stem/progenitor cells through mechanisms involved in inhibition of NF κ B pathway, induction of tumor suppressor p53 and reactive oxygen species (ROS) produc-

tion^[81,82]. DMAPT shows potent *in vivo* biological activity in spontaneous canine acute leukemia and mouse xenotransplantation models^[82]. DMAPT is a novel compound that is specifically target LSCs and now is being evaluated in a phase 1-2 “first in man” in clinical trial in AML in Cardiff University, United Kingdom.

Epigenetic inhibitors

AR-42 (OSU-HDAC42), a novel histone deacetylase inhibitor (HDACi), inhibits NF κ B activity and HSP90 interaction with its various client proteins, leading to robust and selective apoptosis of AML LSCs^[83]. Currently, AR-42 is being tested in advanced or relapsed multiple myeloma (MM), chronic lymphocytic leukemia (CLL), or lymphoma in clinical trials (ClinicalTrials.gov Identifier: NCT01129193).

BRD4 (Bromodomain-containing protein 4) was identified as a promising anti-AML target in a whole-genome RNAi screening^[84,85]. BRD4 is a chromatin “reader” that recognizes and binds acetylated histones. JQ1 is a novel small molecule inhibitor that competes with BRD4 to bind acetyl-lysine recognition motifs^[86]. JQ1 can induce apoptosis in CD34+CD38- and CD34-CD38+ stem- and progenitor cells from both *de novo* AML and refractory AML patients^[87].

3-Deazaneplanocin A (DZNep), is a newly discovered S-adenosyl-methionine-dependent methyltransferase inhibitor^[88]. DZNep inhibits EZH2, disrupts polycomb-repressive complex 2 (PRC2), and preferentially induces apoptosis in cancer cells^[88]. We and another group showed that DZNep promoted cell death in CD34+CD38- AML cells, but not normal CD34+ progenitor cells^[89,90].

Apoptosis pathway modulators

ABT-737, a BCL-2 homology domain 3 mimetic inhibitor, have been shown to target Lin-/Sca-1(+)/c-Kit(+) primitive cells, and progenitor population in a myelodysplastic syndrome (MDS)-AML transgenic mouse model^[91].

Using reversed-phase protein array, Carter BZ and colleagues^[92] found that CD34+CD38- AML stem/progenitor cells expressed increased caspase 8 and increased ratio of cIAP (Baculoviral IAP Repeat Containing 2, BIRC2) to SMAC (second mitochondrial-derived activator of caspases) compared to bulk AML cells. Birinapant is a novel bivalent SMAC mimetic with high affinity for IAP proteins. Treatment with birinapant induced apoptosis of AML stem/progenitor cells involving in activation of DR (death receptor)/caspase-8 complex. In human AML xenograft mouse model, diseased mice treated with birinapant or in combination with 5-azacytidine (5-Aza), decitabine (DAC), survived significantly longer than mice administrated with vehicle control^[92].

Kinase inhibitors

Rapamycin is the first generation of mTOR (mammalian target of rapamycin), a downstream target of

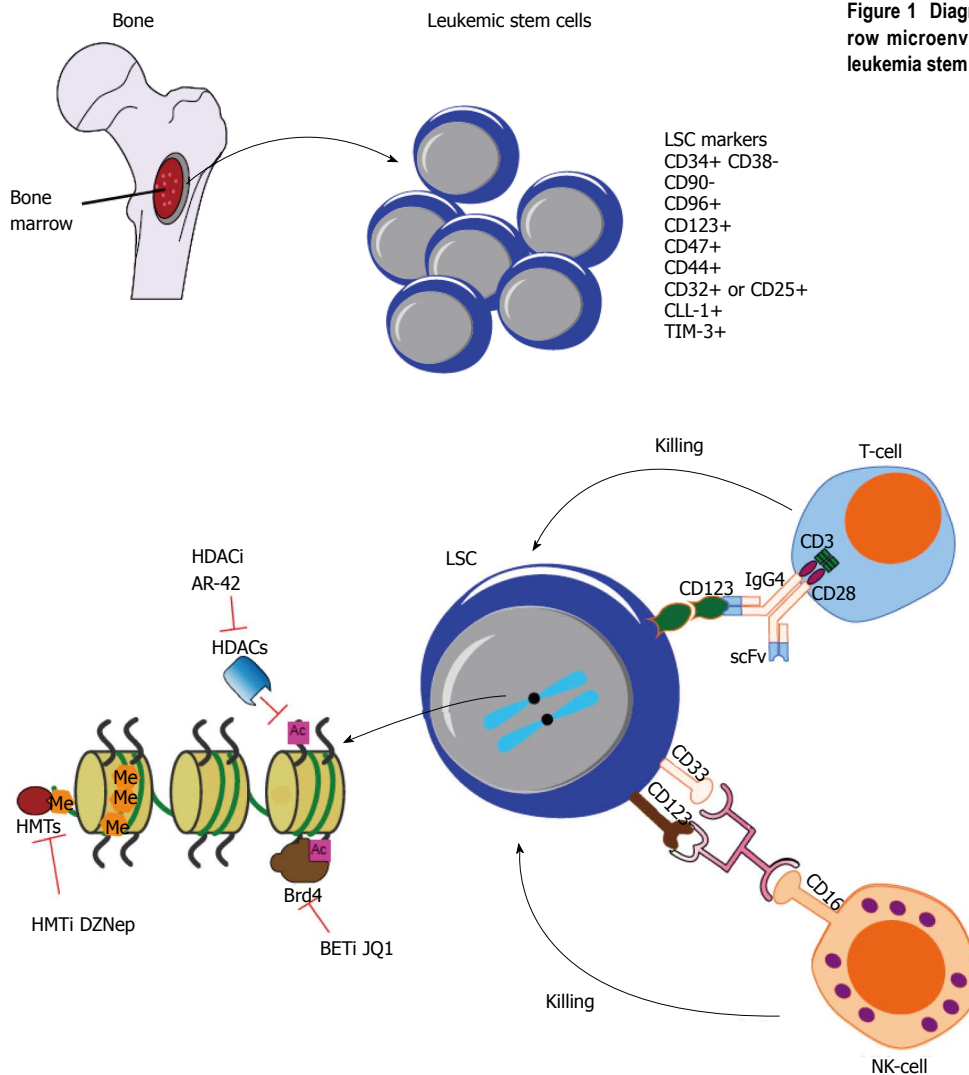


Figure 2 This illustration shows combination therapies aiming to achieve maximal and synergistic anti-leukemia stem cells effect. HDACi: Histone deacetylase inhibitor; HMTi: Histone methyltransferase inhibitor; BETi: Bromodomain and Extra-Terminal inhibitor; Brd4: Bromodomain-containing protein 4; Ac: acetylation; Me: methylation; NK-cell: Natural killer-cell.

phosphatidylinositol 3-kinase (PI3K)-Akt pathway, inhibitor^[93]. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) negatively regulates PI3K-AKT-mTOR activity. Tissue specific deletion of PTEN in hematopoietic cells led the mice to develop AML and acute lymphoid leukemia (ALL) and all mice succumbed to disease rapidly in one month^[94]. One out of 81 Flk-2-Sca-1+Lin-c-Kit+CD48- (enriched for LSCs) from PTEN null AML mice was able to initiate AML in serial transplantation experiments^[94]. A search of “ClinicalTrials.gov” database on 10 July 2014 identified a total of 40 clinical trials that test Rapamycin or its analogs, Temsirolimus (CCI-779) or Everolimus (RAD001), in AML either by alone or in combination with chemotherapy or kinase inhibitors or transplantation. However, it appears that the evaluation of the effect of mTOR inhibitors against LSCs is not included in these trials.

Dasatinib is a multiple kinase inhibitors targeting Abl, Src family and c-Kit. Dos Santos *et al.*^[95] reported that

combination of dasatinib and daunorubicin enhanced the eradication of AML LSCs in mouse xenotransplantation model through increasing p53 activity^[95].

Hematopoietic Cell Kinase (HCK) belongs to the Src family of tyrosine kinases. HACK is mostly expressed in hematopoietic cells, particularly phagocytes. HACK was reported by Saito Y, et al. to overexpress on quiescent, chemotherapy-resistant LSCs compared to normal HSCs^[63]. The same group performed integrated, multiple platform analysis to uncover RK-20449, a pyrrolo-pyrimidine derivative as a potent inhibitor of these LSCs *in vitro* and *in vivo*^[96].

CONCLUSION

The advance in high-throughput and whole genome techniques in conjunction with the development of more immunocompromised mouse strains helps deepen and broaden our understanding of LSCs, the enigmatic frac-

tion of leukemic cells which is the origin of the disease. From the single pattern of CD34+CD38- as phenotypic hallmark for LSCs, a long-list of additional cell surface antigens such as CD123, CD47, CD44, CLL-1, CD96, CD90, CD32, CD25, and TIM-3, has been identified to separate LSCs from normal HSCs (Figure 1). From the notion that LSC is extremely rare, it is now clear that the frequency of LSC among AML patients is highly heterogeneous, ranging from very low to frequent. From the concept that LSCs only reside in CD34+CD38- subpopulation, emerging study reveals that CD34+CD38+ fraction also harbours LSCs. From the idea that one patient only has one population of LSCs, we now understand that some patients may have more than one populations of LSCs.

Along the advance in our understanding of LSC, a growing list of strategies for targeting LSC has been proposed and some of these agents as summarized above have advanced into clinical trials. Currently, monoclonal antibodies targeting CD123 or their related immunoconjugate therapy or CD123 CAR T cell therapy appear to be the front runner leading the way to eliminate LSC and eventually cure AML. The second gold mine for the discovery of drug targets is how LSCs employ “epigenetic machinery” to program or reprogram themselves because epigenetic changes are reversible and epigenetic enzymes are often targetable. The first generation of some of these small molecule inhibitors such as DZNep, JQ1, already showed potent effect in killing LSCs. We shall witness the second generation of these compounds or novel small molecule inhibitors with favourable pharmacological profiles and safety profiles entering clinical trials in the next few years.

However, the real impact on clinical management of AML is far less promising than the remarkable response observed in *ex vivo* cell culture models or xenotransplanted mouse experiments as reported in numerous “sophisticated” studies. In our opinion, although many surface antigens have been identified to be aberrantly expressed on LSCs, it is probably impossible for any single monoclonal antibody targeting one of these surface antigens to eradicate LSCs, given such heterogeneity and dynamics of LSC properties in AML patients. Synergistic therapies in combination with immunotherapy, cell therapy and epigenetic drugs may provide a better opportunity to achieve our ultimate goal of targeting LSCs and curing AML (Figure 2). By using CD123 target as an example, it is hoped that combination of CD123 CAR T cells which bind to CD123 on the surface of LSC or mono- or dual-targeting antibody with small molecule inhibitors targeting epigenetic machinery, such as Brd4 inhibitor or HMTi or HDACi, will be effective for the treatment of AML.

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Advances in haplo-identical stem cell transplantation in adults with high-risk hematological malignancies

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Core tip: Timely donor availability remains a challenge for patients in need of an urgent stem cell transplant. The ability to obtain half matched stem cells from any family member represents a significant breakthrough in the field. This review summarizes some of the current strategies used to substantially improve the outcomes of patients undergoing haplo-identical stem cell transplantation.

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Abstract

Allogeneic bone marrow transplant is a life-saving procedure for adults and children that have high-risk or relapsed hematological malignancies. Incremental advances in the procedure, as well as expanded sources of donor hematopoietic cell grafts have significantly improved overall rates of success. Yet, the outcomes for patients for whom suitable donors cannot be found remain a significant limitation. These patients may benefit from a hematopoietic cell transplant wherein a relative donor is fully haplotype mismatched. Previously this procedure was limited by graft rejection, lethal graft-versus-host disease, and increased treatment-related toxicity. Recent approaches in haplo-identical transplantation have demonstrated significantly improved outcomes. Based on years of incremental pre-clinical research into this unique form of bone marrow transplant, a range of approaches have now been studied in patients in relatively large phase II trials that will be summarized in this review.

INTRODUCTION

Allogeneic bone marrow transplantation (BMT) offers a chance to cure patients that present with high-risk hematological malignancies. These include adults and children with acute myeloid leukemia (AML) in first or latter remission and acute lymphoblastic leukemia (ALL) employing BMT as post remission therapy in first or greater complete remission. Allogeneic BMT can also be considered in eligible patients with chronic lymphocytic leukemia, chronic myeloid leukemia and lymphoma (follicular, large cell, Hodgkin and peripheral T cell). The process of safely performing allogeneic BMT requires the regulated experience of a comprehensive multi-disciplinary team of health care professionals. In principle, the goal is to replace a diseased bone marrow with healthy blood-forming hematopoietic elements from a

fully human leukocyte antigen (HLA)-matched healthy donor. At one time allogeneic BMT was routinely associated with a mortality of greater than 40%. Advances in stem cell acquisition and processing, molecular-level typing of unrelated donors and general supportive care that have reduced infectious complications have collectively improved rates of survival. Essential to success is durable engraftment of donor progenitor cells capable of restoring stable hematopoiesis. In addition to engraftment of hematopoietic progenitors, it is now known that donor immune effector cells (including T-lymphocytes) are required for disease eradication and prevention of relapse^[1-3]. Specific anti-tumor donor lymphocytes engage in an ongoing immune reaction against residual host malignant cells^[4-6]. This graft-versus-tumor (GVT) effect is closely linked to graft-*vs*-host disease (GVHD)^[7-9]. The ability to dissect immune effectors responsible for each process is a element of current BMT research. Recognizing that a successful transplant requires contribution of both donor progenitor and immune effectors has lead to substantial changes in the field. These enhancements include: (1) the design of newer less-toxic preparative regimens^[10-13], and (2) expansion of the sources of donor stem/progenitor cell grafts.

Preparative regimens

Myeloablative transplant conditioning has traditionally been used to “create space” for donor progenitor cells and simultaneously kill residual tumor cells. These preparative protocols employ high-dose chemotherapy often in combination with whole-body irradiation. Significant treatment-related toxicities (TRM) restrict the procedure to young, otherwise-fit patients. Shifting the focus and goals of clinical efficacy from stem cell replacement to maintenance of a transplanted donor immune system has enabled the introduction of less-intense preparative regimens. Milder preparative regimens aim to achieve chimeric engraftment of progenitors as well as donor immune effector cells, including T lymphocytes. Reduced-intensity conditioning (RIC) or non-myeloablative allogeneic BMT is currently performed in a growing number of older patients (up to and beyond 70 years of age) diagnosed with a variety of lymphoid and myeloid neoplasms^[13-16]. These hematological malignancies appear to have mixed susceptibility to the GVT effect. Donor immune effector cells impact therapeutic efficacy but also contribute to serious post-transplant side effects, including severe acute and chronic GVHD (aGVHD and cGVHD, respectively). Patient determinants, including remission status, remission duration and disease type, may govern the choice of preparative regimen as well as the type of graft that will contribute to rate of stable engraftment, immune reconstitution, GVL, and GVHD.

Graft sources

Once exclusively obtained by large volume aspiration from the posterior pelvis of a donor, transplantable hematopoietic progenitor cells can now be obtained directly

from peripheral blood as well as from fresh umbilical cord blood. These stem cell products are transported world-wide in a highly regulated manner. Common terminology are used to describe stem cells derived from a bone marrow harvest (Hematopoietic Progenitor Cells - Marrow, HPC-M), from a mobilized apheresis peripheral blood product (Hematopoietic Progenitor Cells-Apheresis, HPC-A), from umbilical cord blood (Hematopoietic Progenitor Cells -Cord, HPC-C), or for donor lymphocyte infusion (DLI) (Therapeutic Cells-T). Each source of hematopoietic progenitors exhibits differences in cellular composition which leads to specific biological properties that may be of therapeutic benefit or risk depending on the transplant recipient, type of transplant to be performed as well as the type and immediate status of the hematological malignancy. For example mobilized peripheral blood products HPC-A often have a higher number of CD34+ stem/progenitor cells but also more CD3+ T lymphocytes (1-log higher). Higher CD34+ progenitor cell counts may improve time to engraftment and be used in a non-myeloablative setting but also appear to increase the risk of cGVHD^[17-19]. Umbilical cord blood (HPC-C) typically contains a much lower absolute CD34+ progenitor cell count leading to significant delays in engraftment (or rejection); however the immature nature of the donor white blood cells from this source may also reduce the risk of GVHD and allow for some degree of HLA mismatch^[20-22].

Understanding the unique properties of each source of hematopoietic cells helps to determine the anticipated performance for a given transplant recipient. Moreover, efforts to improve efficacy and reduce unwanted toxicities are currently under intense investigation. These include efforts to: (1) increase the dose of CD34+ hematopoietic progenitors in umbilical cord transplantation by combining two separate cord products^[23,24] or by performing *ex vivo* CD34+ stem cell expansions^[25]; (2) reduce the number of T-lymphocytes in HPC-A products by *ex vivo* T cell depletion or by *in vivo* administration of anti-thymocyte globulin (ATG)^[26,27]; and (3) improve engraftment kinetics without cGVHD of HPC-M products by administering G-CSF to the donor prior to marrow harvest^[28]. These advances illustrate the growing ability of practitioners to safely manipulate graft sources for maximum clinical benefit.

Identification of a donor

Finding a suitable bone marrow match is based on the HLA system, comprised of genes on chromosome 6. The major histocompatibility complex (MHC) includes two basic classes involved in antigen presentation and subsequent immune activation. MHC class I involves peptide presentation following intracellular digestion, while MHC class II presents extracellular antigens to host T lymphocytes. HLA-A, HLA-B, and HLA-C comprise class I, and HLA-DR, HLA-DQ and HLA-DP are class II. The proteins encoded by HLA define “self” to the host immune system. One set (haplotype) of HLA

genes are maternal and the other paternal. From this, any given sibling, excluding an identical twin, will have only a 25% chance of being fully HLA-matched. While matched related siblings remain the best source of donor material, this approach has several world-wide limitations including a significant reduction of family sizes (fertility rates of 1.5-2.0 per family across Europe and North America), a policy of one child families, as well as the health status and potential co-morbidities of older sibling donors. Moreover, lack of sibling donor availability is predicted to become a much greater issue due to reduced family size. It is estimated that the likelihood of finding a sibling match will decline from 53.7% in 2002, to 37.1% in 2009 and 16.6% in 2024^[29]. Nonetheless, investigation of family members using low-resolution serological typing (antigen level HLA-A,B, C and allele level HLA-DRB1) remains a standard initial evaluation approach.

If a suitable sibling-match cannot be found, a recipient in need of a transplant will require a search for an unrelated HLA-matched donor. Large national marrow donor programs will canvass for potential volunteers, perform HLA typing and maintain data in an ongoing registry. To be eligible volunteer donors must be in good general health and may be asked to undergo bone marrow harvesting under general anesthesia or daily administration of G-CSF (Filgrastim) followed by large volume leukapheresis. Stem cell donors must be screened to exclude active malignancies, transmissible infectious conditions (HIV, Hepatitis, HTLV-1, West Nile virus, Syphilis), hematological disorders (Sickle Cell Disease), and congenital bleeding disorders. A formal donor assessment will include a comprehensive questionnaire, complete medical history, and medical examination. Once screened and considered eligible, the most pertinent factor that predicts transplant success is donor age. Bone marrow recipients from younger donors (*i.e.*, < 30 years of age) demonstrate improved five-year overall and disease-free survival^[30,31]. This survival benefit appears to be the result of lower rates of GVHD when a younger donor is used. A retrospective analysis by the National Marrow Donor Program (NMDP) on over 6900 HLA-matched transplants performed between 1987 to 1999 was conducted to identify unique donor-specific features associated with transplant outcome^[30]. In this analysis use of a donor aged 18 to 30 years correlated with a lower cumulative incidence of grade III/IV acute GVHD ($P = 0.005$) and lower incidence of chronic GVHD at 2 years ($P = 0.02$). Other studies have suggested a higher rate of chronic GVHD in male recipients transplanted from a multiparous female donor or if mobilized progenitor cells are used^[19,30].

Identifying a potential unrelated donor BMT match generally requires high-resolution (HR) HLA typing of both recipient and donor. Studies suggest that employing a molecular (allele level) typing technique can reduce the incidence of severe GVHD and increase survival to levels similar to that seen with a matched-sibling donor^[32,33]. Algorithms exist that combine a serological preliminary

search (antigen level) with latter confirmatory molecular HR analysis (Figure 1). Efforts to decrease time and cost are dependent on clinical urgency and stability of the primary malignancy^[34]. Patients with common alleles and haplotypes have a higher probability of finding a match and generally require fewer pre-screened potential donors to be selected for HR typing (3-5 donors), while those with rare alleles and haplotypes may require as many as 10 or more. High resolution allele level matching for HLA-A, B, C and DRB1 (8/8 match) results in improved survival^[35-37]. Additional typing at HLA-DQB1 (10/10 match) and DPB1 loci, as well as DRB3, 4, 5 can be considered. Single loci mismatches at DQB1 and DPB1 appear to be tolerated better than at A, B, C or DRB1. Although it is necessary to minimize the number of allele mismatches, a single allele 7/8 or 9/10 alteration can still be considered. Single mismatch at B or C may be less of a concern than mismatches at A or DRB1 in patients undergoing HPC-M, but not HPC-A transplantation^[38,39]. Factors such as the recipient diagnosis, CMV status, age, and sex also need consideration^[30].

The ability to perform world-wide searches and identify volunteer donors has dramatically changed the international landscape of BMT. Superior matching as well as enhanced supportive care has improved the overall outcome of matched-unrelated donor (MUD) BMT such that results appear similar to matched-related donor BMT^[32,33,38]. Despite this, national registries face considerable challenges and limitations. Increasing allogeneic transplant indications puts greater pressure on the number of world-wide searches. Donor attrition and maintenance of a donor registry requires ongoing organized drives that reach out to younger volunteers and maintain a large potential pool of active registrants. This may vary from country to country; in the United States it is estimated that 1 out of 44 is registered, Canada 1 in 100, while Germany has a donor ratio of 1 to 17 (calculated # of registrants/total population). Moreover, within any given registry, certain ethnicities are often significantly underrepresented^[40,41]. Ultimately as many as 25% of all patients requiring BMT will never find a donor and either seek alternative treatments or palliation. This pressing unmet medical need has inspired advances in the use of alternative approaches that include the development of umbilical cord blood (UCB) hematopoietic cell transplant and haplo-identical BMT.

Advances in alternative donor hematopoietic cell transplantation

Umbilical cord blood contains hematopoietic progenitor cells that can be used for allogeneic transplant and immunological reconstitution^[42]. Graft composition is a critical element in predicting the short and long-term engraftment performance, rate of rejection, development of GVHD, or ability to provide GVL and prevention of relapse. Potential advantages of pre-stored UCB units include immediate access without donor attrition and an increased availability for ethnic minorities through

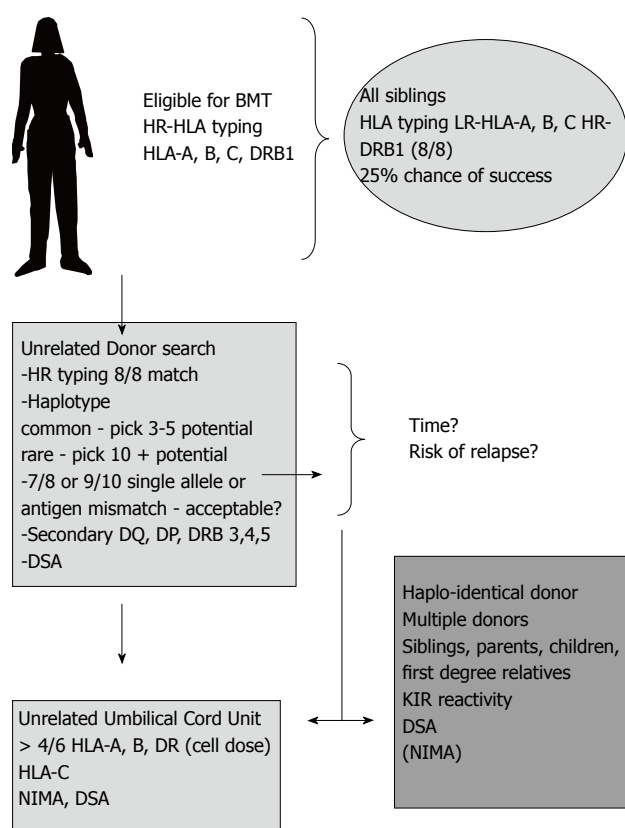


Figure 1 Example of a potential donor search algorithm that aims to expedite finding a suitable stem cell donor. A blend of high and low resolution approaches are employed. In certain situations the likelihood of finding an unrelated donor match is low due to rare alleles and haplotypes. Consideration of a haplo-identical BMT may be considered at an earlier stage. Abbreviations: HR: High resolution (allele level); LR: Low resolution (antigen level); NIMA: Non-inherited maternal antigen; KIR: Killer cell Ig-like receptors; DSA: Donor-specific antibodies; BMT: Bone marrow transplant.

the use of partially matched UCB products. Conversely, donors of unrelated UCB products are not available if future grafts or donor lymphocyte infusions are required. Numerous international cord banks operate under strict standards of testing, storage, and characterization. A minimum target of 3.0×10^7 nucleated cells per recipient weight per unit of cord blood is generally recommended, while flow cytometry-based measurement of CD34+ cells per recipient weight may be more predictive^[42,43]. Most cord products lack sufficient stem cells for most adults or large adolescent recipients. Efforts to accelerate cellular reconstitution following UCB BMT include combining two umbilical cord products (dUCBT) as well as *ex vivo* expansion^[23,25]. When evaluated post-transplant (d 100) typically only one (dominant) cord unit can be identified^[24]. In addition to the risk of graft failure, delayed immune reconstitution and increase in infections or relapse, there are theoretical risks that a potential hematological disease, not yet recognized in the newborn, will be transferred to the BMT recipient.

HLA typing of UCB products requires only low resolution serological testing for HLA-A, B, and molecular typing for DRB1 (6 alleles). Units with matches

of at least 4/6 are potentially acceptable^[44]. Mismatches at DRB1 and C may increase treatment-related mortality. Efforts to safely expand immature progenitors *ex vivo* without increasing differentiation to committed progenitors are moving towards early phase clinical trials^[25].

HAPLO-IDENTICAL BMT IN ADULT PATIENTS

A hematopoietic cell graft (HPC-M or HPC-A) obtained from a family donor that is mismatched at 3/6 loci (HLA-A, B, DRB1) remains a potential for patients who lack a fully matched sibling, 8/8 unrelated donor, or UCB product (patient size, haplotype). Advantages of such a haplo- approach include an expanded potential donor pool that may include parents, siblings, children, and first-degree relatives. Family donors of all ethnicities may be highly motivated, readily available, and willing to donate and be re-mobilized if required. These important theoretical advantages make a compelling case for further development of this approach, especially for patients with high-risk leukemia that are unlikely to maintain a remission during a prolonged unrelated search that may take months. Nonetheless, the ability to safely achieve sustained engraftment of highly HLA-disparate transplanted progenitor cells is a major challenge. Moreover, the risk of lethal aGVHD in the setting of 3/6 HLA disparity is an even greater risk. When GVHD occurs in a fully HLA matched 6/6 sibling transplant it is felt to be the result of minor histocompatibility antigen mismatches, however the nature and extent of immune activation with overt HLA host mismatches will differ in a haplo-identical BMT. Understanding the unique biology, as well as the immediate risk of fatal GVHD, has driven implementation of a variety of relatively effective novel approaches.

Graft-vs-host disease

The essential elements for the development of GVHD include the presence of immunologically competent cells in the hematopoietic cell graft, the presence of transplantation antigens in the host that have not been encountered by the donor, and an inability of the host to destroy the transplanted graft (Billingham's criteria)^[45,46]. Clinical GVHD is generally divided into acute (diffuse maculopapular rash, GI mucosal inflammation, and elevated liver function tests) and chronic GVHD. The diagnosis of chronic GVHD has been recently revised by the National Institutes of Health (NIH) consensus working group report^[47]. Clinical signs involve organs or sites that include skin, nails, mouth eyes, genitalia, GI tract, lung and the musculoskeletal system. Specific abnormalities may be either diagnostic (*i.e.*, poikiloderma, esophageal web, bronchiolitis obliterans) or distinctive (*i.e.*, xerostomia, myositis, keratoconjunctivitis sicca). Diagnosis requires at least one diagnostic category or the presence of least one distinctive manifestation confirmed by biopsy or specialized objective test. While classic acute GVHD occurs within 100 d after transplantation some patients continue

or relapse beyond this time point often during tapered withdrawal of immunosuppressive agents. Similarly, while classic chronic GVHD may occur in the absence of acute GVHD it may also be present along with acute GVHD (overlap syndrome).

The clinical development of the GVHD reaction is complex and involves sequential step-wise immune activation. The primary effector cells are T lymphocytes present in the graft. In the haplo-identical setting, donor T cells may attack disparate non-matched MHC molecules present on the majority of host cells. Donor T cells may be further activated by immunostimulatory cytokines released following tissue damage (gastrointestinal) that results from the preparative regimen^[48,49]. Additional activation may occur at the level of the vascular endothelium, co-stimulatory signals originating from antigen presenting cells, and following release of TNF α and other pro-inflammatory cytokines^[45,46,50]. T cell effector damage appears to be mediated by perforin-based host target cell lysis and Fas-mediated apoptosis^[51,52]. In a standard BMT, treatment options of established GVHD are limited; efforts to prevent GVHD with a combination of a calcineurin inhibitor and methotrexate have proven successful^[53,54]. In this setting about one half of patients will develop significant GVHD requiring additional immunosuppressive therapy (corticosteroids).

With a focus on T-lymphocytes, recent advances have been able to dissect the roles of donor T cell subsets present in the graft^[55]. These include naive T cells, memory T cells, and regulatory T cells. Naive T cells (CD45RA+/CD62L+) may include cells destined to be alloreactive to the host. Memory T cells (CD45RO+/CD62L+/-) include cells that provide protective antimicrobial immunity post-transplant. Regulatory T cells (CD4+/25+/FoxP3) appear to generally dampen other T cell responses and may be useful in attenuating clinical GVHD. Separation of naive T cells responsible for GVHD from donor T cell subsets responsible for GVT remain relatively elusive but are of obvious clinical importance. The ability to customize, harness, or control the fate of these T cell subsets to mitigate GVHD and retain GVT yet provide adequate post-transplant antiviral immunity has considerable clinical potential and is of heightened importance in the setting of high-risk leukemia patients undergoing a haplo-identical BMT. These challenges have led to important advances both in laboratory technologies and clinical application of newer agents.

APPROACHES TO HAPLO-IDENTICAL BMT

Given the potential for development of lethal aGVHD, efforts to entirely eliminate alloreactive donor T cells remain a critical first step. It has been suggested that as few as 3×10^4 T cells per recipient weight are capable of causing clinical GVHD^[56]. Both *in vivo* and *ex vivo* approaches have been developed (Figure 2). *Ex vivo* strate-

gies include immunomagnetic-based positive selection of CD34+ cells or CD3/19 depletion with preservation of NK and gamma-delta T cells^[57,58]. *In vivo* T cell depletion may be accomplished by early administration of post-transplant cyclophosphamide or by aggressive multi-agent anti-GVHD therapies that include anti-thymocyte globulin (ATG), G-CSF, and triple GVHD prophylaxis as well as recent studies using rapamycin (Table 1).

Aversa *et al.*^[59-61] in Perugia, Italy, described a series of incremental approaches to haplo-identical BMT from 1993 to 2006. A series of step-wise approaches focused on patients with high-risk acute myeloid (AML) and lymphoblastic leukemia (ALL). The investigators examined: (1) stem cell sources; (2) graft processing technologies; (3) conditioning regimens; and (4) post-transplant administration of G-CSF. Collectively, the investigative team were able to obtain high doses of CD34+ donor progenitor cells ($> 10 \times 10^6$ /kg, recipient weight) that led to a remarkable rate of successful engraftment. Aversa *et al.*^[61] and Reisner *et al.*^[62] had previously described the ability of purified CD34+ cells to block the action of residual cytotoxic T lymphocytes leading to tolerance^[61]. Moreover when transplanted in very high numbers this “veto effect” could overcome clinical graft rejection by residual host T cells. At the same time newer technologies including CD34-positive selection and use of mobilization agents and peripheral blood progenitor cell collections led to “mega dose” grafts that consistently demonstrated remarkable engraftment of neutrophils and platelets with a low level of graft rejections in a large number of patients. *Ex vivo* T cell depletion was highly effective ($< 0.5 \times 10^5$ CD3+ T cells/kg, recipient wt.) with little or no evidence of significant clinical GVHD even in the absence of prophylaxis. Impressive event-free survival rates of up to 48% were noted in AML patients in first complete remission. Higher rates of relapse were seen in ALL patients. Despite these notable clinical and technological advances, prolonged immune reconstitution (CD4+ T lymphocytes) was problematic and non-relapse mortality in the range of 41%. Infections were mostly cytomegalovirus and fungal in origin. Nonetheless, the ability to use rigorous positive selection of “megadose” CD34+ products and achieve timely multi-lineage engraftment, minimal GVHD, and durable survival in some patients became an important clinical platform for future trials.

Following the work of the Perugia group, Roy and colleagues in Montreal devised a novel strategy to safely “add-back” modified donor lymphocytes to hasten immune recovery and provide anti-viral immunity^[56]. This strategy involved *ex vivo* photo-based depletion of alloreactive T cells derived from a donor-recipient mixed lymphocyte reaction (MLR). Working with a highly potent dibromomorphodamine photosensitizing compound (TH9402) the team demonstrated accumulation of drug in certain cell types including cancer cells and alloreactive T cells. These cells could then be lysed following exposure to a specific wavelength of visible light (514 nm). The mechanism of *ex vivo* cellular lysis was shown

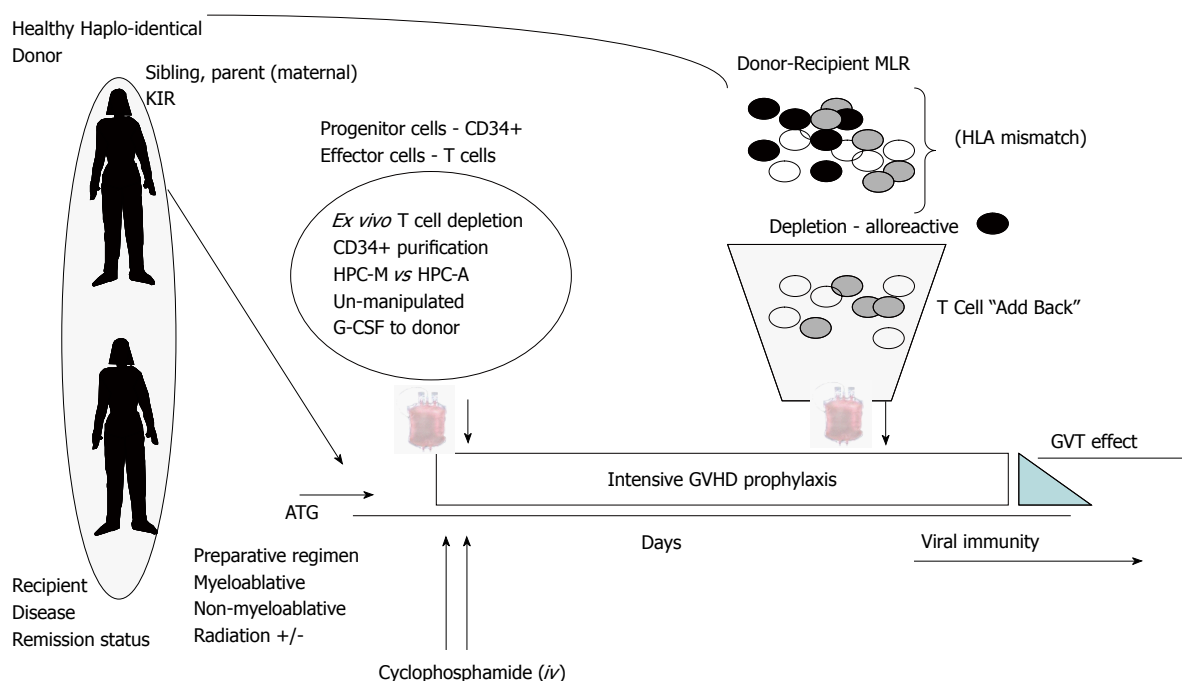


Figure 2 Current approaches to Haplo-identical bone marrow transplant. Recent novel strategies include issues of donor selection, *ex vivo* complete or partial T cell depletion of HPC-M or HPC-A grafts and the use of anti-thymocyte globulin. Additional strategies include *in vivo* administration of “high dose” cyclophosphamide and intensive multi-agent GVHD prophylaxis. Finally, in an effort to overcome severe prolonged immune suppression the addition of donor T lymphocytes that have been purged of alloreactive T cells may be of benefit. Abbreviations: DSA: Donor-specific antibodies; MLR: Mixed lymphocyte reaction; HLA: Human leukocyte antigen; GVT: Graft-versus tumor; KIR: Killer cell Ig-like receptors; GVHD: Graft-vs-host disease; ATG: Anti-thymocyte globulin.

to involve reactive oxygen species. During an MLR reaction in a haplo-identical setting, donor T lymphocytes respond to donor immune cells and take up TH9402. Non-reactive resting T cells capable of anti-viral immunity do not accumulate the agent and are retained in the infused “add-back” lymphocyte product (ATIR). The characterization of retained cells following photo-depletion differed when HLA-matched or haplo-matched pairs were tested. These experiments demonstrated preservation of CD8+ naïve and effector cells in the matched situation and preservation of naïve and central memory cells of both CD4+ and CD8+ phenotypes when haplo-identical pairs were studied. When tested in a clinical trial, administration of relatively high doses of photo-depleted T cells post-transplant did not increase grade III-IV GVHD. Patients receiving higher doses of cells demonstrated decreased rates of infection and improved overall survival (47.4%) at a median of 4 years^[56]. These encouraging results are now being studied in a multi-institutional phase II setting. A similar approach using anti-CD25 immunotoxin MLR-based purging has been studied in several transplant settings. Similar results indicating successful removal of alloreactive GVHD cells and maintenance of virus-specific T cells have been demonstrated^[63].

Ex vivo graft engineering to eliminate GVHD, yet retain GVT and avoid life-threatening infectious complications, is promising but remains investigational, complex, and costly. Kasamon *et al.*^[64], Fuchs *et al.*^[65], Luznik *et al.*^[66], Brunstein *et al.*^[67] in Baltimore have developed a similar *in vivo* platform using post-transplant cyclophosphamide administered shortly following infusion of an un-

manipulated haplo-identical mismatched marrow graft. Similar to the *ex vivo* MLR approach, this strategy exploits the concept of selective depletion of alloreactive immune cells with preservation of resting non-alloreactive cells. Within a period of 24-48 h post-infusion of mismatched HPC-M, alloreactive T cells will rapidly encounter stimulatory host cells. T cells capable of anti-viral immunity will not respond at this stage. Cyclophosphamide will selectively eliminate reactive cells and limit GVHD. Indeed this approach has proven highly feasible in over 200 patients with a range of advanced hematological malignancies. Using a non-myeloablative regimen engraftment was timely (neutrophils 15 d and platelets 24 d) with graft rejections in the range of 13% (autologous marrow recovery). Rates of acute and chronic GVHD were less than 30%. Post-transplant GVHD prophylaxis included tacrolimus and mycophenolate mofetil. Relapse rates were relatively high in a broad range of advanced stage hematological malignancies and overall survival was in the range of 40%-45%.

A recent publication from Di Bartolomero and colleagues demonstrates the feasibility of performing haplo-identical BMT using an un-manipulated G-CSF-primed approach in patients with high-risk malignancy^[68]. In this series, GVHD prophylaxis was intensive and included ATG, cyclosporine, methotrexate, mycophenolate mofetil, and basiliximab (anti-CD25). Engraftment of neutrophils (21 d) and platelets (28 d) were reasonable. The cumulative incidence of serious acute GVHD was 24% and at 2 years extensive cGVHD was only 6%. The overall 3-year overall survival ranged from 33%-54% (high-risk and

Table 1 Summary of clinical approaches to haplo-identical bone marrow transplant

Ref.	n	Preparative regimen	T-cell depletion/engraftment	GVHD prophylaxis	Acute GVHD	Chronic GVHD	TRM	OS
Perugia, Aversa <i>et al</i> ^[59-61]	255	TBI +/- MA ATG	Yes 84%-96%	none	17%	< 5%	41%	47% ²
Peking, GIAC ^[69,70]	250	MA ATG	No G-BM + G-PB 100%	CsA, MTX MMF	45%	31%	12%-48%	56%-71% AML ¹ 25%-60% ALL ¹
Montreal Bastien <i>et al</i> ^[56]	19	TBI +/- MA ATG	Yes T cell "Add Back" 100%	none	20%	25%	15%	47%
Baltimore Studies ^[64-67]	210	NMA	No 87%	Tacro, MMF PTCyclo	27%	13%	15%	40%-45%
Di Bartolomeo <i>et al</i> ^[68]	88	MA 80% NMA 20% ATG	No G-BM 91%	CsA, MTX MMF Basilixumab	24%	6%	36%	33%-54% ¹

¹Survival range including standard and high-risk groups; ²Survival for patients in complete remission. A variety of approaches have been studied that compare MA-myeloablative to NMA-non myeloablative, T cell depletion of graft, and GVHD prophylaxis. Engraftment rates are high, and GVHD can be attenuated through T cell depletion of the graft or by intensive anti-GVHD prophylaxis, including ATG. GVHD: Graft-vs-host disease; TRM: Treatment related mortality; OS: Overall survival; TBI: Total body irradiation; ATG: Anti-thymocyte globulin; CsA: Cyclosporine A; MTX: Methotrexate; MMF: Mycophenolate mofetil; PTCyclo: Post transplant cyclophosphamide; AML: Acute myeloid leukemia; ALL: Acute lymphoblastic leukemia; Tacro: Tacrolimus.

standard risk). A large series published by Huang *et al*^[69] and Wu *et al*^[70] also described a similar approach to haplo-identical transplantation using un-manipulated cell grafts with escalated post-transplant immunosuppression. The Peking "GIAC" approach was studied in 250 patients and highlighted the effects of administration of G-CSF to the donor for collection of combined HPC-M and HPC-A grafts, intense immunosuppression (cyclosporine, methotrexate, mycophenolate mofetil and G-CSF), and ATG. Engraftment was rapid with neutrophils and platelets engrafting at 12 and 15 d, respectively. Grade III-IV acute GVHD was 45% and any cGVHD was 31%. Relapse in standard risk AML and ALL was 19.4 and 21.2%, in high-risk AML and ALL was 29.4% and 50.8%. Treatment related mortality ranged from 11.9% to 48.5% and was dependent on risk and disease type. Ultimately investigators suggested these results were comparable to results obtained using an HLA-matched sibling donor^[69,70]. In this study investigators administered G-CSF to both the donor and recipient post-transplant. Administration of G-CSF following both autologous and allogeneic BMT has been primarily used to reduce the duration of neutropenia and related complications^[71]. In both settings neutrophil recovery is faster resulting in shorter hospitalization for autologous but not allogeneic BMT. Use of G-CSF in allogeneic transplants is otherwise considered safe; however two retrospective studies have raised concern over a possible increase in GVHD^[72,73]. Still others have suggested that pre-treatment of T-lymphocytes with G-CSF results in an anti-inflammatory (type-2) cytokine profile that attenuates experimental GVHD severity^[74].

Towards the future, Fowler *et al*^[75] at the NIH have recently published a compelling phase 2 study using rapamycin-resistant T cells (2.5×10^7 cells/kg, recipient weight) infused 14 d after hematopoietic cell transplantation for treatment of a variety of refractory hematological malignancies. While that study, on 40 patients of a

wide range of ages (18 of whom remained in sustained complete remission up to 84 mo of follow-up), was done in the context of 6/6 HLA-matched sibling donors, it is conceivable that such an approach using their low-intensity conditioning regimen and this specific immune effector product could be adapted to the haplo-transplant setting. Donor lymphocytes in that study demonstrated a consistent and balanced Th1/Th2 profile; incidence probabilities of aGVHD were 20% and 40% at 100 and 180 d post-transplant, respectively.

The studies described above illustrate a broad range of current and future strategies to advance the field of haplo-identical BMT. *Ex vivo* T cell depletion, selective T cell "add back", *in vivo* T cell depletion, and use of intensive GVHD prophylaxis are being actively improved. Feasibility has now been established with reasonable overall survival in a population of high-risk advanced malignancies who lack a traditional matched donor. Previous limitations of graft rejection and unacceptable rates of serious GVHD have been largely overcome. Efforts to enhance GVT and prevent life-threatening viral and fungal infections remain a current focus. Separation and exploitation of the linkage between GVT and GVHD remain a critical next step. It has also been suggested that alloreactive natural killer cells (NK) may be protective against myeloid leukemia relapse. Given that a patient may have several potential haplo-identical donors, it may be possible to choose a donor with heightened NK alloreactivity^[76,77]. In addition, administration of donor-derived regulatory T cells may attenuate GVHD yet facilitate GVT effectors^[78]. Finally a retrospective analysis of 118 acute leukemia patients undergoing haplo-identical BMT using a parent as a donor suggested improved 5-year EFS when the mother was the donor as compared to the father^[79]. When sibling (non-parent) haplo-identical donors were evaluated, the gender of the donor had no effect on outcome. The presence of donor-specific anti-

bodies (DSA) in the recipient may be evaluated to reduce the risk of graft failure^[80].

CONCLUSION

At present, allogeneic BMT remains the only chance of cure for adults and children with advanced hematological disease. Transplant indications and eligibility are expanding. Outcomes are improving with reduced-intensity conditioning, HR molecular typing of unrelated donors as well as improved general supportive care measures. Most, but not all patients in need of this life-saving procedure will have a suitable sibling, matched unrelated, or UCB donor graft. Haplo-identical transplantation offers hope to those high-risk patients who face limited treatment options. Despite ethnicity, an expanded pool of motivated donors could be immediately available. A wide range of strategies are currently being explored. Previous serious pitfalls, including graft rejection, severe GVHD, and prolonged immune suppression are becoming less problematic as the science of the field advances. Novel experimental utilization of T regulatory cells, alloreactive NK cells, and other T cell subsets (T-Rapa cells, for example) hold great promise in this rapidly emerging and much needed field.

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An overview of the role of cancer stem cells in spine tumors with a special focus on chordoma

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Abstract

Primary malignant tumors of the spine are relatively rare, less than 5% of all spinal column tumors. However, these lesions are often among the most difficult to treat and encompass challenging pathologies such as chordoma and a variety of invasive sarcomas. The mechanisms of tumor recurrence after surgical intervention, as well as resistance to radiation and chemotherapy, remain a pervasive and costly problem. Recent evidence has emerged supporting the hypothesis that solid tumors contain a sub-population of cancer cells that possess characteristics normally associated with stem cells. Particularly, the potential for long-term proliferation appears to be restricted to subpopulations of cancer stem cells (CSCs) functionally defined by their capacity to self-renew and give rise to differentiated cells that phenotypically recapitulate the original tumor, thereby causing relapse and patient death. These cancer stem cells present a unique opportunity to better understand the biology of solid tumors in general, as well as targets for future therapeutics. The general objective of the current study is to discuss the fundamental concepts for understanding the role of CSCs with respect to chemoresistance, radioresistance, special cell surface markers, cancer recurrence and metastasis in

tumors of the osseous spine. This discussion is followed by a specific review of what is known about the role of CSCs in chordoma, the most common primary malignant osseous tumor of the spine.

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Key words: Spine tumor; Chordoma; Cancer stem cell; Stem cell marker; Chemoresistance

Core tip: Primary malignant tumors of the spine are relatively rare, less than 5% of all spinal column tumors. However, these lesions are often among the most difficult to treat and encompass challenging pathologies such as chordoma and a variety of invasive sarcomas. The mechanisms of tumor recurrence after surgical intervention, as well as resistance to radiation and chemotherapy, remain a pervasive and costly problem. Recent evidence has emerged supporting the hypothesis that solid tumors contain a sub-population of cancer cells that possess characteristics normally associated with stem cells. These cancer stem cells could be targets for future therapeutics.

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INTRODUCTION

The concept of cancer stem cells that is sufficient for the initiation and maintenance of tumors and underlies treatment resistance has received significant attention in many areas of oncology^[1,2]. Recent studies have provided evidence that cancer stem cells (CSCs) exist in a variety of human tumors, including brain and thyroid tumors, melanoma, breast cancer, prostate tumors and

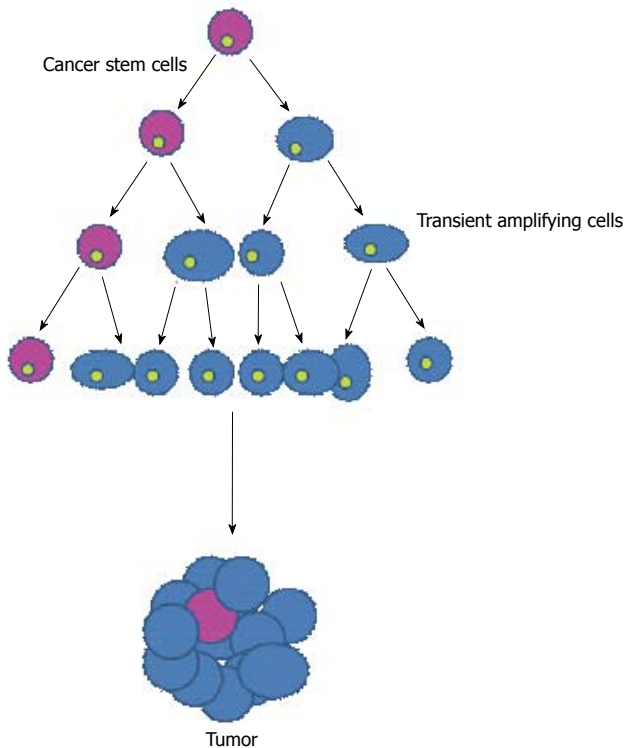


Figure 1 Division model of cancer stem cells. These cells are a small sub-population of cancer cells (< 1%).

gastroenterological cancers^[3,4]. It should be noted that CSCs from different cancers, leukemia and solid tumors might be different in their phenotypical properties and self-renewal pathways; thus, these cells will need to be defined for each disease^[5]. Cancer stem cells are typically recognized by virtue of the expression of cell surface markers. CD133, nestin and recently CD90 have been considered the putative markers of CSCs in malignant cancers, including glioblastoma multiform (GBM). Unlike non-tumor stem cells, tumor stem cells lack the normal mechanisms that regulate proliferation and differentiation, resulting in uncontrolled production and incomplete differentiation of cancer cells^[6]. These cells are considered to be tumorigenic in contrast to the bulk of cancer cells, which are thought to be non-tumorigenic and also responsible for progression, metastasis and relapse after treatments^[5]. The presence of such cells has also been demonstrated in spine tumors^[7]. Spinal tumors are uncommon lesions and affect only a minority of the population but can cause significant morbidity in terms of limb dysfunction and mortality as well^[8,9]. The current theory implies that stem cells may play an important role in tumors of the osseous spine. In support of this hypothesis, there is increasing evidence pointing to the existence of a subset of tumor cells with high tumorigenic potential in many spine tumors^[7]. It has been recently investigated that chordoma cells and cancer stem cells exhibit similar characteristics, including self-renewal, differentiation, metastasis, therapeutic resistance and recurrence of cancer^[10,11]. In studies of chordoma stem cells, the most promising findings concern how stem cells

may offer a reasonable explanation of why this tumor is so difficult to eradicate and suggest how new therapies might be targeted. In this review, we present current evidence regarding the role of cancer stem cells in spine tumors, highlighting new insights and unresolved issues in the identification of this elusive population in chordoma.

THEORY OF CANCER STEM CELLS

The cancer stem cell theory was first postulated about 50 years ago, whereas it is only in the last 10 years that advances in stem cell biology have provided direct evidence supporting this hypothesis^[11,12]. It has been proposed that tumors are organized by a hierarchy of heterogeneous cell populations with different proliferation potentials in which the capability to initiate tumor formation and promote tumor growth exclusively resides in a small subpopulation of cancer cells (< 1%) termed cancer stem cells or tumor-initiating cells (Figure 1)^[13,14]. In general, the most important criteria to define CSCs are cancer-initiating ability on orthotopic implantation, genetic alterations, aberrant differentiation properties, capacity to generate non-tumorigenic end cells and multi lineage differentiation ability^[15,16]. Numerous investigations have suggested that the introduction of key mutations known to cause aberrations in key signaling pathways can transform normal stem cells into tumor-initiating cells^[7,17]. Nevertheless, other experimental evidence has manifested that the introduction of certain oncogenes can transform more differentiated cell types into cancer cells that result in tumors^[18]. Moreover, current studies have considered the role of hypoxia in cancer by demonstrating different responses to hypoxia between heterogenic subpopulations within the tumor^[19,20]. This sensitivity of tumor structure to oxygen status is driven by the hypoxia inducible factor (HIF) proteins in the CSCs population. The HIFs have been determined to be expressed in the cancer stem cell, possibly promoting the stem-like phenotype and driving tumor growth. Notch signaling also has a significant role in the formation of numerous malignancies; therefore, hypoxia may promote Notch signaling in cancer stem cells and maintain them in an undifferentiated state^[21]. Since the presence of cancer-initiating cells within a cancerous mass greatly impairs long-term survival after therapy, it is imperative to understand the characteristics of these cells, including their specific markers and the molecular mechanism for their resistance to conventional therapies^[22].

CANCER STEM CELL MARKERS

Certain barriers complicate the characterization and isolation of CSCs within tumor bulk, particularly chordoma. Among these obstacles are the facts that stem cells are relatively rare and lack a unique morphology that is easily distinguished from its progeny *in vivo*^[23,24]. In an effort to identify specific markers to enrich this population, many groups have used assays based on cell-surface proteins

Table 1 Cell surface markers and transcription factors potentially associated with cancer stem cells

Tumor type	Cell surface markers and transcription factors
Chondrosarcoma	SOX9
Osteosarcoma	CD133, CD117, CD44, CD105, ABCG2, CXCR4, ICAM-1, STRO-1, OCT 3/4, Nanog, STAT3, SOX2
Ewing's sarcoma	CD133/Prominin-1, OCT4, SOX2, Nanog
Multiple myeloma	CD19, CD20, CD27 ⁺ , CD138 ⁺ , SOX2
Giant cell tumor	CD105, CD37, CD166, CD117, CD113, CD44, CD73, CD166, FGF-R3
Chordoma	CD133, CD15, OCT4, klf4, C-myc, SOX2, SSEA-1, Nanog, Brachyury

ICAM-1: Intercellular adhesion molecule 1; STAT3: Signal transducer and activator of transcription 3.

such as CD20, CD24, CD34, CD90, CD44, CD133, stage-specific embryonic antigen 1 (SSEA-1), nestin, integrin $\alpha 6$, epithelial-specific antigen, efflux activity (side-population cells) and more recently, label-retention (Table 1)^[25-27]. It is highly noticeable that the expression of CSC surface markers is tissue type-specific, even tumor subtype-specific^[28]. In addition, aldehyde dehydrogenase (ALDH1) is a marker used initially for the enrichment of normal stem cells and has recently been used to identify CSC in colon, breast and lung cancers^[14,29,30]. Noticeably, one of the biggest challenges of using ALDH as a marker of CSC is the arbitrary nature of the 2% or 3% cut-off of cells with the highest and lowest ALDH activity^[31]. Expression of stem cell genes such as (sex determining region Y)-box 2 (SOX2), octamer-binding transcription factor 4 (OCT4) and NANOG is also used as a marker of tumor-initiating cells. These genes are found in embryonic stem cells and appear to be essential for maintenance of an undifferentiated state, pluripotency and self-renewal^[32].

ROLE OF CSCS IN CANCER METASTASIS

Metastasis, frequently a final and fatal step in the progression of solid malignancies, encompasses several fundamental biological events: cancer initiation, breach of the basement membrane barrier, vascularization, invasion, detachment, embolization, survival in the circulation, arrest, extravasation, evasion of the host defense and progressive growth^[33,34].

According to cancer stem cell theory, CSCs are favorable seeds of metastasis. Brabletz *et al.*^[35] first proposed the hypothesis of migrating CSCs, which possess both an element of stemness and mobility^[36]. Evidence has been offered that epithelial-to-mesenchymal transition (EMT) represents a crucial step towards invasiveness and metastasis, and is strongly associated with poor clinical outcome in a variety of tumors^[37]. Importantly, EMT endows human mammary epithelial cells with CSCs-like properties which are characterized by their CD44^{high}/CD-24^{low} phenotype through up-regulating Mena, member of the Ena/VASP family which plays a significant role in tumor metastasis^[36,38]. In fact, induction of EMT in immortalized human mammary epithelial cells led to the

expression of stem cell markers, gain of mesenchymal characteristics, and phenotypes associated with CSCs^[39]. These observations established a direct link between EMT and the acquirement of properties of migratory stem cells. Moreover, it has been found that up regulation of some micro RNAs and down regulation or absence of some of them have been observed in metastatic CSCs. For instance, over-expression of miR-30 in breast CSCs xenograft reduced lung metastasis, whereas blocking miR-30 expression increased metastasis *in vivo*^[40]. Target genes with an established role in tumor cell invasion, migration and other steps in the metastatic process have been identified for many miRNAs, including matrix metalloproteinases, human epidermal growth factor receptors, bone morphogenetic proteins, Phosphatase and tensin homolog (PTEN), ZEB1, ZEB2 or E-cadherin^[41]. Additional evidence demonstrated that a subpopulation of migrating CD133⁺CXCR4⁺ cancer stem cells is essential for tumor metastasis. Hermann *et al.*^[42] have also shown that CD133⁺CXCR4⁺ subsets determined the migrating phenotype of pancreatic cancer, although both CD133⁺CXCR4⁺ and CD133⁺CXCR4⁻ pancreatic cancer stem cells were able to form pancreatic cancer when transplanted into athymic mice^[33]. In addition, the CXCR4/stromal cell-derived factor-1 axis could mediate metastasis of the distinct subpopulation of CSCs. On the basis of the provided histological evidence for the existence of CXCR4⁺ CSC in the invasive front of human tumor specimens, it has been hypothesized that a specific subset of CXCR4⁺ CD133⁺ CSC plays an important role in tumor metastasis^[43].

MECHANISMS OF CSCS RESISTANCE TO THERAPY

Chemoresistance

It has become increasingly evident that CSCs are uniquely resistant to standard chemotherapeutic agents compared with their non-stem cell. There are several criteria attributed to the role of CSCs in chemoresistance: (1) entrance into a long-term latent state; (2) CSCs activate DNA damage response; and (3) dormant CSCs are concealed in vascular niche with lower concentrations of reactive oxygen species.

CSCs highly express multi-drug resistance due to up-regulation of cellular efflux pumps^[5,44,45]. Although there is a growing body of literature considering the role of CSCs in the chemoresistance for different malignancies, including breast, lung, GBM, head and neck, and pancreatic cancer, there is a paucity of literature focusing specifically on this subject for spine tumors^[46-50]. For instance, Jiang *et al.*^[51] investigated the expression of CD133 in primary Ewing's tumors and cell lines to see if there was a correlation between CD133 expression and chemoresistance. Similarly, functional studies of CD133⁺ and CD133⁻ fractions derived from Ewing's sarcoma family tumors (ESFT) cell lines demonstrated that, although CD133⁺ cells could be isolated from all ESFT cell lines,

only CD133⁺ cells isolated from the STA-ET-8.2 cell line displayed evidence of stem cell characteristics and chemoresistance^[51]. Noticeably, CD133⁺ glioblastoma cell chemoresistance may be caused by an over expression of genes important in drug resistance, such as BCRP1, DNA-mismatch repair (MGMT), and inhibition to cell apoptosis [FLICE-like inhibitory protein (FLIP), B-cell lymphoma 2, B-cell lymphoma-extra large]^[52,53]. Bleau *et al.*^[54] reported that down-regulation of autophagy-related proteins play a significant role in the resistance of CD133 glioma cells to temozolomide. The role of CSCs in chemoresistance has also been investigated in tumors (most notably breast and lung cancer) that commonly metastasize to the spine^[55,56]. For example, it is well established that tumorigenic breast cancer cells expressing high levels of CD44 and low or undetectable levels of CD24 may be resistant to standard dose chemotherapy (docetaxel, doxorubicin, cyclophosphamide and trastuzumab) and therefore responsible for cancer relapse^[28,57]. Moreover, several studies implicate adenosine triphosphate-binding cassette super family as one type of multi drug resistant proteins, which can pump chemotherapy drugs out of the cell and lead to chemoresistance^[33,58]. ABCG2 is one of the most important members of this family and represents a purified marker of cancer stem cell transporters and its substrates include many commonly used drugs in cancer chemotherapy^[59]. Despite these finding demonstrating the relationship between cancer stem cells and chemoresistance, further studies are essential to provide direct evidence supporting the existence of chemotherapy-resistant CSCs in order to develop alternative strategy for targeted therapy.

Radioresistance

Radiation therapy is crucial in the treatment of the majority of spine tumors, whether in combination with chemotherapy and/or surgical resection^[60]. There is considerable evidence to suggest the role of CSCs in the resistance of a wide panel of tumors to radiation therapy^[61]. Diehn *et al.*^[62] suggested that human and mouse cancer stem cells contained lower levels of reactive oxygen species (ROS) compared with their non-tumorigenic progeny. Thus, the heterogeneity of (ROS) levels in a subsets of CSCs may contribute to their radioresistance^[33,63]. Based on this evidence, it is possible that the poor tumor control associated with chordoma may be due to hypoxic effects and/or cancer stem cells which are resistant to ionizing radiation and chemical agents^[64,65]. Recent clinical data suggest that the combination with topoisomerase II inhibitor razoxane improve the effectiveness of chordoma radiotherapy^[64,66]. Bao *et al.*^[67] have recently shown that CD133⁺ positive glioma cells survive ionizing radiation by preferentially activating DNA damage response and also inhibiting the cell cycle checkpoint kinases Ch1 and Ch2 sensitized the resistant cells to radiotherapy^[68]. As a result, CD133⁺ positive cell fraction seems to be responsible for acquiring radioresistance and presumably is one of the main sources of

tumor recurrence after radiotherapy^[69]. More importantly, in CD133 positive glioma stem cells, the expression of the autophagy-related proteins LC3, ATG5 and ATG12 was increased as a response to radiation^[70]. Noticeably, other stem cell mechanisms, including notch, hedgehog, PTEN and epidermal growth factor receptor (EGFR), may also have a role in CSCs radioresistance^[71-75].

ROLE OF CSCS IN PRIMARY SPINE TUMORS

Chondrosarcoma

Chondrosarcoma is the most common primary malignant bone tumor of chondrogenic origin, typically occurring in the fourth and fifth decades of life^[76,77]. Molecular lesions and aberrant oncogene expression in the p16INK4a/pRb pathway may be characteristic of this tumor^[78]. Chondrosarcoma is most commonly observed in the petrous portion of the temporal bone, as well as in petro-occipital, spheno-occipital, and sphenopetrosal synchondrosis areas^[79]. The treatment of chondrosarcoma is usually limited to wide-margin surgical resection and conventional radiation therapy and chemotherapy have not been proven to be effective^[80,81]. Recent studies demonstrated that resistance to chemotherapy may be attributed to multidrug resistance-1 and to P-glycoprotein expression^[82]. Based on histopathology, chondrosarcoma is able to divide into primary subtypes, conventional, dedifferentiated, clear cell and mesenchymal, and only 12% of all skull base chondrosarcomas show mesenchymal characteristics^[83,84]. Importantly, chondrosarcomas with mesenchymal features are associated with an approximately tenfold increase in 5-year mortality compared to those with conventional histopathological traits^[79]. The novel findings suggest that this tumor differentiates along the chondrocytic lineage and normal differentiation of mesenchymal stem cells into chondrocytes is accompanied by sequential production of characteristic extracellular matrix proteins^[85,86]. Transcription factor SOX9 is also involved in the activation of chondrogenesis from mesenchymal chondroprogenitor cells in an adult organism during fracture repair. Furthermore, increased expression of SOX9 and the prechondrogenic splice variant type IIA collagen in chondrosarcomas provides further evidence that these tumors may originate from a multipotent stem cell committed to differentiation along the chondrogenic pathway^[7,87].

Osteosarcoma

Osteosarcoma, the most common primary malignant tumor of bone, is among a group of mesenchymal tumors identified by clinical, histological and molecular heterogeneity, and karyotypes with a high degree of aneuploidy^[88]. It is an aggressive bone tumor of osteoblastic origin which primarily affects children and young adults^[89,90]. Despite modest advances in surgical resection techniques and chemotherapy regimens, long-term survival rates for osteosarcoma have had no significant improvement,

stable at approximately 65%, attributable to the aggressive malignant potential and early metastasis^[91]. Lesions frequently occur in the metaphyses of long bones, which express the major pool of mesenchymal stem cells^[92]. Recent studies suggest the existence of stem-like cells in primary osteosarcomas and cell lines derived from human osteosarcoma in a subpopulation of cells capable of self-renewal^[14,93]. These cells have been detected in spherical clones under anchorage-independent, serum-starved culture conditions as side population cells based on efflux of Hoechst 33342 dye or using cancer stem cell markers^[94,95]. The identification of CSCs in human osteosarcoma has been more difficult than in tumors originating from other types of tissues^[96,97]. Because of differences in mesenchymal origin, the markers that have been characterized and developed for epithelial, hematological and neural cancers are not necessarily useful for isolation of CSCs from human osteosarcoma^[14]. There is preliminary evidence that osteosarcoma stem cells express the mesenchymal stem cell markers CD133, CD117, ABCG2, CXCR4, ICAM-1 and nucleostemin, as well as key marker genes Oct3/4, Nanog, Signal transducer and activator of transcription 3 (Stat3) and Sox2^[91,98]. It has been also reported that osteosarcoma stem cells express more anti-apoptotic proteins, including Bcl-2, FLIP, apoptosis inhibitor XIAP, IAP-1, IAP-2 and survivin than normal osteosarcoma cells^[99]. In addition, Wang *et al.*^[90] have shown that CSCs could be identified in the established human osteosarcoma OS99-1 cell line based on high ALDH activity^[90]. More importantly, cells with elevated ALDH activity preferentially represented the stem cell markers Nanog, Oct3/4 and Sox-2 compared with cells with low ALDH1 activity^[7]. Tang *et al.*^[100] and Mohseny *et al.*^[101] have reported that mesenchymal stem cells or osteoprogenitor cells, because of disruption in the osteoblast differentiation pathway, develop osteosarcoma. Different pathways, including Hedgehog signaling, notch signaling pathway, Wnt/ β -catenin and Mitogen-activated protein kinases, may also be involved in the determination of the fate of osteosarcoma stem cells^[11,102,103].

Ewing's sarcoma

The ESFT is the second most frequent solid bone and soft tissue malignancy of childhood and young adults^[104,105]. Genetically, this tumor is associated with specific chromosomal translocations that result in the formation of fusion genes encoding proteins composed of the transactivation domain of EWS and the DNA binding domain of one of five ETS family transcription factors, including ERG, ETV1, FLI1, FEV and ETV4^[106]. The EWS-FLI1 fusion protein is a favorable candidate for targeted therapy as its expression is limited to tumor cells and is critical for initiation and maintenance of the tumor^[107]. Further evidence suggests that the regulation of EWS-FLI1 in hypoxic environments may occur at the posttranscriptional level, which is supported by the observation that HIF-1 α -activated genes, such as vascular endothelial growth factor, Aldolase-C, Glucose transporter-1, CA9 and Insulin-like growth factor-binding

protein 3, were increased under hypoxia^[20]. Interestingly, the histological features of Ewing's sarcoma suggest that this tumor may arise from a neural crest stem cell exhibiting mesenchymal features or from a mesenchymal stem cell that is neural derived^[108]. It has been also investigated that human and mouse bone marrow (BM) mesenchymal stem cells expressing EWS-Flt-1, when engrafted into NOD/SCID mice, induce a malignancy with similar pathological characteristics to Ewing's sarcoma^[105,109]. Other evidence implicates a significant role of cancer stem cells in Ewing's sarcoma pathogenesis. For instance, it has been reported that the cell surface glycoprotein CD133 is a marker of tumor-initiating cells in ESFT. In fact, functional studies of CD133⁺ and CD133⁻ fractions derived from ESFT cell lines provide new insight into the biology of these tumors and constitutes the first identification of CSCs in a human sarcoma^[110]. Notably, although CD133⁺ cells could be isolated from all ESFT cell lines, only CD133⁺ cells isolated from the STA-ET-8.2 cell line exhibited evidence of stem cell features and chemoresistance^[51]. Recently, Suvà *et al.*^[111] demonstrated that the cell surface marker CD133/Prominin-1, which has been associated with CSCs in glioblastoma, can also be used to isolate a subpopulation of Ewing's sarcoma cells. Awad *et al.*^[107] reported that the subpopulation of ESFT cells that express the highest levels of ALDH have some characteristics of stem cells, including the capacity to generate a heterogeneous population, *in vitro* clonogenic activity and *in vivo* tumorigenic activity. The current standard treatment for ESFT is chemotherapy with intercalated loco regional management with surgery for patients with localized disease^[112]. Accumulating data demonstrated that Ewing's sarcoma stem cells are resistant to two of the standard agents used to treat ESFT, doxorubicin and etoposide, suggesting that these cells have relatively higher transport protein activity than the bulk population, and chemoresistance is reversed by verapamil, an inhibitor of ABC transport proteins^[107].

Multiple myeloma

Multiple myeloma (MM) is a clonal B-cell malignancy characterized by clonal expansion of malignant bone marrow cells engaged in the production of a unique monoclonal immunoglobulin^[113]. This tumor has a reported incidence of 5 per 100000 persons and is the cause of 1% of all cancer-induced deaths^[114]. More than 70% of multiple myeloma patients may present with bone disease as the onset symptom or develop osteolytic lesions, osteoporosis or spinal compression fractures during the development of the disease^[115]. This is a result of either erosion of bone caused by direct infiltration of plasma cells or secretory factors released by plasma cells resulting in an imbalance in bone metabolism^[7]. Analysis of the immunoglobulin gene sequence itself has provided significant insights into the stage of normal B cell development that gives rise to this tumor^[116]. Several key observations provide evidence for the role of cancer stem cells in multiple myeloma and these CSCs have characteristics similar to those of memory B cells^[117]. It

has been demonstrated that CD138⁺ multiple myeloma plasma cells cannot undergo long-term proliferation but rather arise from clonogenic CD138neg B cells^[118]. It has been investigated that CD138⁺ cells isolated from both established multiple myeloma cells lines and from clinical BM samples give rise to colonies and could be successfully replicated, whereas CD138⁺ cells did not. In contrast to CD138⁺ cells, CD138⁻ MM cells from human BM were capable of successful engraftment into NOD/SCID mice, indicating their potential for self-renewal^[7,119]. In addition, CD138⁻ MM stem cells isolated from cell lines expressed CD19 and CD20 molecules characteristic of B lymphocytes^[119]. Ghosh and Matsui investigated the functional role of Hedgehog signaling on multiple myeloma stem cells and found that pathway activation by Hedgehog ligand induced the expansion of less differentiated CD138neg cells, whereas pathway inhibition using a monoclonal neutralizing antibody against Hedgehog ligands or the naturally occurring small molecule inhibitor cyclopamine limited subsequent clonogenic growth^[116,119,120]. Moreover, the embryonic stem cell-associated antigen SOX2 may represent another potential antigen expressed by multiple myeloma stem cells^[121]. Potentially curative treatment of MM consists of standard chemotherapeutic agents (dexamethasone, lenalidomide, bortezomib, cyclophosphamide, thalidomide) followed by autologous or allogeneic stem cell transplantation^[7,114]. Despite the availability of novel therapies, multiple myeloma remains incurable for the vast majority of patients, suggesting that cancer stem cells with the growth capacity to mediate relapse are relatively resistant to these clinical strategies. It has been shown that circulating clonotypic B cells may persist following systemic treatment and their frequency increases during clinical relapse^[122]. These findings suggest that these cells are drug resistant and mediate tumor regrowth and supports our data that multiple myeloma stem cells are not inhibited by these drugs^[123].

GIANT CELL TUMOR

Giant cell tumors (GCTs) are the second most common primary sacral tumor after chordomas, with a generally benign course and frequently located at the meta-epiphyseal region of long bones, including the distal femur, proximal tibia and the radius^[124]. Benign GCTs mostly account for expansive osteolytic defects associated with significant bone destruction and represent a high recurrence rate^[125]. GCTs are characterized by multinuclear giant cells scattered among a mass of mononuclear cells^[126]. The currently favored hypothesis indicates that giant cell tumors of bone contain a subpopulation of cells localized in the stromal component of the tumor that is spindle shaped and expresses antigens related to the mesenchymal stem cell^[127]. Interestingly, this subpopulation has been identified to express mesenchymal stem cell markers like CD73, CD105 and CD166, as well as the mesenchymal markers FGFR3 (fibroblast growth factor receptor 3), collagen type IIa and CD34⁺ antibody^[128]. Evidence

has also been offered that giant cell tumor stromal cells show differentiation features of mesenchymal stem cells in the form of CD105 (SH2) and CD73 (SH3, SH4) markers, in addition to expressing markers of early osteoblastic differentiation (Thy 1.1, Stro1)^[129]. In support of this hypothesis, Lan *et al.*^[130] confirmed the heterogeneity in stromal cells (SCs) of GCTB, showing that there were at least two different subsets of cells: Stro-1⁺ and Stro-1⁻. Both subpopulations can be further subtyped using additional markers such as CD117, CD133 and CD44. Taking this into account, up-regulation of these markers (CD117, CD113 and CD44) in Stro-1⁺ SCs further implies that the Stro-1⁺ subset is enriched with TSCs and may suggest that CD117, CD113, and CD44 may have key roles in the function of tumor stem-like cells in GCTs. Recent clinical studies have used interferon alpha-2b, denosumab and bisphosphonates to treat inoperable Giant cell tumor of bone (GCTB), but the optimal treatment and medical management of this tumor in the spine and sacrum has not been well established^[130,131]. These agents mainly inhibit angiogenesis or osteoclast-induced osteolysis in GCTB rather than eliminating the neoplastic SCs.

Chordoma

Chordoma is the most common primary malignant bone tumor of the spine that accounts for 1%-4% of all bone malignancies^[132]. The median age is 58.5 years but skull-base presentations affect a younger age and may even appear in children and adolescents^[133]. Chordoma is believed to arise from vestigial notochordal remnants or ectopic notochordal tissue and can occur along the whole length of the spine^[134]. Chordomas were characterized by their physaliferous features and immunoreactivity for S-100 and epithelial markers such as epithelial membrane antigen and cytokeratins. In fact, the term chordoma was first introduced by Ribbert in the 1890s, in view of the notochord hypothesis^[135]. It has been shown that notochordal cell nests topographically correspond and distribute to the sites of occurrence of chordoma. Perhaps the discovery of brachyury transcription factor in familial chordoma was the most compelling evidence of the notochordal hypothesis^[136]. Brachyury is highly expressed in chordoma but not in a wide variety of normal or neoplastic tissue and therefore could be a novel discriminating biomarker for this tumor^[137]. In addition, brachyury regulates several compelling stem-cell genes and has been implicated in promoting epithelial-mesenchymal transition in other human carcinomas^[138]. Chordomas are traditionally considered to be slow-growing, radioresistant tumors that are locally aggressive and invasive^[10]. Chordomas have the potential for metastases, with extension to the lungs, bone, brain, skin and liver and also the high tendency for local recurrence^[139]. Despite advances in radiotherapy techniques, including charged particle (proton beam) radiotherapy for cranial disease, surgery remains the mainstay of chordoma management^[140]. Some case studies reported that complete radical resection produces better local control compared with subtotal resection

and chemotherapy. However, due to the anatomical location of these tumors, gross total resection can be very challenging^[64,134]. It is suggested that PI3K/AKT/TSC1/TSC2/mTOR pathway and EGFR are potential therapeutic targets for chordoma and the combination with topoisomerase II inhibitor razoxane enhances the effectiveness of radiation therapy in this tumor^[141].

There is increasing evidence suggesting that the poor tumor control associated with chordoma may be due to hypoxic effects and/or cancer stem cells which are resistant to ionizing radiation and chemical agents in an *in vivo* tumor environment^[64]. More recently, a positron emission tomography study also revealed that a substantial volume of chordoma is hypoxic. Therefore, it is reasonable to consider that chordoma tumors contain a large fraction of hypoxic area. More evidence is now available that cancer stem-like cells may be present in chordoma, contributing to its aggressive nature. Aydemir *et al.*^[10] have shown for the first time that chordoma cells (U-CH1 cells) and tissues express all the common stem cell markers, including oct4, klf4, c-myc and sox2, and embryonic stem cell markers SSEA-1 and nanog, according to the gene expression analysis. Moreover, they have revealed that chordoma cells are enriched by cancer stem-like cell markers, namely CD133 and CD15, which are able to live in a nonadherent soft agar medium, demonstrating a self-renewal capability. Importantly, these cells could be differentiated into another mesenchymal lineage (an osteogenic lineage) when treated with an osteogenic differentiation agent, indicating that a subpopulation of chordoma cells may possess cancer stem-like characteristics. Further evidence for the existence of a cell population with stem cell properties in human chordoma has been recently reported by Hsu *et al.*^[7] (27th Annual Meeting of the AANS/CNS Section on Disorders of the spine and peripheral nerves). They have established a stable chordoma cell line that is morphologically identical to classical chordoma with expression of brachyury, S-100 and keratin. Chordoma sarcospheres were found to be self-perpetuating and exhibited higher expression of the functional stem cell marker ALDH1 compared to typical chordoma cells. Moreover, sarcospheres were able to successfully differentiate into neuroepithelial and mesodermal cell types. The mechanism that controls the formation of chordoma is not clearly understood. However, the resistance of chordoma to chemotherapy and radiation therapy, as well as the high rate of recurrence after surgical resection, suggests that CSCs may play a role in the pathogenesis of this tumor. Therefore, understanding the molecular pathways that control the maintenance and differentiation ability of normal and cancer stem cells may contribute to new strategy for treating chordoma.

CONCLUSION AND FUTURE PERSPECTIVES

Since the discovery of oncogenes and tumor suppressor genes established the genetic nature of cancer, the cancer

stem cell hypothesis is probably the only novel notion that has provided distinct new views in cancer biology in the last decade. Although knowledge of the identification and characterization of CSCs are quickly expanding, little is known about the cellular and molecular mechanisms underlying their distinct functions. Recently, emerging studies have focused on the roles of CSCs in the pathogenesis of many tumors, including spine tumors. However, there have been scarce numbers of publications focusing on the potential role of CSCs in chordoma. Perhaps the rarity of this tumor coupled with a lack of cell lines available to facilitate investigation explains the paucity of literature on this subject.

To sum up, this study is the first of its kind to review further evidence supporting the role of CSCs in spine tumors, specifically in chordoma. Consequently, with a growing appreciation for the essential role of CSCs in tumorigenesis and metastasis, there is significant interest in developing novel therapeutic approaches to effectively target this unique subpopulation.

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Glioblastoma stem cells: Molecular characteristics and therapeutic implications

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environment-mediated mechanisms to overcome current therapeutic approaches. They are, therefore, very important therapeutic targets. Although the functional criteria defining GSCs are well defined, their molecular characteristics, the mechanisms whereby they establish the cellular hierarchy within tumors, and their contribution to tumor heterogeneity are not well understood. This review is aimed at summarizing current findings about GSCs and their therapeutic importance from a molecular and cellular point of view. A better characterization of GSCs is crucial for designing effective GSC-targeted therapies.

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Key words: Glioblastoma; Glioblastoma stem cells; Self-renewal; Differentiation; Molecular markers; Therapy resistance

Core tip: Stem-like cells in glioblastoma, a malignant brain tumor, have increased tumorigenic capacity, generate tumor lineages and exhibit marked resistance to current therapies. A better understanding of these stem-like cells is necessary for designing new effective treatments. This review discusses the molecular characteristics of these cells and their therapeutic importance.

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Abstract

Glioblastoma Multiforme (GBM) is a grade IV astrocytoma, with a median survival of 14.6 mo. Within GBM, stem-like cells, namely glioblastoma stem cells (GSCs), have the ability to self-renew, differentiate into distinct lineages within the tumor and initiate tumor xenografts in immunocompromised animal models. More importantly, GSCs utilize cell-autonomous and tumor micro-

GLIOBLASTOMA MULTIFORME

Glioblastoma Multiforme (GBM), classified by World Health Organization (WHO) as grade IV astrocytoma, is a deadly primary brain malignancy with more than 10000

new cases in the United States annually (<http://www.cb-trus.org>). Despite the aggressive treatment options involving surgery and concomitant chemoradiotherapy, median survival is 14.6 mo^[1]. The fact that survival has improved by only a few months over the past 50 years highlights the need for a better understanding of the disease and the design of informed therapies^[2].

GBM is a highly heterogeneous tumor with distinctive histologic hallmarks including high cell density, intratumoral necrosis, vascular hyperplasia and invasion through brain parenchyma^[3]. This heterogeneity is also displayed at the microscopic level, where a cellular hierarchy is dominated by the presence of stem-like cells, namely glioblastoma stem cells or GSCs^[4]. In this review we will discuss the molecular and phenotypic characteristics of GSCs and their therapeutic implications.

CANCER STEM CELL HYPOTHESIS AND GLIOBLASTOMA STEM CELLS

Within multi-cellular systems, cells specialize to undertake different responsibilities, in order to maintain homeostasis. As a consequence of this specialization, every cell is not equal in its self-renewal and differentiation ability. Some cells are more stem-like, meaning that they can self-renew and give rise to different progeny through more restricted intermediate progenitors (Figure 1A)^[5]. The extent of self-renewal is dictated by the developmental stage that cells are in and varies from tissue to tissue. For example, in tissues such as the gastrointestinal tract or hematopoietic system, where cellular turnover is high, adult stem cells self-renew more often, compared to more quiescent tissues such as the brain^[6,7]. On the other hand, as cells differentiate, their self-renewal ability decreases and they adopt properties related to their tissue (Figure 1A)^[8]. The differences in differentiation potential define a cellular hierarchy within these systems, where stem cells represent the top of this hierarchy. Lineage restriction and differentiation during physiological processes are mostly believed to be irreversible. However, pathologic conditions or experimental manipulations can cause de-differentiation^[4,9]. Therefore, it is important to understand how cellular hierarchy is established and maintained in tumors in order to understand tumor biology.

Guided from research in liquid tumors, the idea of cancer cells with stem-like properties has revolutionized the field of cancer biology^[10,11]. Although initially thought to be controversial, cancer stem cells (CSCs) are a proven concept for many liquid and solid tumors, including GBM.

In liquid tumors, cellular hierarchy is very well defined by the expression of surface markers. These hierarchically distinct populations were easily isolated by Fluorescence-Assisted Cell Sorting (FACS) *via* the expression of surface markers and their tumor formation ability was assessed *in vivo*^[10]. These surface markers were then investigated in many solid tumors and some of them are still among the best-studied CSC markers.

Glioblastoma cells need to fulfill specific criteria to be classified as GSCs. In particular, they should be able to: (1) *self-renew* (Figure 1A); (2) differentiate into distinct lineages, a property termed *multipotency* (Figure 1A); and (3) *initiate tumors* in animal models, which recapitulate the original disease phenotype and heterogeneity (Figure 1A and B)^[12,13]. Self-renewal is assessed with *in vitro* tumorsphere formation assay, a system borrowed from neural stem cell culture. In this assay, single cells are plated in suspension and their sphere formation ability is evaluated over serial passaging, which is an indicator of long-term self-renewal^[14]. *In vivo* self-renewal is assayed by serial xenograft tumor formation experiments^[11-13] (Figure 1B). The differentiation potential of GSCs is assessed *via* analysis of tumor-derived lineages *in vitro* and *in vivo*^[15-17].

Evidence for GSCs first came from Dirks and colleagues, who isolated cells from human GBM samples based on expression of the cell surface glycoprotein CD133 (Prominin1/PROM1)^[12,13]. They showed that these cells initiated orthotopic tumor xenografts in immunodeficient mice more efficiently than cells that did not express CD133.

Although the functional criteria defining GSCs are completely defined, the molecular characteristics of these cells are not understood. As expected by the heterogeneous histology of GBM, there is extensive cellular heterogeneity within GBM cells, and GSCs as well. The complex interplay of signaling pathways and lack of universal molecular markers identifying GSCs further complicate the study of these cells. More importantly, GSCs are resistant to chemoradiotherapeutic approaches and are, therefore, believed to cause tumor recurrence^[18-20]. Thus, it is of major importance to understand the biology of these cells and their contribution to tumorigenesis, in order to overcome the problems current therapeutic approaches encounter. This review will focus on GSC markers, their molecular signatures and the signaling pathways important for their biology. Finally, we will discuss the therapeutic importance of these cells.

MOLECULAR MARKERS

CD133, a pentaspan transmembrane protein of unknown function, is one of the best-studied GSC markers to date. CD133 expression has been observed during embryonic development, as well as in adult neural stem cells and ependymal cells. However, CD133 knockout mice only have a mild retinal phenotype^[21-23]. When isolated and injected into immunodeficient animals, CD133+ GBM cells are more tumorigenic than CD133- cells and produce xenograft tumors that phenocopy the original patient tumor^[13]. Furthermore, knockdown of CD133 with shRNA impairs GSC self-renewal^[24]. However, the facts that CD133- cells can also generate tumors and that some tumors do not have a CD133+ population suggest that CD133 is not a universal GSC marker^[25-31].

GSCs were also expected to share common markers with neural stem cells, their normal counterparts, based

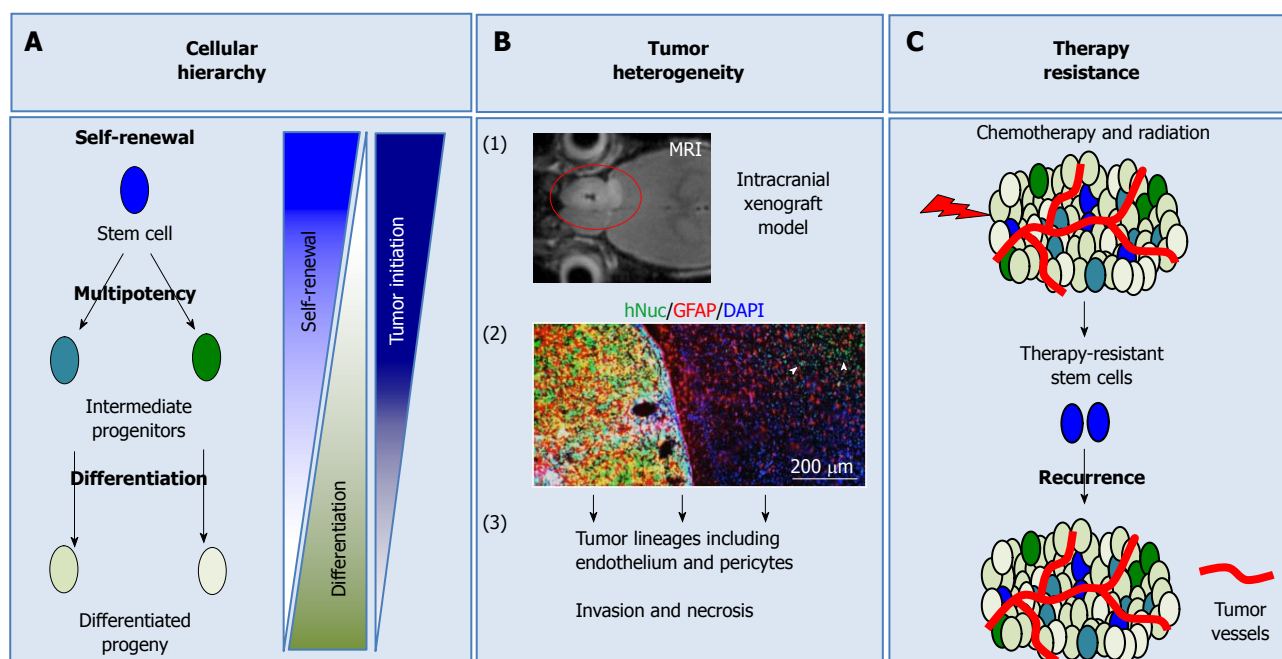


Figure 1 Biological significance of glioblastoma stem cells. A: Glioblastoma stem cells (GSCs) have the ability to self-renew and differentiate into distinct lineages through different intermediate progenitors, a property termed multipotency. Co-existence of cells with different differentiation capacities defines the cellular hierarchy within the tumor; B: GSCs have the ability to initiate tumors more efficiently than differentiated cells. Tumor initiation ability can be tested via intracranial xenograft models in immunodeficient animals. (1) These tumors can be imaged with Magnetic Resonance Imaging (MRI); (2) Microscopic analysis shows that xenografts maintain the histologic heterogeneity of the patient tumor, including the invasion of normal surrounding brain (arrowheads) (hNuc: human nuclear antigen marking human tumor cells in mouse brain, GFAP: Glial Fibrillary Acidic Protein, DAPI: nuclear counterstain); and (3) GSCs promote tumor heterogeneity by giving rise to distinct tumor lineages including tumor endothelium and pericytes, and maintain the phenotype of the parent tumor; C: GSCs are resistant to current therapeutic approaches causing relapse of the tumor.

on the concept of stem cells sharing common signaling pathways. With this rationale, expression of neural stem cell markers was analyzed in GBM tumors. GSCs were shown to have increased expression of Nestin, an intermediate filament expressed in neural stem cells in neurogenic niches^[18,32,33]. Besides Nestin, GSCs are enriched for Sox2, a transcription factor associated with multipotency and pluripotency^[34,35].

Comparative gene expression analysis led to identification of more GSC markers, including Oct4, SSEA-1/CD15, Bmi-1, Musashi-1, Nanog, integrin- α 6, L1CAM, A2B5 and ABC-type transporters, whose expression defines the side population (SP) on flow cytometric analysis, through the ability to extrude Hoechst dye^[25,35-40]. Interestingly, some of these markers are expressed in embryonic stem cells, suggesting GSC overlap not only with NSCs but also with less differentiated stem cells as well. However, none of these markers are universal. Furthermore, the intracellular localization of some of these markers makes them less desirable candidates for selective therapeutic targeting.

SIGNALING PATHWAYS REGULATING GSC BIOLOGY

In addition to oncogenic pathways globally important to tumor biology, signaling pathways that are important for maintenance of self-renewal and regulation of differentiation receive attention in cancer stem cell biology (Table

1). In the context of GSCs, pathways known to regulate neural development are of major interest. Various signaling pathways influence GSC biology by either maintaining self-renewal or regulating differentiation. However, certain pathways can regulate either self-renewal or differentiation in the appropriate context (Table 1).

Self-renewal

Studies of pathways involved in GSC self-renewal gained momentum when Fine and colleagues started culturing tumor cells in serum-free conditions^[41]. By using the mitogens epidermal growth factor (EGF) and fibroblast growth factor (FGF), they limited differentiation and promoted GSC self-renewal. These mitogens act through their receptor tyrosine kinases (RTKs) and induce activation of downstream pathways such as the Phosphoinositide 3-kinase/Akt (PI3K/Akt) and Mitogen-Activated Protein Kinase (MAPK), to induce proliferation, survival and tumorigenicity^[41,42]. Furthermore, blocking the PI3K/Akt pathway has been shown to impair GSC self-renewal and tumorigenicity. Finally, knockdown of CD133 in GSCs causes downregulation of Akt phosphorylation, further highlighting the role of the PI3K/Akt pathway in GSC biology^[43,44].

Originally identified in genetic screens in *Drosophila* as a master regulator of neurogenesis, Notch signaling plays diverse roles in nervous system development, including maintenance of self-renewal and regulation of fate decisions in neural and glial lineages^[45-47]. Upon bind-

Table 1 Major signaling pathways and their roles in glioblastoma stem cell biology

Signaling pathway	Function	Ref.
Self-renewal		
Notch Signaling	Maintenance of GSCs Tumorsphere formation Tumorigenesis Asymmetric division	[50-57]
TGF- β Signaling	Regulation of self-renewal Maintenance of perivascular GSCs	[34,58]
Sonic Hedgehog Signaling	Promotion of self-renewal and migration Upregulation of stem cell associated genes Tumorigenesis	[56,61-66]
Wnt/ β -catenin Signaling	Self-renewal and maintenance of GSCs Tumorigenesis Associated with bad prognosis	[15,66-71]
PI3K/Akt Signaling	Promotion of GSC self-renewal in vitro Proliferation and survival of GSCs Tumorigenesis	[41-44]
MAPK Signaling	Proliferation and survival of GSCs	[41]
Differentiation		
BMP Signaling	Inhibition of asymmetric division Differentiation and proliferation block	[72-74]
Notch Signaling	Trans-differentiation to tumor-derived endothelium	[16]
TGF- β Signaling	Trans-differentiation to vascular pericytes	[17]

GSC: Glioblastoma stem cell; TGF: Transforming growth factor.

ing to its ligands (Delta-like and Jagged), heterodimeric Notch receptors (Notch1-4) get cleaved by γ -secretase in the cytoplasm, releasing the Notch intracellular domain (NICD). NICD translocates into the nucleus where it acts as co-activator for transcription of the *Hes* and *Hey* families of genes^[48]. These genes are transcriptional repressors of neurogenic genes, thereby causing maintenance of stemness in activated cells^[49]. In GBM, Notch signaling is involved in several distinct processes in tumorigenesis, by regulating both self-renewal and differentiation of GSCs^[16,50,53]. Blockage of Notch signaling with γ -secretase inhibitors inhibits self-renewal, as assayed by tumorsphere forming ability, and causes depletion of the CD133+ GSC population^[54-56]. Furthermore, Numb, which prevents NICD from travelling to the nucleus and thus inhibits downstream signaling upon Notch activation, was shown to be asymmetrically distributed within GSCs and to promote asymmetric division. Asymmetric division of GSCs gives rise to two distinct daughter cells: a stem cell (GSC); and a more restricted and differentiated cell^[57]. These findings support a role for Notch signaling in the maintenance of GBM's stem cell compartment.

Inhibitors of Notch pathway components represent promising therapeutic candidates in GBM. However, the overlapping roles with normal neural and other adult stem cell maintenance raises the question of toxicity. Of note, there are ongoing phase II trials with Notch inhibitors in GBM patients (www.clinicaltrials.gov).

Transforming growth factor- β (TGF- β) signaling promotes GSC self-renewal through regulation of distinct mechanisms. First, it was shown to act through SRY-Related HMG-Box transcription factors Sox2 and Sox4, factors important for GSC biology, to induce self-renewal^[34].

Second, blockage of TGF- β signaling decreases perivascular CD44^{high}/Id1^{high} GSCs, *via* repression of inhibitors of DNA-binding proteins Id1 and Id3^[58].

Sonic Hedgehog (Shh-Gli) signaling, which is highly important for brain and spinal cord patterning during embryonic development, also plays crucial functions in GSC maintenance^[59,60]. It has been shown to promote GSC self-renewal and expression of stem cell genes, whereas its blockage leads to apoptosis, delay in tumorigenesis and inhibition of GSC self-renewal and migration^[56,61-66].

The Wnt/ β -catenin pathway induces proliferation of progenitor cells within gliomas^[15,67]. Some reports suggest that Wnt signaling is important for GSC self-renewal. Overexpression of Wnt ligands, Wnt3a and Wnt1, is observed in GSCs^[67]. Other Wnt pathway components were shown to promote GSC self-renewal and tumorigenicity. Some of pathway's downstream effectors such as β -catenin, Lgr5, Dishevelled 2 and Frizzled 4 are associated with negative prognosis^[66,68-70]. FoxM1, which promotes nuclear localization of β -catenin, was also shown to be critical for GSC maintenance and tumorigenesis^[71].

Differentiation

Bone morphogenic protein (BMP), a member of TGF- β superfamily, functions as a differentiation signal within GBM, as opposed to the previously discussed roles of other members of the TGF- β family in maintenance of self-renewal^[34,72]. The difference between BMP and TGF- β 's effects on GSC biology can be ascribed to distinct signaling cascades, even though they belong to the same superfamily of ligands. Also important for astrocytic differentiation in development, BMP4 treatment inhibits asymmetric division of GSCs, thereby blocking their self-renewal and depleting the stem cell compartment of the tumor^[73,74]. Treatment with BMP4 leads to differentiation and proliferation block. However, a subset of GSCs manages to escape this differentiation cue *via* epigenetic silencing of BMP receptor 1B (BMPR1B)^[74].

Although highly important for self-renewal, reports also suggest that Notch signaling is important for trans-differentiation of GSCs into tumor-derived endothelium^[16]. Similarly, TGF- β was shown to induce GSC differentiation into vascular pericytes, supporting vessel formation and leading to further tumor growth^[17].

MicroRNAs

An additional level of complexity in GSC biology is exhibited by regulatory non-coding RNAs, which are fine tuners of gene expression. Among them, microRNAs (miRNAs) have the ability to modify gene expression levels by specifically binding mostly to the 3'-UTRs of genes and causing their degradation through the RNAi machinery^[75]. Besides being highly important for regulation of pluripotency and reprogramming, miRNAs play important roles in GBM tumorigenesis and GSC biology. Similar to other molecular markers enriched in GSCs, miRNAs regulating neural stem cell biology are also of main interest in GSC biology. miRNAs upregulated

in GBM and particularly in GSCs have anti-apoptotic, anti-differentiation, pro-proliferative and pro-invasion properties^[40,76,77]. On the other hand, miRNAs promoting differentiation were shown to be downregulated in GBM, including miR-124, which is important for neural differentiation^[78-81].

STEM CELL NICHE AND TUMOR MICROENVIRONMENT

To better understand the interplay of different signaling pathways mentioned above and how they regulate GSC biology, we need to study the niches in which GSCs reside. Besides providing crucial signals for GSC maintenance, stem cell niches and the tumor microenvironment are critical factors in the response to therapy.

Vascular niche

Endothelial cells provide signals required for self-renewal of neural stem cells and many other adult stem cell populations^[82]. Similar to their normal counterparts, GSCs reside in a perivascular niche, where they maintain close contact with CD34+ endothelial cells^[83-85]. This close contact facilitates presentation of Notch ligands on the surface of endothelial cells. These ligands activate Notch signaling in GSCs, thereby promoting self-renewal^[85].

The perivascular niche is also subject to bidirectional cues coming from GSCs. CD133+ GSCs express higher levels of vascular endothelial growth factor (VEGF), leading to angiogenesis and increased vascularity of the tumor, when compared to their CD133- counterparts^[86].

New evidence for trans-differentiation of GSCs into endothelial cells and pericytes further suggests that GSCs play a central role in maintaining the tumor microenvironment and their own niches, when presented with appropriate signaling cues^[16,17].

Necrotic niche

As mentioned earlier, GBM is characterized not only by extensive vascular hyperplasia but also pronounced intratumoral necrosis. One of the main histologic hallmarks of GBM is a phenomenon called pseudopalisading necrosis (PPN), where densely packed tumor cells surround a necrotic area^[87]. Although the etiology and biological significance of these areas are not well understood, they are believed to be regions of active tumor growth and neo-vascularization. Considering the importance of hypoxia in promoting self-renewal in embryonic stem cells and NSCs, pseudopalisades represent plausible niches for GSCs^[88,89]. This hypothesis is further supported by studies showing immunoreactivity for CD133 in pseudopalisades^[90]. Furthermore, hypoxia leads to activation of angiogenesis and neo-vascularization through the upregulation of VEGF in GSCs^[91,92]. Some evidence also suggests that hypoxia reprograms CD133- GSCs to become CD133+ and induces Notch signaling, whose importance for GSC biology was mentioned above^[88,89].

Keeping these findings in mind, the possibility of a necrotic niche for GSCs is biologically intriguing and represents a therapeutic challenge for systemic drug delivery methods, since these areas are devoid of blood vessels.

Invasion

The most malignant feature of GBM is its invasion of brain parenchyma. GBM cells infiltrate normal brain tissue and can be found centimeters away from the tumor core^[93]. The vast majority of recurrence after surgery and chemoradiotherapy occurs within 2 cm of the resection cavity suggesting that these invading cells also have tumorigenic capacity^[94-96].

Expression of C-X-C chemokine receptor type 4 (CXCR4) and its ligand, stromal derived factor 1 α (SDF-1 α), which are important regulators of invasion of GBM cells, is enriched in GSCs^[91]. This signaling pathway also mediates recruitment of GSCs towards endothelium, causing further invasion, differentiation and endothelial cell proliferation *via* VEGF expression^[92].

GSCs AS THERAPEUTIC TARGETS

Standard care for GBM is surgical resection, followed by concomitant temozolomide, an alkylating agent, and radiotherapy. GSCs represent important therapeutic targets because they have intrinsic machinery that overcomes current chemoradiotherapeutic approaches (Figure 1C). Some of the molecular mechanisms underlying GSC resistance to chemoradiotherapy are discussed below.

Chemotherapy resistance

GSCs are believed to resist chemotherapy *via* several distinct mechanisms. One such mechanism involves the active transport of chemotherapeutic agents to the extracellular space *via* ABC-type transporters on the cell surface. This mechanism also defines the side population (SP) of GBM cells on flow cytometry, through the exclusion of Hoechst dye^[97]. Enrichment of stem cell markers such as CD133, CD117, CD90, CD71 and CD45 is observed in cells resistant to lethal doses of chemotherapeutic drugs^[98]. Furthermore, CD133 expression is increased in recurrent tumors. Transcriptional analysis of CD133+ GSCs showed that these cells have increased expression of anti-apoptotic genes, suggesting that GSCs have intrinsic mechanisms of chemoresistance^[36].

In line with these observations, more compelling evidence came from Parada and colleagues, who showed that a restricted Nestin+ GSC population was able to regenerate tumors after temozolomide treatment. Selective ablation of this population led to tumor growth arrest, consistent with the notion that GSCs resist conventional chemotherapy and cause relapse^[18].

Another mechanism for chemoresistance lies in the cell cycle profiles of GSCs. Most chemotherapeutic agents target actively cycling cells. However, GSCs are mostly dormant or slow-cycling cells, thereby resisting such therapies^[99].

Radioresistance

In addition to their chemoresistance, GSCs evade radiation, with radiation-resistant clones showing increased expression of GSC markers. More importantly, the Notch and TGF- β signaling pathways, which were mentioned earlier as critical for GSC self-renewal, promote radioresistance as well^[51,100]. GSCs have increased DNA repair capacity. CD133+ GSCs selectively activate Chk1 and Chk2 kinases upon radiation, making them less susceptible to radiation-induced apoptosis^[19].

CONCLUSION

In this review, we have summarized recent advances in understanding the biology of GSCs. We have focused on molecular markers commonly used to identify GSCs and signaling pathways that regulate important GSC characteristics, such as self-renewal, differentiation and therapy resistance. Due to their high tumorigenic potential and resistance to current therapies, GSCs represent critical drug targets. However, the lack of universal markers identifying GSCs, the complexity of signaling cascades regulating GSC biology and the large overlap between tumorigenic pathways active in both GSCs and normal stem cells complicate the development of GSC-targeted therapeutics. A better understanding of GSC biology and their contribution to cellular hierarchy and tumor heterogeneity is crucial for designing effective new therapies against gliomas and other brain malignancies.

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Training stem cells for treatment of malignant brain tumors

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tients. Here, we perform a literature review to identify the problems in the field. Given the lack of efficacy of most stem cell-based agents used in the treatment of malignant brain tumors, we found that stem cell distribution (*i.e.*, only a fraction of stem cells applied capable of targeting tumors) are among the limiting factors. We provide guidelines for potential improvements in stem cell distribution. Specifically, we use an engineered tissue graft platform that replicates the *in vivo* microenvironment, and provide our data to validate that this culture platform is viable for producing stem cells that have better stem cell distribution than with the Petri dish culture system.

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Key words: Stem cells; Malignant brain tumors; Engineered tissue graft; Organotypic slice model

Core tip: Neural stem cells can target malignant brain tumors in preclinical models; however, clinical trials show dismal efficacy. We reviewed the literature and found that only a small fraction of applied stem cells can move toward tumors while the majority of stem cells cannot reach the target tumor. To fill in the gap in stem cell technology, we propose a solution to train stem cells in a native tissue environment, allowing them to move through tissue barriers and arrive at the target tumor.

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Abstract

The treatment of malignant brain tumors remains a challenge. Stem cell technology has been applied in the treatment of brain tumors largely because of the ability of some stem cells to infiltrate into regions within the brain where tumor cells migrate as shown in preclinical studies. However, not all of these efforts can translate in the effective treatment that improves the quality of life for pa-

INTRODUCTION

Malignant brain tumors are devastating to patients

Billions of dollars have been spent since United States

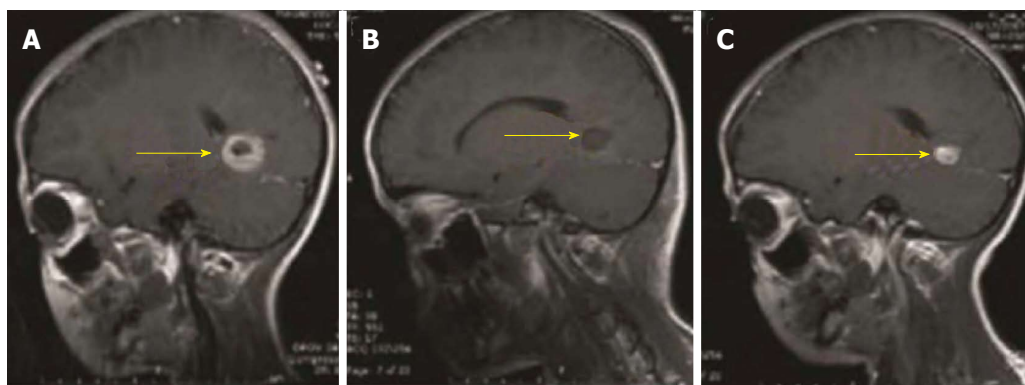


Figure 1 Magnetic resonance imaging graphs illustrate the presence, removal, and reappearance of a glioblastoma patient (yellow arrow: tumor mass). A: Pre-operation, visualizing the presence of the tumor; B: Post-surgery, visualizing disappearance of the tumor; C: 3-mo post-surgery, visualizing the reappearance of the tumor.

President Richard Nixon declared the “war on cancer.” Understanding the molecular biology of cancer led to gain better survival in certain cancers, such as childhood leukemia. However, survival in solid tumors has not improved since the 1970s. New studies revealed that unexpected factors such as intratumoral heterogeneity^[1] and clonal evolution force us to realize that classical therapies cannot fully address the tumor subclonal switch mechanism that allow tumors to escape therapy^[2]. This includes chemotherapy drug temozolomide-driven evolution of recurrent glioma^[3] into a restricted subclonal cell population of drug-resistance^[4]. Ineffective cancer treatment results in mortality and economic burden: one-third of 2007 healthcare dollars (total: \$686 billion) was spent on 1.4 million cancer patients in the United States^[5-7]. Some pediatric malignant brain tumor patient costs \$67887, which is 200 times as much as a demographical control, \$277^[5,8]. It is devastating, considering that the fortunate survivors suffer cognitive changes, cognitive deficiency that challenges the quality of life of both patients and their care givers^[9].

LIMITATION OF CURRENT STANDARD TREATMENT

Cancer treatment is largely unsuccessful due to current blindfolded anti-cancer strategic and tactical issues in the fight. Surgical resection allows glioma patients survive the traumatic attack; however, surgery alone cannot clear the residual infiltrative glioma. Malignant brain tumors disseminate widely to distant regions of normally functioning tissues^[10]. Thus, surgery in conjunction with chemotherapy and radiation therapy still cannot eradicate residual tumors^[11,12].

ADVERSE SIDE EFFECTS OF STANDARD THERAPIES

Chemotherapy and radiation therapy do not strictly discriminate tumor cells from normal cells, resulting in

adverse effects. Survivors of current standard brain tumor treatment show neurological, cognitive, endocrine sequelae, and metabolic side effects^[11,13-20]. These side effects result from the cumulative effects of pre-treatment injury caused by the growing tumor, the adverse impact of surgery and from adjuvant therapeutics (chemotherapy and radiation therapy)^[21]. The surgical removal of the initial tumor followed with adjuvants (radiation plus chemotherapy) may awaken the dormant clones of the primary tumor and these cells then grow to form a secondary tumor (Figure 1) as the dormant cells go through switch-board signaling to become dominate clones of cancer^[2]. These glioma residues grow back, leading to recurrent incurable and metastatic cancer. Adjuvant therapies (Local radiotherapy, chemical sensitizers, gene therapy) did not provide any survival advantage in clinical trials.

GENETIC PROFILING

Genetic profiling shows the potential genetic risk factors for patients and a way to predict how a patient may react with a given tumor treatment. Across 12 tumor types in 2928 out of 3277 patients, The Cancer Genome Atlas Network (TCGA) analyzed 10281 somatic alterations^[22]. This TCGA data set predicts patient survival when applying therapies useful in one cancer type to other cancer types. This molecular profile-based prediction of therapeutic efficacy may imply a new classification system different from the previous organ-based tumor classification system^[23].

For example, the analysis of somatic mutations in glioblastoma multiforme (GBM)^[24] helped establish Pro-neural, Neural, Classical, and Mesenchymal subtypes^[24]. Each subtype, with its own molecular stratification (*PDGFRA*, *IDH1*, *EGFR*, and *NF1* gene), can exhibit specific drug targets that minimize adverse effects and enhance efficacy. Another study shows that recurrent H3F3A mutations are further characterized into six methylation patterns^[25]. The methylation patterns help design epigenetic-pattern-specific targeted therapies^[25]. Molecular changes in *BRAF*, *RAF1*, *FGFR1*, *MYB*, *MYBL1*, *H3F3A*,

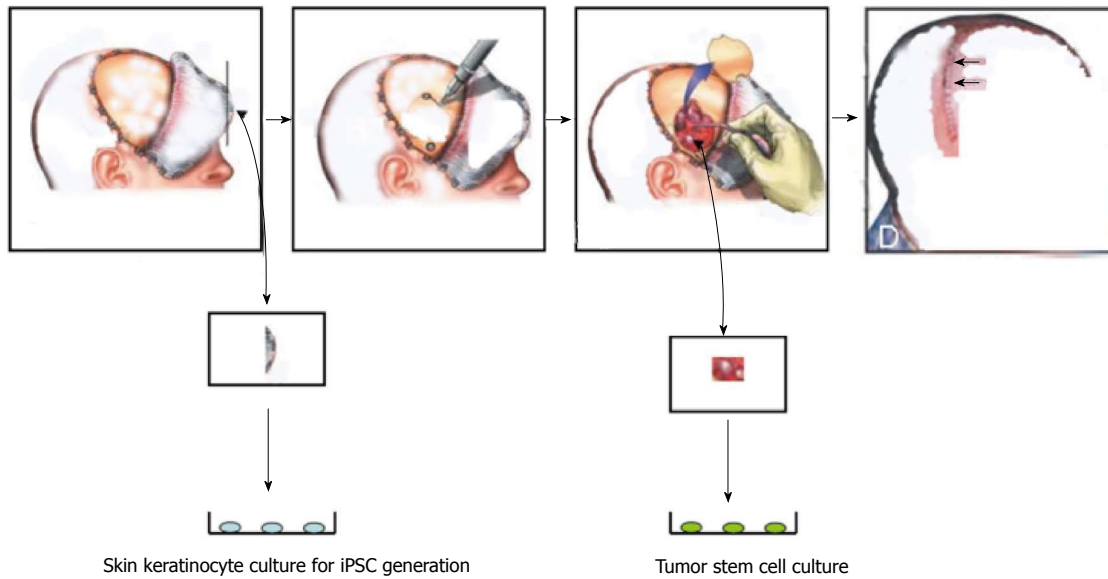


Figure 2 Personalized treatment of brain tumors by using autologous stem cells (induced pluripotent stem cells) through the induced pluripotent stem cells strategy for treating brain tumors. During surgery, a piece of skin is obtained to generate induced pluripotent stem cells (iPSCs) while tumor cells are processed to obtain tumor stem cells (TSCs). The iPSCs are used to take therapy specific to autologous TSCs.

and ATRX were identified in 151 low-grade gliomas (LGGs)^[26]. Another study defined recurrent activating mutations in FGFR1, PTPN11, and NTRK2 genes in LGGs^[27]. The mutations imply some targeted therapies, *e.g.*, specific inhibitors against FGFR1 autophosphorylation can block MAPK/ERK/PI3K, preventing cancer cells from proliferating.

These mutations can help focus targeted therapies for patients. Temozolomide (TMZ) and radiation, increase survival for patients with Classical or Mesenchymal subtypes but not with Proneural subtype^[24]. However, chemotherapy can activate chemoresistant cancer cells. TMZ drives a subset of endogenous cells out of their quiescent subventricular zone to develop to a new tumor^[4]. Evidence shows that TP53, ATRX, SMARCA4, and BRAF mutations in the initial tumor but were undetected at recurrence, suggesting new mutations occur upon drug-driven tumor evolution. TMZ-activated RB (retinoblastoma) and Akt-mTOR (mammalian target of rapamycin) mutations led to recurrent tumors^[3]. New strategy to address these therapy-driven detrimental effects in a real-time manner is needed.

EMERGING THERAPIES

Neural stem cells (NSCs) possess the tumor-tracking capacity as shown in preclinical models^[28]. NSCs modulate the brain tumor microenvironment^[29-32]. Other candidate stem cells include HSCs^[33], BM-MSC^[34], and induced pluripotent stem cells (iPSC)^[35]. Because iPSC technology enables autologous transplantation allowing immune compatibility with a host immune system (Figure 2), iPSCs are proposed for replacement therapy in certain diseases^[36]. However, potential immune rejection of these autologous iPSCs remains to be tested in clinical trials^[37].

These stem cells could be engineered as delivery vehicles for therapeutic agents^[38] such as antibody^[39], oncolytic adenoviral virotherapy^[40], and prodrug therapy^[41]. NSCs inhibit glioma proliferation *in vivo* and *in vitro*^[42]. Intracranial tumors activate endogenous NSCs to migrate towards neoplastic target lesions^[43,44].

Evidence shows that BM-MSCs work in the same fashion as NSCs^[34]. MSCs exhibit tropism towards gliomas^[45-47]. MSCs locally produce IFN- β that suppresses cancer cells^[48].

CLINICAL TRIALS SHOW DISCREPANCIES

Serving to reconstitute hematopoietic and immune function, some stem cells act as a salvage therapy for surgery, radiation therapy, and high dose chemotherapy. For example, patients rely on autologous hematopoietic stem cell transplantation to replenish immune capacity against recurrent cancer after surgery and chemotherapy^[49]. Currently, 240 studies on “stem cell therapy of cancer” exist in mostly Phases I / II clinical trials (See <http://clinicaltrials.gov>, accessed on August 22, 2014) using HSCs and BM-MSCs. Interestingly, genetically modified NSCs orchestrate flucytosine and leucovorin calcium in treating gliomas [ClinicalTrials.gov identifier NCT02015819 (2014)]. The genetically modified NSCs carry the gene for *Escherichia coli* (*E. coli*) flucytosine that sensitizes cancer to chemotherapy while leucovorin calcium helps stop cancer cells from dividing. The project of ClinicalTrials.gov Identifier NCT01540175 aimed at replenishing an immune system (T cell, B cell, and NK cell compartment) on autologous transplant to the baseline values, representing an innovation that is expected to replace

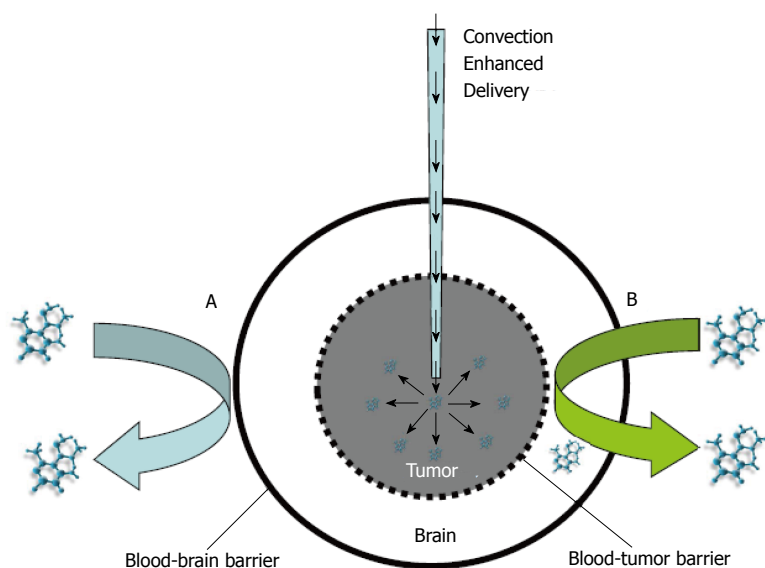


Figure 3 Convection enhanced delivery of therapy to overcome two barriers of brain tumors. A: Systemic delivery of drugs blocked from entry into the brain by the blood brain barrier; B: Drug delivery inhibited by the brain-tumor barrier. This convection enhanced delivery can be used to deliver neural stem cells locally onto a tumor.

the conventional HSC transplantation. Phase I trials using tumor dendritic vaccines evaluated the side effects of vaccine therapy on recurrent GBM (ClinicalTrials.gov Identifier: NCT00890032 - tumor cells/dendritic cells; ClinicalTrials.gov Identifier: NCT01171469-tumor stem cells; assessed on August 22, 2014), a potential that a real-time anti-cancer system is established *in vivo* to monitor cancer growth. These everlasting vaccines are expected to set up an immune response to stop cancer.

Discrepancies of efficacy occurred in all these clinical trials and efforts have been made to explain what roadblocks are in the way for achieving consistent efficacy. Roadblocks for stem cells to reach the site of the tumor include the blood brain barrier (BBB) and the brain tumor barrier (BTB) (Figure 3). Most intravenously administered NSCs cannot cross BBB and BTB but only a few do^[7]. These roadblocks must be removed to clear that path for success of stem cell therapy for cancer^[7]. Specifically, we need to cultivate potentiated stem cells to be potent to tranverse these roadblocks.

THE NEED TO FIND WAYS OF IMPROVING THE POTENCY OF STEM CELLS

What qualities for stem cells could allow therapeutic effectiveness? The ideal stem cells should provide: (1) long-distance inter-organ autopilot traveling to surgically inaccessible tumors, ideally when administrated by peripheral intravenous injection; (2) accuracy in eliminating tumors without adversely affecting normal organs; (3) capability of suppressing primary and metastatic tumor; and (4) memory so that recurrence never occurs.

Components of an inter-organ movable vehicle for targeting cancer

(1) The therapeutic agent shows the maximum anti-cancer efficacy with the minimum adverse effect; (2) The vehicle should protect the therapeutic agent for its potency

and specificity; and (3) The vehicle possesses the ability to home in on targets.

Stem cell therapy provides the essential components of such a defined therapeutic agent, as fellows.

The therapeutic agent: Therapeutic benefits of stem cells include (1) regenerative action; (2) neuroprotective modulation; and (3) immune regulation. The BM-MSCT transplantation induces survival and proliferation of host neurons through secreting BDNF, β -NGF, and adhesion molecules^[50]. Stem cells can serve as a “Trojan Horse” for transplantation of cancer drugs^[50,51].

The autopilot vehicle: NSCs can detect a target (homing) *via* chemokines produced by tumors (Figure 4, Li *et al.*^[7] 2008), the capacity like a self-driving vehicle. Following this chemokine gradient, NSCs can move through tissue barriers such as the blood brain barrier and brain tumor barrier (Figure 3) to reach their target tissue. We need to determine the therapeutic window of stem cell development, the window of stem cell development that is capable for targeting tumors^[52]. If stem cells develop outside of a window period, thereby lose the ability of migrating toward tumors because their migration-required molecules are down regulated^[53].

Delivery system: Stem cell delivery for cancer remains to be defined. For brain tumors, we can use a stereotactic injection for a specific brain region. Mooney and colleagues show that NSCs can facilitate the tumor-selective distribution of nanoparticles, a drug-loading system that is promising in cancer therapy^[54]. We can apply CED (convection-enhanced delivery) to deliver stem cells across the blood-brain barrier and the brain-tumor barrier (Figure 3). We need further to track down stem cell migration *in vivo* by using a real-time tracking system as we discussed previously^[55], a way that can address possible adverse effects.

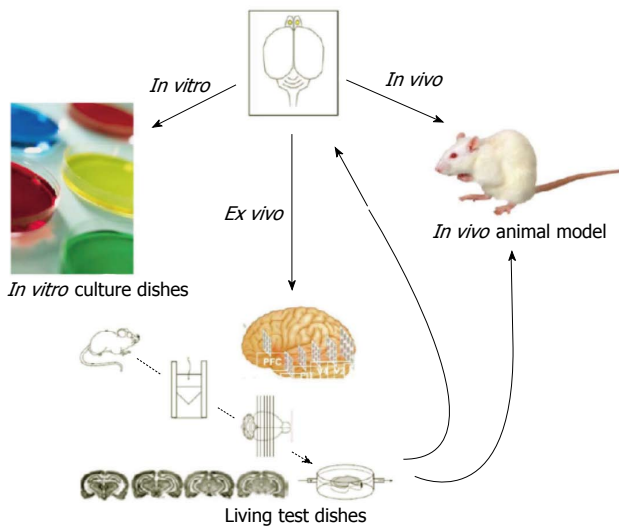


Figure 4 Three ways to drug testing: *In vitro* Petri dishes, *in vivo* animal model and *ex vivo* engineered tissue graft. An engineered tissue graft has an intrinsic character of native brain environment.

A PROBLEM IN STEM CELL TRANSPLANTATION AND ITS SOLUTION

Only marginal effects can be observed in stem cell therapy despite exciting potency shown in some animal models^[52]. In fact, it is a game of number wrestling between good stem cells and tumor cells^[52,53]. Current stem cell experiments in mouse models involve transplantation of millions of stem cells, with only some migrating toward tumors, a few surviving at the tumor site, and rare engraftment^[52,53]. The rest of the non-migratory stem cells are detrimental to a recipient, because these can induce the formation of heterogeneous tumor and inflammation. Thus, we must enable stem cells to pass certain uniform quality control standard so that they can fulfill their designed purpose of targeting brain tumors.

TRAIN STEM CELLS IN AN ORGAN-SPECIFIC MICROENVIRONMENT

The low number of stem cells capable of migrating toward tumors derived from Petri dish culture system as shown in preclinical and clinical studies may result from the following differences: (1) the source of stem cells; (2) methods of stem cell culture; (3) differentiation status (percentage of differentiated cells); (4) the age of the stem cells in culture; and (5) the nature of a tumor^[34].

We found that culture matrix makes a difference in stem cell characteristics. NSCs behave differently in coated Petri culture plates (Figure 5). NSCs show much more neurite growth on Matrigel-coated Petri polystyrene plates than on other adhesion molecule-coated plates (Collagen I, Collagen IV, or Laminine). Nevertheless, none of these adhesion molecules can generate uniform populations of stem cells. We have designed an engineered tissue graft model as a universal training platform

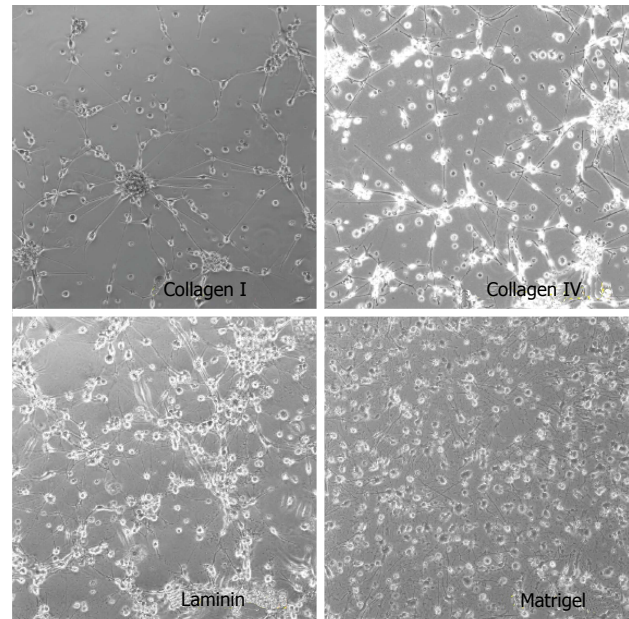


Figure 5 Stem cells cultured on Petri dishes coated with different matrix, showing non-physiologically relevant morphology with a few neurite growth.



Figure 6 An engineered brain tumor tissue graft in culture, showing tumor lesions (black dots) that attract stem cells to engraft.

to address the issue of heterogeneity of cultured stem cells^[7]. An engineered tissue graft (ETG) provides a native organ microenvironment closer to an *in vivo* model and very different from an *in vitro* Petri dish (polystyrene plates) system (Figure 4)^[56]. This ETG model can be generated from patient specific brain tumor specimens for autologous characterization of therapeutic *in vivo-like* trials of a new drug (Figure 6). This ETG material was made according to our patented technology - an ETG generated by seeding brain tumor stem cells onto slice cultures of patients' pathological brain tissue harvested during tumor resection - which preserved the pathological micro-environment^[52].

Such a culture platform can train stem cells to fulfill the purpose of targeting brain tumor cells as they help generate uniform neurite formation in culture that is essential for brain-tumor-targeted migration (Figure 7). These ETG-based matrix produced cells express molecu-

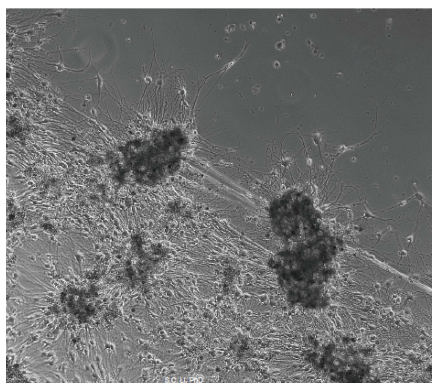


Figure 7 Neural stem cells cultured on the engineered tissue graft showing abundant neurite formation and neuronal morphology.

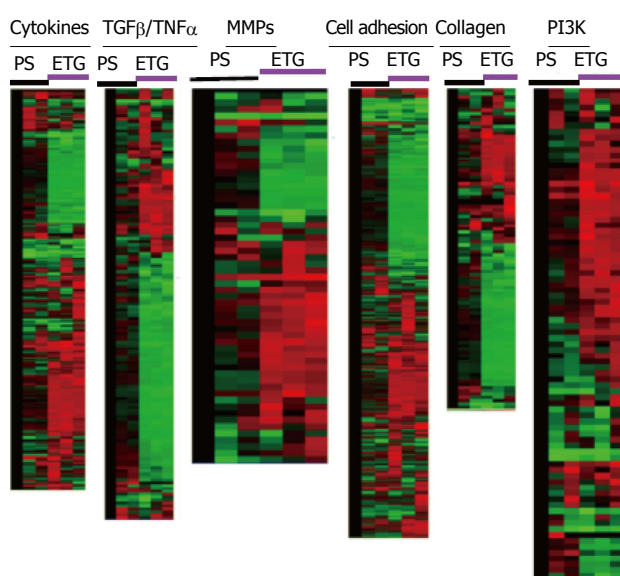


Figure 8 Data analysis of Affymetrix Gene Chip arrays for pediatric derived brain tumor stem cells grown on engineered tissue graftmatrix-like surface or polystyrene dish. The cells were grown on engineered tissue graft (ETG) matrix-like surface or polystyrene dish (PS) for 7 d for gene chip array analysis showing gene clusters on different functional group of signaling pathways. Notice that red color represents the highest expression, green color for medium expression, and black for lowest expression. MMPs: Matrix-remodeling matrix metalloproteinases; TNF: Tumor necrosis factors; TGF: Transforming growth factor.

lar markers different from cells cultured on polystyrene plates (PS) as shown in gene arrays (Figure 8). We can obtain a morphologically uniform population of stem cells in an ETG microenvironment (Figure 9). Optimizing the chemokine responsiveness (chemokine receptors expressed by stem cells) and upregulating matrix-remodeling matrix metalloproteinases (MMPs) are essential: Both chemokine receptor and MMPs are well expressed in cells with ETG but not with Petri dish culture system^[7]. Additionally, to overcome the problem of immune response, we have designed autologous iPSCs (induced pluripotent stem cells) for certain patient tumors (Figure 3), a dual system that can mutually promote each other for better efficacy. These trained stem cells can act as an autopilot

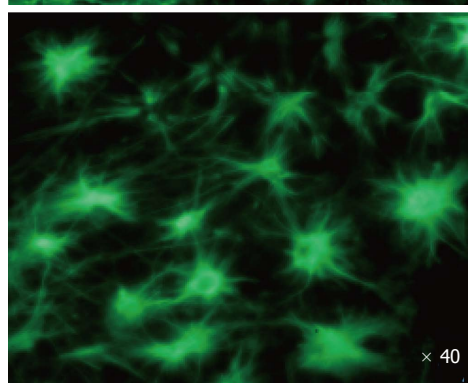
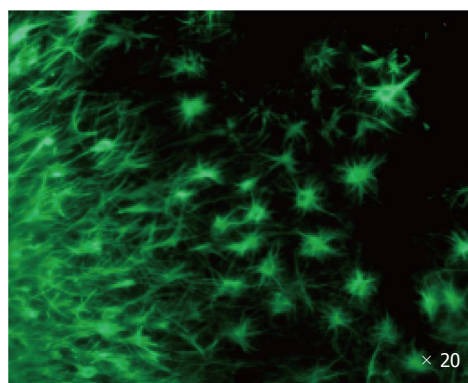


Figure 9 An engineered tissue graft is used as the designer matrix to train stem cells to target a specific tumor as shown for production of a morphologically homogeneous population of stem cells.

vehicle that is self-driven to its target (Li *et al.*^[7], 2008, Figure 5). This ETG can be engineered to mimic the *in vivo* fluidic microenvironment with the continuous flow of physicochemical buffer, the microfluidic system that can be coupled with real-time imaging for analysis of cell development as the quality control (QC) as detailed in a recent report^[57]. In the future, a QC system should be implemented for the structural and functional characterization of stem cell production before using for transplantation. This ETG could be scaled for automatic studies.

CONCLUSION

Stem cell therapies of brain tumors are being investigated preclinically; however, little efficacy has been found in clinical trials. We reviewed the literature and found that heterogeneous stem cell populations were made using artificial matrices, a roadblock to achieve consistent efficacy. We provide an ETG as a uniform platform to train stem cells for attacking tumor cells, which may address the discrepancies of current clinical trials.

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Ovarian cancer stem cells: Can targeted therapy lead to improved progression-free survival?

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Abstract

Despite significant effort and research funds, epithelial ovarian cancer remains a very deadly disease. There are no effective screening methods that discover early stage disease; the majority of patients are diagnosed with advanced disease. Treatment modalities consist primarily of radical debulking surgery followed by taxane and platinum-based chemotherapy. Newer therapies including limited targeted agents and intraperitoneal delivery of chemotherapeutic drugs have improved disease-free intervals, but failed to yield long-lasting cures in most patients. Chemotherapeutic resistance, particularly in the recurrent setting, plagues the disease. Targeting the pathways and mechanisms behind the development of chemoresistance in ovarian cancer could lead to significant improvement in patient outcomes. In many malignancies, including blood and other solid tumors, there is a subgroup of tumor cells, separate from the bulk population, called cancer stem cells (CSCs). These CSCs are thought to be the cause of metastasis, recurrence and resistance. However, to

date, ovarian CSCs have been difficult to identify, isolate, and target. It is felt by many investigators that finding a putative ovarian CSC and a chemotherapeutic agent to target it could be the key to a cure for this deadly disease. This review will focus on recent advances in this arena and discuss some of the controversies surrounding the concept.

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Key words: Epithelial ovarian cancer; Cancer stem cells; Chemoresistance; Targeted therapy; Chemotherapy; Recurrent ovarian cancer

Core tip: Ovarian cancer stem cells (CSCs) are difficult to isolate, identify, and target. However, they are often thought to be the source of development of chemoresistance. Finding a therapeutic target in ovarian CSCs and identifying the mechanisms associated with the development of chemoresistance may lead to a long-lasting cure for patients with epithelial ovarian cancer.

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INTRODUCTION

It is estimated that over 14000 women in the United States will die with ovarian cancer and more than 22000 women will be newly diagnosed with the disease in 2013^[1]. Women with early stage disease often have vague symptoms such as bloating, back pain, and fatigue leaving most women undiagnosed until later stages of the disease. Standard treatment of ovarian cancer consists of

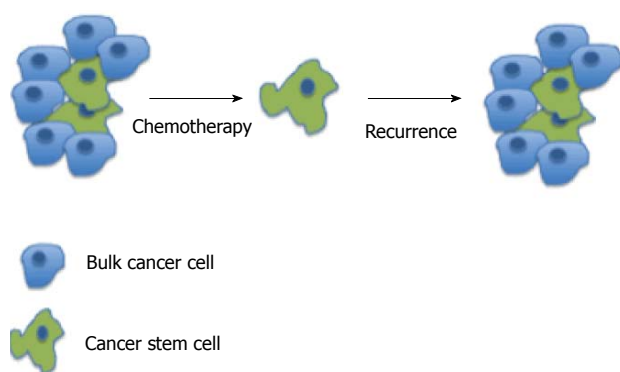


Figure 1 Death of bulk cancer cells by chemotherapy, but not cancer stem cells leads to recurrence.

surgical resection of disease followed by taxane and platinum-based chemotherapy which yields a partial response rate of greater than 80% and a complete response rate of 40%-60% in patients with advanced disease^[2]. Although initial response rates are promising, the recurrence rate is approximately 70% and five-year survival is 45% in patients with advanced disease^[3]. While it appears that the majority of ovarian cancer cells are initially chemosensitive as evidenced by the high initial chemotherapy response rates, the high recurrence rates suggest development of chemoresistance. Some believe that a population of cells are not killed by chemotherapy, or they repopulate after exposure to chemotherapeutic agents. These cells have been called ovarian cancer stem cells (CSCs).

CSCS

It has been theorized that CSCs exist in certain malignancies, particularly the blood cancers and basal-like breast cancer. For the blood cancers, identifying CSCs has been in progress since the first stem cells were identified^[4]. In acute myeloid leukemia, CSCs have been proven to be an immature abnormally differentiated cells that have the ability to self-renew^[5]. It is felt by some investigators that these CSCs exist to promote tumor growth and metastasize to other organs. They have an increased tumorigenicity and differentiating capacity compared to other cells. The majority of solid tumor cells, may not have a differentiation capacity or the ability to develop chemoresistance but offer support to angiogenesis or signaling pathways. The CSCs (progenitor cells) are typically a small portion of the tumor and give rise to differentiated progeny that comprise the bulk of tumors (Figure 1), and are capable of unlimited growth^[6,7]. CSC markers have been shown to be upregulated in cells growing in tumorspheres compared to single cells suggesting that CSCs are enriched in this population. In ovarian cancer, this spheroid form of tumor cells is thought to be involved in the dissemination of cancer in the peritoneal cavity. This suggests that CSCs are involved in metastasis intra-abdominally. CSCs are generally thought to have the ability to self-renew, differentiate, and metastasize to form secondary and tertiary tumors^[8]. It has been shown

that primary treatment with chemotherapeutic agents results in increased drug-resistant CSCs and this leads to recurrence^[9]. Unlike some of the blood cancers which have known normal stem cells, there is no known normal ovarian stem cell^[6]. This obviously complicates the identification of specific ovarian CSCs. The majority of evidence in favor of ovarian CSCs exists from the identification of markers of "stemness" as identified in other malignancies. Still, many researchers are investigating the existence of specific ovarian CSCs.

OVARIAN CSCS

The isolation of ovarian CSCs is fraught with difficulty, like that of many other solid tumors. For isolation to occur, a single-cell suspension must be made from a solid tumor while sustaining viability. While there may be a large volume of tumor or ascites, the actual CSCs are a rare population of that tumor; unlike blood tumors, there is no specific marker for an ovarian CSC. The first model for this process was described by Bapat *et al*^[10] in 2005. They collected ascites from a patient sample and were able to develop 19 immortalized tumor sphere-forming clones. Two of these were passaged into nude mice and grew into tumors that closely resembled the parental tumor. A single transformed clone was able to be isolated that demonstrated increased aggressiveness from the parent tumor. This experiment was some of the first evidence to show heterogeneous growth properties of tumor cell subpopulations in ovarian cancer. Also, these tumor cells demonstrated the ability to self-renew by continuing to form tumors even after serial transplantation.

CSC MARKERS

There is no specific ovarian CSC marker and researchers have relied on markers of "stemness" identified from other malignancies. Some of these proteins used as CSC markers include CD44, CD133, CD117, ALDH1A1, and EpCAM (Table 1). There are many other proteins that have been used as markers of "stemness" but are not as well defined in ovarian cancer. Discovered as a marker for breast development and breast carcinoma, CD44 is a hyaluronate receptor^[11] that is involved in cell-cell and cell-matrix interactions and ultimately affects cellular growth, differentiation, and motility^[12,13]. Zhang *et al*^[14] found that CD44+/CD117+ cells had increased chemoresistance to taxane and platinum-based chemotherapy as well as the ability to self-propagate. Similarly, Alvero and colleagues showed that CD44+ cells were enriched in ovarian cancer patient ascites and once isolated and xenografted gave rise to tumor with both CD44+ and CD44- cells suggesting they can differentiate and self-renew^[15]. Orian-Rosseau described various strategies to target the CD44 receptor, which included binding to hyaluronic acid and osteopontin, a protein involved in interleukin production and overexpressed in ovarian cancer, as well as contributing to receptor tyrosine kinase activation^[16].

CD133 is a transmembrane glycoprotein that is ex-

Table 1 Cancer stem cell markers and significance

Cancer stem cell marker	Expression	Significance
CD44	Hyaluronate receptor	Cell growth, differentiation, motility, increased chemoresistance, self-propagation
CD133	Transmembrane glycoprotein	Increased tumor formation, increased chemoresistance, regeneration of original tumor cells
CD117	Tyrosine kinase receptor	Cell signaling, apoptosis, cell differentiation, proliferation, cell adhesion
ALDH1A1	Cell protector from aldehydes	Regeneration of tumor cells, chemoresistance
EpCAM (CD326)	Transmembrane glycoprotein	Cell adhesion, cell proliferation, tumor formation, epithelial to mesenchymal transition
CD24	Transmembrane glycoprotein	Cell adhesion, aggressive phenotype, metastasis

pressed in normal hematopoietic and epithelial stem cells, and has also been described as a CSC marker in solid tumors. Ferrandina *et al.*^[17] showed that the amount of CD133 positive cells was higher in ovarian carcinoma than in normal ovarian tissue. In 2009, Baba and colleagues reported the ability of CD133+ cancer cells to generate both CD133+ and CD133- cells, similar to what Alvero had seen with CD44+ cell spore^[18]. CD133 has also been shown to be involved in increased tumor formation, increased chemoresistance, and the ability to recapitulate the original heterogeneous tumor^[19].

CD117, also known as c-kit or stem cell growth factor receptor, is a proto-oncogene encoded by the KIT gene. It is a type of tyrosine kinase receptor involved in cell signal transduction. It has been shown to be involved in apoptosis, cell differentiation, proliferation, and cell adhesion^[20]. CD117 was shown by Kusumbe *et al.*^[21] to have high expression in ovarian cancer cells. Interestingly, cells expressing CD117 appear to be highly tumorigenic as it only takes approximately 10³ cells to be able to self-renew, differentiate, and regenerate tumor in mouse models^[22]. The Wnt/ β -catenin pathway which has been implicated in the development of chemoresistance is activated by CD117^[23].

ALDH1A1 is a member of the ALDH group of proteins, which contains 19 enzymes that function as cell protectors from carcinogenic aldehydes^[24]. Landen *et al.*^[25] declared it a putative CSC marker and showed its association with chemoresistance in ovarian carcinoma. Cells that are double positive for CD133 and ALDH1A1 have a greater ability to develop tumors in mouse models as compared to CD133+/ALDH1A1 - or ALDH1A1 +/CD133 - cells^[26]. Recently, Shank *et al.*^[27], showed that metformin decreased the population of ALDH+ cells in ovarian cancer cell lines as well as decreased the formation of tumor spheres in patient tumors. *In vivo*, they also presented that metformin would restrict the growth of whole tumor cell line xenografts^[27].

EpCAM (CD326) is a transmembrane glycoprotein involved in cell adhesion. EpCAM has been shown to have oncogenic signaling properties which result in cell proliferation and tumor formation^[28]. Higher expression of EpCAM has also been seen in metastatic ovarian tumors^[29] and it is involved in epithelial to mesenchymal transition leading to metastasis^[30].

Another glycoprotein identified as an ovarian CSC is CD24 which is a cell membrane glycoprotein involved in cell adhesion. In 2005, the movement of CD24 from the cell membrane to the cytoplasm in borderline ovarian tu-

mors was associated with microinvasion and omental implants as well as shorter survival time in adenocarcinoma of the ovary^[31]. Moulla *et al.*^[32] also demonstrated that the transition from membrane to cytoplasmic CD24 expression was associated with a more aggressive phenotype in borderline tumors.

CLINICAL SIGNIFICANCE

While it is interesting to utilize proteins to identify CSCs in various tissues, the clinical significance of these markers is still being determined. In 2012, Meng and colleagues reported on CD44+/CD24- cells in ovarian cancer cell line studies and patient ascites samples. Ovarian cancer cell line studies confirmed that increased numbers of CD44+ cells increased chemoresistance. Patient ascites samples with > 25% CD44+ cells had significantly decreased median progression-free survival (6 mo *vs* 18 mo, $P = 0.01$) as well as propensity to recur (83% *vs* 14%, $P = 0.003$)^[33]. Zhang and colleagues studied 400 ovarian cancer tissue samples for CD133 positivity. They found associations between CD133+ and higher grade ovarian tumors, advanced stage disease, and decreased response to chemotherapy. They also found that CD133+ tumors are associated with decreased overall survival ($P = 0.007$) and shorter disease free interval ($P < 0.001$)^[34]. In a study by Chau *et al.*^[23], they evaluated 3 patient samples in a xenograft mouse model and it was found that there was increased chemoresistance in patients with CD117+ tumor cells. In 65 ovarian cancer patients with advanced stage disease, greater than 20% of ALDH1A1+ cells correlated with decreased progression-free survival (6 mo *vs* 14 mo, $P = 0.035$)^[25]. Recently, Zhu *et al.*^[35] reported on overexpression of CD24 in epithelial ovarian cancer and found that it was an independent variable associated with a low survival rate, increased metastasis, and decreased survival time.

Recent studies have indicated an enriched population of CSCs in ovarian cancer patients with recurrent carcinoma as compared to patients with primary cancer. Rizzo *et al.*^[36] noted an increased percentage of side population cells (generally accepted to be CSCs) in the ascites of patients with first recurrence after platinum-based chemotherapy as compared to ascites of chemo-naïve patients. Steg *et al.*^[37] compared 45 matched primary and recurrent ovarian cancer patient samples for expression of stem cell markers including ALDH1A1, CD44, and CD133. Primary samples showed low densities of the markers,

but samples collected after primary therapy showed higher densities of ALDH1A1, CD44, and CD133 due to the death of the non-stem cells. Stem cell markers were also examined in this study and 14% of recurrent tumors showed overexpression of these markers compared to primary tumors.

TARGETING OF OVARIAN CSCS

Stem cell markers have been implicated in chemoresistance and recurrence of ovarian cancer; therefore, it is reasonable to evaluate agents that could target these cells. CD44 has been studied with phase I trials in head and neck cancer *via* an antibody drug conjugate, BIWI 1^[38]. There have also been several monoclonal antibodies designed to target CD44 in squamous cell cancers which could be extrapolated to adenocarcinomas^[39]. CD44+ cells have been targeted in an intraperitoneal (IP) mouse model with cisplatin via a conjugate of hyaluronic acid and cisplatin which was then internalized more efficiently than CD44+ cells in ovarian cancer cell lines (A2780 and OV2008). Li and Howell^[40] also demonstrated decreased growth in IP inoculated A2780 ovarian cancer cells treated with a hyaluronic acid-cisplatin conjugate when compared to free cisplatin. A hyaluronic acid-paclitaxel (HA-TXL) conjugate to target CD44+ cancer cells has also been studied in an IP mouse model with ovarian cancer cell lines (SKOV3ip1 or HeyA8) and showed significantly reduced tumor weights and nodules^[41]. Similarly, CD133 has been targeted by IP administration of an anti-CD133 targeted toxin (dCD133KDEL), in an ovarian cancer cell line (NIH:OVCAR5-luc) in a mouse model, which resulted in significant decrease in progression of CD133 expressing tumors^[42].

Noguera *et al*^[43] evaluated imatinib mesylate, a CD117 specific inhibitor, in low grade recurrent platinum resistant tumors of the ovary in a single site phase II trial. Thirteen patients were enrolled and 48% of those had c-kit positive tumors. Eleven patients were eligible for evaluation of response, and though well-tolerated, no antitumor activity was seen in these low-grade tumors^[43]. An anti-EpCAM monoclonal antibody, catumaxomab, was evaluated in a phase II/III trial in 258 patients with malignant ascites from epithelial cancer, half of which were ovarian carcinomas. When compared to paracentesis alone for treatment of ascites, addition of catumaxomab increased the median time to next paracentesis (11 d *vs* 77 d, $P < 0.0001$). Patients who received catumaxomab also had decreased signs and symptoms of ascites. The safety profile was acceptable^[44]. Catumaxomab was evaluated in conjunction with steroid premedication (Catumaxomab Safety Phase IIIb Study with Intraperitoneal Infusion in Patients with Malignant Ascites Due to Epithelial Cancer) as well as in retreatment with IP therapy (SECIMAS), but results from these studies have not yet been posted (www.clinicaltrials.gov). It is also being evaluated in combination with cytotoxic chemotherapy in a phase II trial [ENGOT-ov8]^[45].

Another method of targeting CSCs is to target their signaling pathways, which include Notch, Wnt/ β -catenin, TGF- β , and Hedgehog pathways. McAuliffe and colleagues demonstrated this concept with the Notch pathway and platinum resistant ovarian cancer^[46]. In particular they looked at Notch3, and showed that it was overexpressed in ovarian CSCs and was correlated with increased platinum resistance. A pan Notch inhibitor, gamma-secretase inhibitor (GSI), when used in combination with cisplatin, had a synergistic cytotoxic effect, and led to decreased numbers of CSCs (12.8% side population cells in the control, 2.31% with Notch inhibitor alone, and 0.81% with GSI and cisplatin). A Notch ligand, Jagged 1, was targeted in taxane-resistant ovarian cancer cell lines by Steg *et al*^[47]. They showed that targeting Jagged1 induced chemosensitivity to docetaxel *in vivo* and reduced tumor weights. They implicated the Hedgehog pathway in these experiments with Jagged1 by showing that rather than the chemoresistance being mediated by MDR1 as expected, it was GLI2, a Hedgehog downstream marker, that was downregulated. Another study with Jagged1 found that inhibition of the Wnt/ β -catenin signaling pathway reduced its expression^[48]. Wnt/ β -catenin pathways have previously been demonstrated to produce self-renewal in ovarian cancer and appear to be a driving force behind ovarian cancer progression^[49]. The Hedgehog signaling pathway has been implicated in the growth regulation of spheroid-forming cells in ovarian cancer. This was demonstrated by Ray *et al*^[50], in four ovarian cancer cell lines (ES2, TOV112D, OV90, and SKOV3) where spheroid volume was increased up to 46-fold with Hedgehog agonists. Cyclopamine, a Hedgehog inhibitor, was used to prevent further growth of spheroid-forming cells in these cell lines and showed up to a 10-fold reduction in growth in ES2 cells^[50]. Multiple groups are actively working to target these signaling pathways in hopes of altering ovarian cancer chemoresistance and recurrence.

CONTROVERSIES

Although there is growing evidence that ovarian CSCs are relevant, there are still many who debate the existence of these cells. At the forefront of this debate, remains the fact that a specific ovarian CSC marker has not been identified. None of the markers discovered are exclusively found in ovarian cancer cells. CD133 is recognized as the putative CSC marker for many human solid tumors, however, signaling pathways that regulate its behavior remain unknown^[51]. Some studies presented in this review may be showing that CSCs are more “tumorigenic” based on ability of preferential or improved grafting. It will give much more credence to the argument if some of the pathways or markers being targeted show significant clinical results.

FUTURE DIRECTIONS

If progression and development of chemoresistance is due to the ovarian CSCs, then specific therapy for CSCs

must be developed. In ovarian cancer, the use of monoclonal antibodies to many surface markers for CSCs has proven of some potential value. The most utilized monoclonal antibody, bevacizumab, an anti-vascular endothelial growth factor agent, has been shown to improve progression-free survival in advanced ovarian cancer^[52]. Recently, CSCs have been implicated in the hypoxic environment that bevacizumab creates, but this relationship has not yet been well defined^[53]. In addition to those mentioned previously, the anti-CD44 antibody, A3D8 was shown to produce significant apoptosis and arrest of cell cycle in the S phase for the SKOV3 ovarian cancer cell line by Du *et al.*^[54] and may represent a therapeutic option. Patients taking metformin for diabetes have previously been reported to have improved survival and some groups postulate that this relationship is due to the downregulation of CSC growth. A phase II trial is currently underway to evaluate this relationship (NCT01579812) (www.clinicaltrials.gov). There are over 3000 results when searching for clinical trials related to CSCs on Clinicaltrials.gov. While the majority of these are not specific for ovarian cancer, many are for breast cancer or other solid tumors, which have traditionally led to findings applicable to ovarian cancer.

CONCLUSION

It appears that ovarian CSCs are involved in chemoresistance and likely contribute to an overall poor prognosis in ovarian cancer patients. Researchers continue to study the role of ovarian CSCs and develop targeting agents for specific identification and therapeutic treatment. Clinical trials are ongoing for agents targeting ovarian CSCs and data from these trials will be important to determine future research directions aimed at improving survival in women with ovarian cancer.

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Histone modifications: Targeting head and neck cancer stem cells

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, and is responsible for a quarter of a million deaths annually. The survival rate for HNSCC patients is poor, showing only minor improvement in the last three decades. Despite new surgical techniques and chemotherapy protocols, tumor resistance to chemotherapy remains a significant challenge for HNSCC patients. Numerous mechanisms underlie chemoresistance, including genetic and epigenetic alterations in cancer cells that may be acquired during treatment and activation of mitogenic signaling pathways, such as nuclear factor kappa-light-chain-enhancer-of activated B cell, that cause reduced apoptosis. In addition to dysfunctional molecular signaling, emerging evidence reveals involvement of cancer stem cells (CSCs) in tumor development and in tumor resistance to chemotherapy and radiotherapy. These observations have sparked interest in understanding the mechanisms involved in the control of CSC function and fate. Post-translational modifications of histones dynamically

influence gene expression independent of alterations to the DNA sequence. Recent findings from our group have shown that pharmacological induction of post-translational modifications of tumor histones dynamically modulates CSC plasticity. These findings suggest that a better understanding of the biology of CSCs in response to epigenetic switches and pharmacological inhibitors of histone function may directly translate to the development of a mechanism-based strategy to disrupt CSCs. In this review, we present and discuss current knowledge on epigenetic modifications of HNSCC and CSC response to DNA methylation and histone modifications. In addition, we discuss chromatin modifications and their role in tumor resistance to therapy.

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Key words: Head and neck squamous cell carcinoma; Chromatin remodeling; Histone deacetylases inhibitor; Histone acetylation; Cancer-initiating cell; Epigenetic target; Epigenetic marker; Oral squamous cell carcinoma; Tumor resistance

Core tip: Stem cells are long-lived, therefore their genome is subject to more stress from genetic mutations and epigenetic factors than their short-lived, differentiated progeny. Recent evidence strongly indicates that a subpopulation of tumor initiating cells, termed "cancer stem cells", play a fundamental role in tumor heterogeneity, growth, and preservation. Cancer stem cell behavior is influenced by epigenetic events comprised primarily of DNA methylation and histone modifications that dynamically regulate gene expression and silencing.

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INTRODUCTION

There are approximately 560000 cases of head and neck cancer diagnosed worldwide each year and approximately 300000 deaths annually. This cancer type occurs in the head and neck region, involves the nasal and oral cavity, pharynx, and larynx and primarily occurs as squamous cell carcinoma (HNSCC)^[1-4]. Although HNSCC has well recognized risk factors, including tobacco use, excess alcohol consumption, and infection by high risk papillomaviruses^[5,6], we do not fully understand the mechanisms underlying its malignant progression^[5]. Our understanding of the molecular biology of HNSCC has significantly improved in the last few decades, contributing to the development of novel therapies targeted against pro-survival signaling circuitries, including the epidermal growth factor receptor (EGFR), vascular endothelial growth factor, receptor tyrosine kinases, interleukins, and phosphoinositide 3-kinase (PI3K) pathways, among others. Unfortunately, the long-term survival rate for HNSCC patients, which is 50% at five years after diagnosis, has remained consistent over the past thirty years^[3,7-9]. The incidence of HNSCC is much higher in developing nations, where it is the third most common malignancy in Asian countries compared to the sixth most common malignancy in Western countries^[10-12]. This discrepancy in incidence of HNSCC is associated with varying risk factors, such as chewing Betel quid in the Asia-Pacific region compared to consumption of tobacco and alcohol and/or human papillomavirus infection outside Asia^[1,13-17].

The poor long-term survival rates in HNSCC patients may be due to diagnosis of disease at an advanced-stage and development of chemoresistance^[8,18]. Numerous mechanisms underlie chemoresistance, including genetic and epigenetic alterations in cancer cells that may be acquired during treatment^[19,20] and the activation of mitogenic signaling pathways, such as nuclear factor kappa-light-chain-enhancer of activated B cell NFκB, that result in reduced apoptosis^[21]. Furthermore, the recurrence of cancers depend on a subpopulation of cancer stem cells (CSCs) that possess the unique and exclusive ability to self-renew and differentiate into nontumorigenic heterogeneous cell types that maintain the tumor^[7,22-24]. Therefore, many factors play a critical role in the maintenance of tumor heterogeneity and CSC behavior, including the tumor microenvironment, genomic instability and the effect of genetic mutations and epigenetic changes on gene expression^[22,25-27].

In a significant number of HNSCC, tumor progression results from mutations in genes, such as *TP53*, *CDKN2A*, *HRAS*, *PTEN*, and *PIK3CA*. This causes alterations in cell signaling cascades (*e.g.*, PI3K/mTOR, NFκB, ERK, p53), resulting in aberrant cell growth, migration, and survival^[3,8,23,28,29]. Epigenetic changes also play a key role in regulating gene expression through histone modifications, DNA methylation, miRNA silencing and DNA repair mechanisms [HMT (Histone methyltransferases), HAT (Histone acetyltransferases), HDAC (Histone deacetylases) ncRNA (non-coding RNA), and lncRNA

(long non-coding RNA)]^[30-33]. Consequently, by identifying the molecular mechanisms that drive progression and recurrence of HNSCC, novel cancer therapeutics can be developed to improve the effectiveness of treatment and the rate of long-term survival in patients. In this review, we highlight the current understanding on cancer stem cells and the effects of epigenetic modifications on tumor behavior. We also discuss the latest findings on pharmacological manipulation of epigenetic circuitries that may result in the development of novel therapeutic strategies that target cancer stem cells.

CANCER STEM CELLS

Because normal stem cells are long-lived, their genome is subject to more stress from genetic mutations and epigenetic factors than their short-lived, differentiated progeny. The majority of oncogenic mutations in stem cells perturb central cellular processes that regulate cellular division, DNA damage repair, and signal transduction pathways^[24,25,34]. Certain HNSCC-related phenotypes that arise from mutations in oncogenes and tumor suppressor genes, such as *PIK3CA*, *TP63*, *PTEN*, *EGFR*, and *MET*, result in limitless replication potential, insensitivity to apoptotic signals, angiogenesis, invasion and metastasis^[28,35-38]. Therefore, tumors arise when stem cells lose their ability to regulate and maintain tissue form and function and when they show reduced control over apoptosis, cellular senescence and cellular proliferation. Additionally, although tumors are a population of malfunctioning cells, they are commonly characterized by histological features that resemble normal tissue^[39]. Similarly, hematopoietic cancers are comprised of identical neoplastic cells, but solid tumors from HNSCC consist of non-identical cells, resulting in phenotypic heterogeneity^[25,27,40-42]. Within the polyclonal tumor, there is a cellular hierarchy in which a small subpopulation of neoplastic cells with the highest potential for tumorigenesis and self-renewal are positioned at the top. The remaining bulk of the tumor primarily consists of well-differentiated non-tumorigenic cells that are susceptible to chemotherapy and radiation^[43-45]. In addition to HNSCC, solid tumors of the breast, brain, colon, lung and prostate also demonstrate a diverse array of cellular heterogeneity that increases genomic instability and adaptability of the tumor to its microenvironment^[25,46,47]. Recent evidence strongly indicates that a subpopulation of tumor initiating cells, termed “cancer stem cells” play a fundamental role in tumor heterogeneity, growth, and preservation^[25,44,48,49]. The cancer stem cell hypothesis, first conceptualized by Bonnet *et al.*^[44] in 1997, established that a subpopulation of human leukemic cells, positive for CD34 and negative for CD38 cell surface markers, initiates human acute myeloid leukemia in Non-obese diabetic/Severe combined immunodeficient (NOD/SCID) mice. The following observations support the cancer stem cell hypothesis: (1) only a subpopulation of tumor cells within a tumor mass grow in immunodeficient mice; (2) the subpopulation of

tumor cells generate both CSCs and heterogeneous non-tumorigenic cancer cells; and (3) cancer stem cells self-renew, as revealed by serial transplantation assays^[22,44,50]. The frequency of CSCs is relatively low in HNSCC, lung squamous cell carcinoma, lung adenocarcinoma, and human pancreatic adenocarcinoma, but xenotransplantation assays greatly increase their frequency^[51].

Cancer stem cell surface markers

CSCs were first discovered in solid tumors in 2003^[52], and the isolation of CSCs in HNSCC, based on the CD44+ cell surface marker, occurred in 2007^[18]. In that study, approximately 70% of NOD/SCID mice receiving CD44+ tumor cell xenografts showed tumor formation compared to 1% of mice receiving CD44- xenografts. In addition to their association with CSCs in HNSCC^[53-56], CD44+ cells also play a role in chemoresistance. Genes associated with chemoresistance, including ABCB1, ABCG2, CYP2C8 and TERT, are upregulated in CD44+ cells compared to CD44- cells^[57]. Furthermore, CD44+ HNSCC cells express high levels of B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1), a self-renewal and oncogenic protein associated with poor survival and tumor aggressiveness^[18,58-62]. Different isoforms of CD44 differentially modify the behavior of HNSCC. For instance, the v3, v6, and v10 isoforms of CD44 promote HNSCC tumor migration, invasion, and metastasis^[63,64] and confer chemoresistance in other solid tumors, attributes commonly associated with the chemo- and radio-resistant fractions of cancer stem cells^[65]. Therefore, CD44 is used to identify CSCs, and it promotes many of the biological characteristics associated with cancer “stemness”. These characteristics include tumorsphere formation in suspension, unrestricted cellular proliferation, enhanced migration, tumor invasion, and resistance to chemotherapy and ionizing radiation therapy. CD24 and CD133 (also known as Prominin 1) are also CSC cell surface markers^[66-68].

The increased enzymatic activity of aldehyde dehydrogenase 1 (ALDH1) is commonly used to identify normal pluripotent cells and tumor cells harboring “stemness” potential in various solid tumors, including HNSCC^[51,69-73]. ALDH is a detoxifying enzyme involved in the oxidation of intracellular aldehydes and was initially described for its role in hematopoietic stem cell self-renewal *via* reduction of retinoic acid activity^[76,77]. The presence of ALDH1-positive tumor cells correlates with poor clinical outcome in breast cancer^[69], ovarian cancer^[78], papillary thyroid carcinoma^[79], and pancreatic adenocarcinoma^[80], among other solid tumors^[70,81-83].

It is believed that HNSCC progression and invasion, in addition to resistance to non-surgical therapies, may be regulated by the rare population of CSCs^[18,43,84,85]. Therefore, to effectively treat this type of cancer, we must develop a therapy that can target and eliminate CSCs.

EPIGENETICS OF HEAD AND NECK CANCER AND ITS STEM CELLS

Basic concepts of epigenetic regulation

DNA methylation: When exploring the molecular mech-

anisms underlying cancer, DNA methylation is the most commonly studied epigenetic alteration^[86-88]. DNA methylation patterns occur in early and precancerous stages and most frequently discovered in tumors compared to normal tissues^[89,90]. Methylation occurs sporadically and is globally distributed in mammals throughout the genome at cytosine-phospho-guanine (CpG) dinucleotide sequences, as revealed by immunofluorescent labeled 5-methylcytosine. Without considering CpG-rich islands (approximately 1 kilobase in length), there is a low, but global level of methylation in specific CpG sequences throughout the entire mammalian genome^[26,91]. Therefore, aberrant DNA methylation of these CpG islands or specific sequences can lead to oncogenic activation *via* silencing of tumor suppressor gene expression^[92,93]. Hypomethylation is associated with activation of oncogenes, while hypermethylation is associated with the silencing of tumor suppressor genes. Both mechanisms induce genomic instability and play a dominant role in tumor initiation and progression^[90,94]. The most common types of DNA methylation in tumors are hypermethylation of CpG islands and global hypomethylation^[89]. Hypermethylated CpG islands are often associated with gene promoters; thus, methylation results in a transcriptionally inactive gene. In contrast, methylation of DNA sequences further from promoter sequences has less of an effect on transcription^[26].

Histone modifications: In addition to DNA methylation, the chromatin architecture can be remodeled by a network of protein mediators called histones that play an important role in gene regulation by compacting DNA. Histones can be post-translationally modified at the amino-terminal ends by acetylation, methylation, phosphorylation, sumoylation, ubiquitination, and ADP-ribosylation^[95]. These modifications result in gene transcription through the uncoiling of chromatin or gene silencing through compacting DNA^[96]. HAT, HMT, and HDAC are key co-factors that modify histones and produce the epigenetic changes observed in cancer. Histone acetylation, deacetylation and methylation are the major marks associated with transcriptional activity. Histone acetylation results in chromatin decondensation, promotion of transcription, and inhibition of DNA methylation, and is often correlated with the formation of euchromatin. In contrast, histone deacetylation is the predominant epigenetic influence in transcriptional gene silencing^[95,97,98]. In general, histone modifications modulate a diverse array of biological processes, including gene regulation, DNA repair, mitosis and meiosis *via* chromosome remodeling^[99].

Histone acetylation and deacetylation: Dysregulation of the exquisite interplay between acetylation and deacetylation controlled by HAT and HDAC is coupled to the initiation and progression of cancer, cellular plasticity, inflammation, and dynamic transformation in metabolic cascades^[100,101]. In addition to the histone sub-

strate peptides described in^[102], HAT is associated with non-histone proteins, transcription co-factors, such as p53, p65, c-MYC, NFκB, STAT3 (signal transducer and activator of transcription 3) and BRCA1 (breast cancer 1), among others^[30,103]. In particular, acetylation of the p53 tumor suppressor and pro-apoptotic protein by the CBP (CREB-binding protein)/p300 family of HATs has been extensively reviewed in^[104,105]. Modification of p53 is associated with increased DNA binding affinity, transcriptional activity^[106,107] and protein stability^[108]. Similar to p53, CBP/p300 is associated with the pro-proliferative and oncoproteins previously listed, and its expression impacts a variety of human diseases, such as leukemia^[109,110], lung cancer^[111], colon cancer^[112], bladder cancer^[113] and prostate cancer^[114-116]. CBP/p300 is also associated with transcription factors involved in heart disease^[117,118], diabetes^[119,120] and neurological disorders^[121,122].

Histone methylation: Histone methylation is the third major epigenetic process that affects transcriptional activation *via* chromatin remodeling. Similar to previously described post-translational histone modifications, methylation and demethylation of amino acids at different sites on histones either promotes or prevents transcriptional activity^[123]. For example, methylation of lysine residues is associated with transcription and DNA repair, but methylation of arginine residues is only associated with transcription^[95,124,125]. Histone H3 is methylated at different lysine sites, including K4, K9, K27, K36, and K79, that experience various methylated states, including mono-methylated, dimethylated, and trimethylated. Therefore, the epigenetic modification of the chromatin depends on the location and state of methylation^[126,127]. K9 and K27 methylation is associated with heterochromatin formation and inactive transcription. In contrast, K4 methylation is associated with euchromatin formation and active transcription^[128,129].

HAT and HDAC inhibitors: The development of HAT inhibitors (HATi) are in the early stages of preclinical studies. Although drugs that regulate HDAC activity are being used for cancer treatment, there is great interest in developing HAT inhibitors as a potential treatment for cancer and other human diseases^[130]. Several natural compounds effectively inhibit HAT activity. For example, Marcu *et al*^[131] demonstrated that curcumin inhibits HAT activity by promoting proteasome-dependent degradation of CBP/p300 in both prostate cancer cells and in HDAC inhibitor-induced peripheral blood lymphocytes. In addition, epigallocatechin-3-gallate and plumbagin are selective inhibitors of CBP/p300^[132-134]. The potential for HDAC inhibitors (HDACi) to serve as cancer chemotherapeutics has been examined in clinical trials due to the role of HDAC in genome stability, proliferation, differentiation, apoptosis, and metabolism. A current list of HDACi under clinical investigation can be found in a review by Li *et al*^[135] that focuses on HDAC and its clinical implications in cancer therapy.

In summary, epigenetic modifications constitute the next frontier in tumor biology research. Post-translational modification of histones dynamically influences gene expression independent of alterations to the DNA sequence. These mechanisms are often mediated by histone linkers, proteins associated with the recruitment of DNA-binding proteins, HDAC I and II interacting proteins and transcriptional activators, coactivators or corepressors. Therefore, histones are molecular markers of epigenetic changes^[136].

Epigenetic regulation of HNSCC

In HNSCC and other carcinomas, the combination of genetic and epigenetic factors affect gene expression, resulting in altered downstream cellular signaling pathways that regulate tumor growth, anti-apoptosis, DNA repair, resistance to extrinsic factors, angiogenesis, and epithelial-mesenchymal transition (EMT)^[31,137-140]. Although both genetics and epigenetics may affect the initiation and progression of HNSCC, epigenetic factors regulate gene expression in the absence of genomic mutations^[19,141,142]. Therefore, epigenetics is defined as a stable heritable phenotype passed on through either mitosis or meiosis, resulting in changes in chromosome characteristics without inducing genome alterations, as proposed by Conrad Waddington in the early 1940s^[143-145].

Tumor development is a multi-stage process that requires the accumulation of numerous genetic mutations and often results in gain-of-function in oncogenes and loss-of-function in tumor suppressor genes^[146-150]. In addition to genetic mutations, tumor development and progression is extensively influenced by changes in gene expression independent of alterations in the DNA sequence, a mechanism known as epigenetic modification. Epigenetic events are comprised primarily of DNA methylation and histone modifications that dynamically regulate gene expression and silencing^[19,31,141,142,151]. These dynamic processes occur within the chromatin that is packed into the nucleus through interactions with core histone proteins.

The effect of chromatin on cellular behavior depends on how tightly DNA is spooled around H2A, H2B, H3 and H4 core histones^[152]. Together, histones and DNA form nucleosomes, the fundamental units of chromatin. Gene expression is driven by the ability of chromatin to fold and unfold in a process that requires rapid acetylation/deacetylation of the histone core, resulting in alterations in the cellular response to environmental cues^[153].

DNA methylation in HNSCC: In Demokan *et al*^[89] extensive review^[89] of DNA methylation in head and neck cancers, they provide a list of the most frequently methylated genes. In this list, the hypermethylated genes include the following: (1) Adenomatous polyposis coli (APC), which is the most common gene methylated in HNSCC^[154,155]; (2) p16, a cell cycle controller encoded by the *CDKN2A* gene, which plays a critical role in inducing cellular senescence in tumor cells and is downregulated

via promoter hypermethylation^[156-167]; and (3) p14, also known as ARF, that in combination with p16 is involved in regulating the cell cycle and in activating the p53 tumor suppressor gene by inhibiting MDM2^[168]. Surprisingly, in 96 human samples of oral squamous cell carcinoma, methylation of p14ARF is associated with a good prognosis, methylation of MINT1 and MINT31 is associated with poor prognosis, and DCC methylation is associated with increased bone invasion by squamous cell carcinoma from the gingiva^[169]. Notably, Carvalho *et al.*^[159] and Ogi *et al.*^[169] also identified methylated MINT31 as an independent predictor of outcome and showed its association with the T4 disease group, according to the Union for International Cancer Control classification. *RASSF1A* is a tumor suppressor gene that is frequently silenced in tumors, including HNSCC. *RASSF1A* is involved in the maintenance of genomic stability and is highly mutated in poorly differentiated HNSCC compared to moderate and well-differentiated HNSCC^[154, 159, 160, 163, 165, 167, 170, 171]. *RASSF2* is a novel Ras-associated protein that negatively regulates Ras signaling^[172]. *RASSF2* binds directly to K-Ras in a GTP-dependent manner promoting apoptosis and cell cycle arrest; however, *RASSF2* weakly interacts with H-Ras. In solid tumors, including human colorectal cancer and HNSCC, *RASSF2* is frequently silenced by DNA methylation at 5' CpG islands^[167, 173].

Other interesting genes methylated in head and neck cancer include EDNRB, a member of the G protein-coupled receptor family that encodes endothelin receptor type B protein; EDNRB is methylated in 97% of primary HNSCC tissues^[174]. EDNRB is involved in the development and function of blood vessels, cellular growth and mitosis^[174]. Another gene methylated in HNSCC is RARB, which encodes retinoic acid receptor beta and restricts cell growth by altering gene expression. Hypermethylation of RARB results in loss of function and reduced control of transcription^[154, 162, 163, 167, 175, 176]. Currently, only a few methylated genes can predict the clinical outcome of HNSCC patients. It is unknown how methylated genes correlate with cancer therapy, patient response and tumor progression and behavior. Methylation analysis techniques have revealed that methylation patterns are not affected by external factors and are increased during cancer progression. Therefore, as with stem cell surface markers, increased sensitivity and specificity of quantitative methodologies for DNA methylation analyses will allow scientists to develop prognostic tools for clinical evaluation of head and neck cancer.

Histone methylation in HNSCC: Mancuso *et al.*^[177] showed that the level of H3K4 methylation is significantly different in normal mucosa compared to oral squamous cell carcinoma (OSCC) tissues, with dimethylated K4 increased and trimethylated K4 decreased. A similar trend was observed in oral leukoplakias compared to the pathological sample^[177]. H3K9 and H3K27 are targets for methylation by enhancer of zeste homolog 2 (EZH2), a member of the Polycomb-group family, resulting in gene silencing *via*

chromatin condensation^[178-181]. Interestingly, overexpression of EZH2 is associated with malignancy and prognosis of a variety of cancers, including breast^[182, 183], prostate^[184-186], gastric^[187], hepatic^[188], bladder^[189, 190] and oral squamous cell carcinoma^[129, 191]. Wei *et al.* showed that increased expression of EZH2 is associated with dysplasia and malignant transformation. Similarly, Kidani *et al.*^[191] revealed that overexpression of EZH2 is associated with tumor progression, malignancy and poor prognosis in OSCC. Collectively, these data reveal that different histone methylation patterns can greatly influence gene expression in cancer, thereby affecting malignant behavior.

Histone acetylation in HNSCC: Early evidence suggested that histones and their modifiers are involved in sophisticated processes that modulate tumor behavior and cellular phenotype. We recently reported that chromatin folding in HNSCC during tumor response to environmental cues dynamically modulates tumor behavior and cellular phenotype^[151]. We found that HNSCC cell lines are hypoacetylated compared to normal mucosa controls (Figure 1A). Furthermore, we found that endothelial cell-secreted factors, but not fibroblast cell-secreted factors, are able to trigger the acetylation of histones in tumor cells (also referred to as tumor histones) (Figure 1B). In fact, paracrine-induced histone modifications resulted in enhanced expression of Bmi-1, a transcriptional repressor upregulated in a variety of cancers and associated with tumor aggressiveness, and poor survival along with the expression of vimentin, a canonical marker of EMT (Figure 1B)^[192-199]. Similar to our *in vitro* findings, human HNSCC samples presented coexpression of acetylated histone 3 and vimentin in the proximity of normal endothelial cells (Figure 1C-white dashed line) next to the tumor invasion front in human HNSCC samples (Figure 1C-yellow dashed line). Therefore, acetylation of tumor histones are associated to changes in cellular behavior, phenotype and associated to increased invasion. In fact, malignant tumors derived from epithelial cells (carcinomas) are known to undergo EMT that precedes local invasion and metastasis of cancer cells^[200-204]. EMT is characterized by the loss of cell adhesion, increased motility, aggressive behavior, acquisition of an elongated fibroblastoid morphology and expression of vimentin^[200, 205, 206], similar to what we observe with pharmacological inhibition of HDAC in HNSCC cell lines (Figure 2-HN6 and HN13 cells). Interestingly, cellular morphology is not altered and vimentin is not induced in normal epithelial cells (NOK-SI) treated with HDAC inhibitors, suggesting that hyperacetylation of chromatin differentially modulates normal and neoplastic cells (Figure 2). However, changes in the acetylation of HNSCC chromatin also triggered an unexpected phenotype, which was the loss of CSCs. HNSCC treated with Trichostatin A, a histone deacetylase inhibitor, lose the ability to generate and maintain tumor spheres and experience rapid reduction in the enzymatic activity of ALDH1 (Figure 3)^[151]. It has been suggested that epigenetic signals play a major role

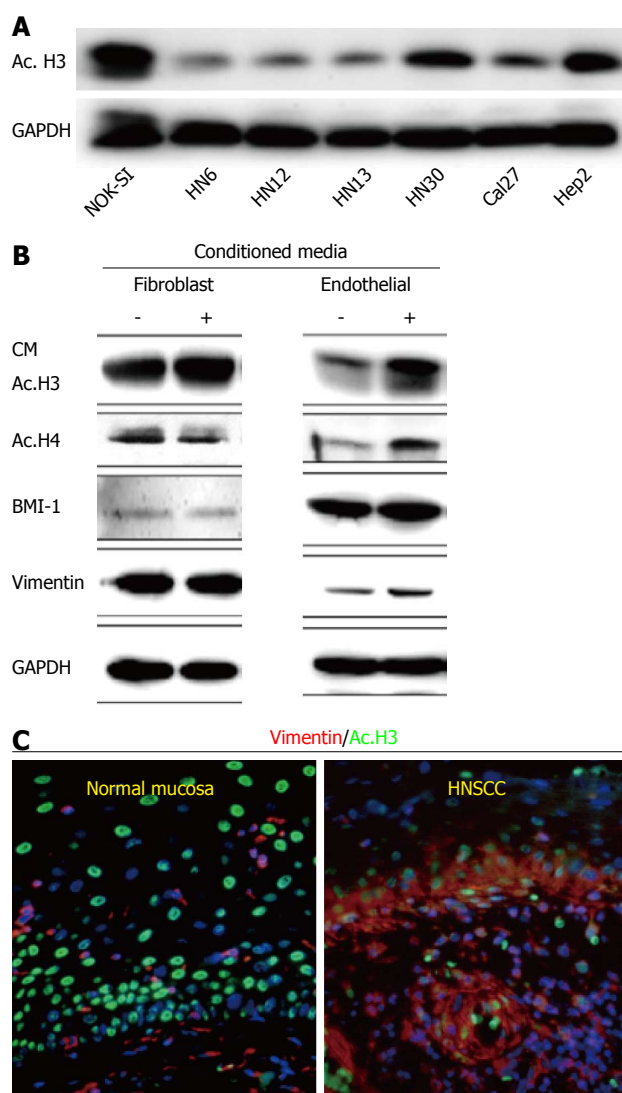


Figure 1 Data represents acetylation status of histone 3 in Head and Neck Squamous Cell Carcinoma by Giudice *et al.*^[151]. A: Tumor cells present hypoacetylation of histone 3 (ac.H3) in a panel of Head and Neck Squamous Cell Carcinoma (HNSCC) compared to control cells (NOK-SI); B: Endothelial cell-secreted factors are capable of inducing ac.H3 while fibroblast cell-secreted factors cannot. Also, endothelial cell-secreted factors induce increased expression of BMI-1 and vimentin compared to the fibroblast counterpart; C: Representative examples of human samples of normal oral mucosa and HNSCC. Note acetylated tumor cells (Ac. H3-FITC) with high levels of the epithelial-mesenchymal transition marker vimentin (TRITC) are localized at the invasion front of HNSCC (arrow). Normal mucosa display acetylated cells distributed throughout the epidermis but do not express vimentin. ac. H3: Acetyl histone 3; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; CM: Conditioned Media; BMI-1: B lymphoma Mo-MLV insertion region 1 homolog; NOK-SI: Normal oral epithelial keratinocytes.

in stem cell control through deacetylation of histones, which promotes chromatin condensation and reactivation of stem cell-like transcription programs^[34]. These striking findings suggest that chromatin acetylation selectively disrupts the physiological requirements for maintenance of CSC. Indeed, chromatin acetylation has long been known to induce cellular differentiation and restrict cellular transformation of normal cells^[34,207,208].

In summary, histone modifications *via* methylation,

acetylation and deacetylation play a critical role in transcriptional activation and gene expression. Aside from the physiological maintenance of cellular homeostasis, aberrant alterations in histone methylation proteins and/or an imbalance in the HAT/HDAC network results in dysfunctions in cellular processes, such as proliferation, differentiation, DNA repair and apoptosis. Importantly, post-translational histone modification and DNA methylation can have similar patterns in the same cancer type. For example, a study by Piyathilake *et al.*^[209] revealed that patterns of global DNA and histone methylation are similar in different human mucosal tissues (*e.g.*, normal, dysplastic and squamous cell carcinoma). Using immunohistochemical analysis, they also found that global DNA methylation and H3 methylation at lysine 4 and lysine 9 are significantly higher in dysplastic lesions and carcinoma cells compared to normal oral epithelium^[209]. Therefore, when developing methods and techniques for identifying epigenetic markers in premalignant cells, we must consider analyzing both global DNA and histone methylation levels concurrently in the progression of cancer. In conclusion, the previously described epigenetic alterations are closely associated with tumorigenesis and malignancy in many types of cancers. As a result, genomic instability affects numerous intracellular signaling cascades. We will discuss the NF κ B signaling pathway in the next section.

TUMOR HISTONE MODIFICATIONS: EVIDENCE FOR AN EPIGENETIC MECHANISM RESPONSIBLE FOR ACQUIRED TUMOR RESISTANCE TO THERAPY

NF κ B is an epigenetic modifier that plays a major role in malignant transformation^[210], and this pathway serves as a target for epigenetic drugs^[211-213]. We, along with others, have previously reported that constitutive activation of NF κ B signaling is often observed in HNSCC, suggesting a common epigenetic mechanism in HNSCC biology^[214,215]. Indeed, activation of NF κ B signaling in HNSCC induced chromatin compaction and acquisition of resistance to chemotherapy^[216]. NF κ B is active following its translocation to the nucleus, a process that is regulated by the I κ B kinase (IKK) complex. I κ B proteins are targeted for degradation by phosphorylation, which permits nuclear translocation. Nuclear NF κ B binds to target DNA sequences and modulates the expression of target genes involved in immune response, cell growth, and cell survival^[217]. Targeted inhibition of NF κ B through IKK α and IKK β silencing resulted in disrupted accumulation of nuclear phospho-p65, increased acetylation of histone 3 and accumulation of BRCA1. Collectively, we showed that NF κ B epigenetically modulates chromatin organization and recruits BRCA1 to the nucleus. Indeed, histone 3 is acetylated following loss of NF κ B, resulting in decondensation of tumor chromatin and sensitization of

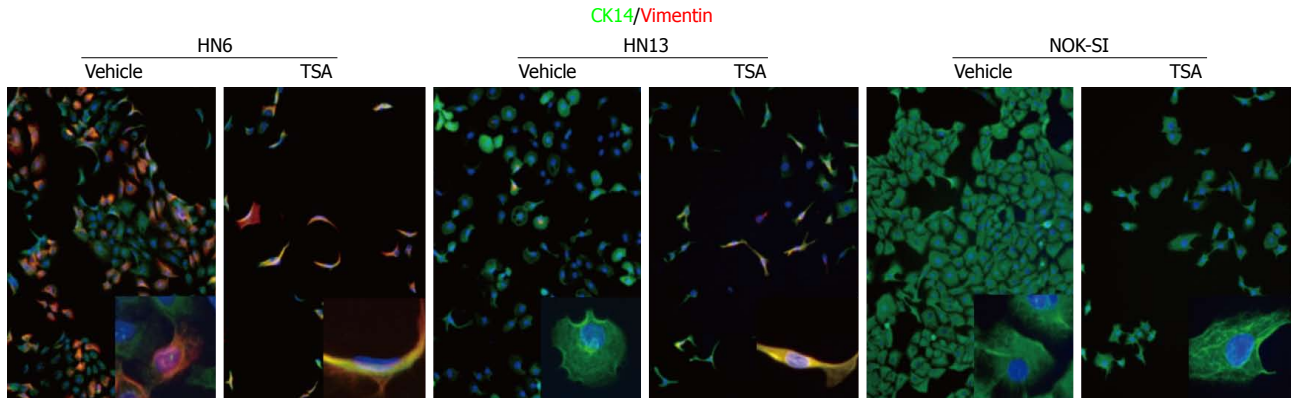


Figure 2 Figure from Giudice *et al.*^[15] depicting chemically-induced chromatin acetylation leading to activation of the epithelial-mesenchymal transition phenotype. Inhibition of HDAC induces vimentin expression in HNSCC cells and EMT. Vehicle treated HNSCC cells (HN6 and HN13) present an epithelioid shape and express CK14. Administration of TSA result in acquisition of a fusiform morphology and expression of vimentin. Normal keratinocytes (NOK-SI) are not sensitive to EMT upon administration of TSA. TSA: Trichostatin A; CK14: Cytokeratin 14; EMT: Epithelial-mesenchymal transition; HDAC: Histone deacetylases; HNSCC: Head and Neck Squamous Cell Carcinoma; NOK-SI: Normal oral epithelial keratinocytes.

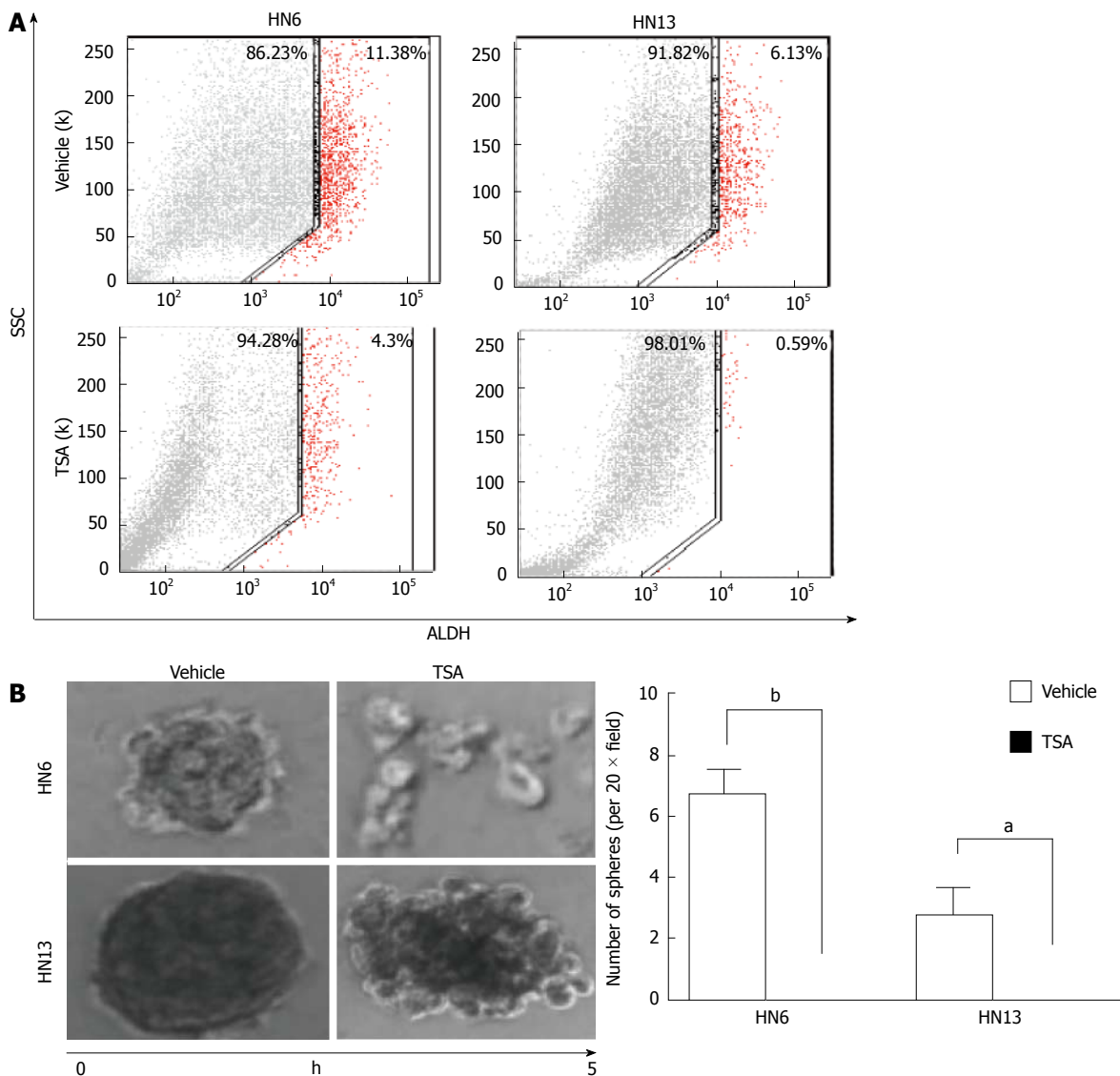


Figure 3 Data from Giudice *et al.*^[15] showing the impact of histone deacetylase inhibitor on the population of CICs. A: Fluorescence-activated cell sorting of ALDH+ cells demonstrates that HNSCC cell lines have a high number of CICs, and that administration of TSA reduced total number of ALDH+ cells; B: HDACi (TSA) disrupts tumor spheres as depicted in representative images of tumor spheres and by quantification of spheres (HN6 ^b $P < 0.01$, HN13 ^a $P < 0.05$). TSA: Trichostatin A; ALDH: Aldehyde dehydrogenase; SSC: Side scatter of light; HDACi: Histone deacetylase inhibitors; HNSCC: Head and neck squamous cell carcinoma.

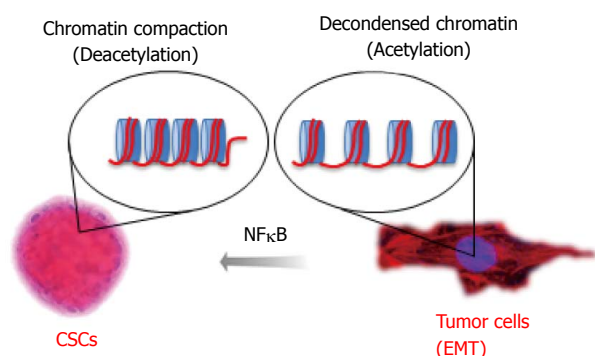


Figure 4 Data from Almeida *et al* proposing the mechanism for nuclear factor kappa-light-chain-enhancer of activated B cells driven resistance to chemotherapy in head and neck squamous cell carcinoma. Chromatin undergo normal compaction and decondensation through the acetylation of core histones organized in nucleosomes. Acetylation of tumor histones driven by expression of NFκB influences tumor behavior and plasticity of Cancer Stem Cells. NFκB: Nuclear factor kappa-light-chain-enhancer of activated B cells; EMT: Epithelial to mesenchymal transition.

head and neck tumors to chemotherapy. This indicates that the effect of NFκB on chromatin organization directly influences tumor response to therapy. As proof of concept, administration of HDAC inhibitors recapitulate the effects of NFκB targeted inhibition by promoting chromatin decondensation and sensitizing tumor cells to chemotherapy, resulting in increased sensitivity of tumor cells to chemotherapy (Figure 4).

In addition to chemoresistance, activation of NFκB signaling increases the number of tumor spheres, indicating a broader role of NFκB as an epigenetic switch in CSCs. Notably, NFκB signaling is required for the development of tumor spheres in breast, cervical and head and neck cancers^[218] (Almeida and Castilho, submitted). We established that by controlling tumor histones, we can dynamically regulate the behavior and number of HNSCC and its CSCs^[151]. Epigenetic signals may play a major role in stem cell control through deacetylation of histones, which promotes chromatin condensation and reactivation of stem cell-like transcription programs^[34]. Aligned with previous reports^[219-221], we showed that HNSCC tumor cell lines have a subpopulation of CSC, as detected by elevated ALDH activity, and clonogenic potential^[151]. This subpopulation of CSCs is highly tumorigenic and can self-renew, as observed by serial transplantation assays^[37]. By inhibiting HDAC and inducing acetylation of tumor histones, we found that CSCs lose their “stemness”, as evidenced by a reduction in ALDH+ cells and progressive disruption of tumor spheres. These findings indicate that HDAC inhibition disrupts the physiological requirements for CSC maintenance. Indeed, chromatin acetylation induces cellular differentiation and restricts cellular transformation^[207,208].

Altogether, HNSCC behavior appears dependent on dynamic changes in chromatin organization and subsequent gene transcription. Unlike stable DNA modifications mediated by methylation, acetylation of histones

dynamically alters gene expression, thereby influencing tumor behavior following changes in the microenvironment as observed during administration of secreted factor from endothelial cells^[151] and expression of tumor aggressiveness markers^[222-225].

CONCLUSION

The role of epigenetic modifications in HNSCC warrants further investigation. Compared to histone modifications, the role of DNA methylation in regulating gene expression is better characterized. Nonetheless, recent studies have correlated the effects of histone acetylation in the dynamic process of tumor adaptation to its microenvironment and the acquisition of a resistant phenotype^[151]. The identification of the NFκB signaling pathway as an epigenetic modulator of tumor behavior and resistance to chemotherapy further improved our knowledge in the intricate molecular mechanism of HNSCC and further clarified our understanding of the NFκB signaling pathway^[216]. Novel therapeutic strategies can now be developed that target epigenetic alterations driven by histone modifications, and the NFκB signaling may serve as an ideal adjuvant target for therapy. The development of personalized therapies specific for tumor subtypes, in this case tumors with active NFκB signaling, holds the promise of preventing tumor resistance and sensitizing tumors to chemotherapy. Recent advances in genome sequencing, including next-generation sequencing (NGS), have also improved our understanding of altered molecular signaling in HNSCC. NGS was used to identify single-base changes and larger structural variants characterized by insertions, deletions, translocations and viral insertions in HNSCC^[3,226]. Interestingly, NGS also revealed that HNSCC have a significant number of mutations in histones, histone modifiers, transcriptional activators and coactivators, and transcription regulators, further emphasizing the complexity of tumor signaling^[30]. Collectively, emerging knowledge about tumor behavior and how it correlates with dynamic changes in gene expression mediated by epigenetic events have substantially clarified the concept that successful therapeutic strategies will require targeting of both genetic and epigenetic pathways.

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Role of liver stem cells in hepatocarcinogenesis

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Abstract

Liver cancer is an aggressive disease with a high mortality rate. Management of liver cancer is strongly dependent on the tumor stage and underlying liver disease. Unfortunately, most cases are discovered when the cancer is already advanced, missing the opportunity for surgical resection. Thus, an improved understanding of the mechanisms responsible for liver cancer initiation and progression will facilitate the detection of more reliable tumor markers and the development of new small molecules for targeted therapy of liver cancer. Recently, there is increasing evidence for the "cancer stem cell hypothesis", which postulates that liver cancer originates from the malignant transformation of liver stem/progenitor cells (liver cancer stem cells). This cancer stem cell model has important significance for understanding the basic biology of liver cancer and has profound importance for the development of new strategies for cancer prevention and treatment. In this review, we highlight recent advances in the role of liver stem cells in hepatocarcinogenesis.

Our review of the literature shows that identification of the cellular origin and the signaling pathways involved is challenging issues in liver cancer with pivotal implications in therapeutic perspectives. Although the dedifferentiation of mature hepatocytes/cholangiocytes in hepatocarcinogenesis cannot be excluded, neoplastic transformation of a stem cell subpopulation more easily explains hepatocarcinogenesis. Elimination of liver cancer stem cells in liver cancer could result in the degeneration of downstream cells, which makes them potential targets for liver cancer therapies. Therefore, liver stem cells could represent a new target for therapeutic approaches to liver cancer in the near future.

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Key words: Liver cancer; Liver stem cells; Hepatocarcinogenesis; Tumorigenic transformation; Transdifferentiation

Core tip: Liver cancer is an aggressive disease with a high mortality rate. However, the concept of liver cancer origin is controversial. Recently, there is increasing evidence for the "cancer stem cell hypothesis", which proposes that liver cancer originates from the malignant transformation of liver stem/progenitor cells (liver cancer stem cells). This cancer stem cell model has important significance for understanding the basic biology of liver cancer and has profound importance for the development of new strategies for cancer prevention and treatment. This review discusses current knowledge concerning the role of liver stem cells in the hepatocarcinogenesis of primary liver cancer.

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INTRODUCTION

Liver cancer is one of the most common tumors and rep-

resents the second leading cause of cancer-related death worldwide. Its incidence continues to increase while the prognosis remains gloomy^[1]. Management of liver cancer is strongly dependent on the tumor stage and underlying liver disease. Unfortunately, most cases are discovered when the cancer is already advanced, missing the opportunity for surgical resection. For patients with unresectable or metastatic disease, however, no systemic treatment has been found to prolong survival in randomized studies and no systemic chemotherapy provides a sustained remission^[2]. Although Llovet *et al.*^[3] showed that sorafenib, an oral multikinase inhibitor, prolonged the median survival and the time to progression in patients with advanced hepatocellular carcinoma (HCC), most of the recent phase III trials of multi-targeted tyrosine kinase inhibitors (TKIs) have obtained disappointing results^[4-6]. Thus, an improved understanding of the mechanisms responsible for liver cancer initiation and progression will facilitate the detection of more reliable tumor markers and the development of new small molecules for targeted therapy of liver cancer^[3].

Primary liver cancer (PLC) is a form of liver cancer that begins in the liver. The molecular mechanism associated with initiation and progression of PLC remains obscure. HCC is the most common type of PLC, representing more than 80% of the cases of PLC. Cholangiocellular carcinoma (CCC), the second most common PLC, accounts for approximately 15% of PLC cases worldwide^[7]. Combined HCC and cholangiocarcinoma (cHCC-CC) is an uncommon subtype of PLC that displays components of both HCC and CCC and now accounts for 0.4% to 14.2% of all PLC cases, with significant variations from country to country^[8-10]. Although all three subtypes of PLC begin in the liver, they show very different biological characteristics that have remained unexplained until now.

Stem cells are undifferentiated biological cells with the capacity to undergo extended self-renewal through mitotic division (to produce more stem cells) and to differentiate into mature cells. There are two broad types of stem cells in mammals: embryonic stem (ES) cells that are found in the inner cell mass of blastocysts, and adult stem cells that are found in various adult tissues. In adult organisms, stem cells are responsible for tissue renewal and repair, replenishing aged or damaged tissues^[11]. Fifty-six years ago, Wilson and Leduc suggested that liver stem cells (LSCs) are present in the adult liver^[12]. Later, accumulating evidence suggested that LSCs play a pivotal role in the initiation and progression of PLC. This review summarizes and discusses current knowledge regarding the role of LSCs in the hepatocarcinogenesis of PLC.

LSC CANDIDATES

The liver is known to comprise two epithelial cell lineages, hepatocytes and cholangiocytes, which are known to originate from hepatoblasts during embryonic development. LSCs are bi-potential stem cells that are able to dif-

ferentiate towards the hepatocyte and the biliary lineages. Under normal physiologic conditions, LSCs are quiescent stem cells with a low proliferating rate, representing a reserve compartment^[13]. Upon acute injury, the mature hepatocytes and cholangiocytes, which can be considered conceptually as unipotent stem cells, acquire unexpected plasticity by direct dedifferentiation into LSCs, compensating for the loss^[14,15]. However, when the mature epithelial cells of the liver are continuously damaged or in cases of severe cell loss, LSCs are activated as a consequence and contribute to liver regeneration^[13]. There are two possible sources of liver stem cells: endogenous or intrahepatic LSCs and exogenous or extrahepatic LSCs (Figure 1)^[13,16].

Intrahepatic LSCs

Included in the intrahepatic LSC compartment are the adult liver stem/progenitor cells (referred to as oval cells), which are present in great numbers but with a short term proliferation capacity. In 1956, the term oval cell was first assigned by Farber^[17], who observed a population of nonparenchymal cells in the portal area of the rat liver after being fed ethionine, and described them as small oval cells with scanty, lightly basophilic cytoplasm and pale blue-staining nuclei. Over the past several decades, oval cells have been shown to be localized within the canals of Hering (the most peripheral branches of the intrahepatic biliary tree)^[18,19], interlobular bile ducts^[20], or in the periductular/intraportal zone of the liver^[21]. These cells are called into action when hepatocytes/cholangiocytes are insufficient or unable to respond. Numerous investigators have concluded that oval cell activation was the first step in liver regeneration in response to certain types of injury^[18,22,23].

In addition, it has been reported that mature hepatocytes have the capacity to dedifferentiate into LSCs through a transient oval cell-like stage both *in vitro* and *in vivo*, which indicates that mature hepatocytes are direct contributors to the LSC pool^[14]. Moreover, some investigators observed that liver regeneration also can proceed from a novel cell type, the small hepatocyte-like progenitor cells (SHPCs), which are phenotypically distinct from fully differentiated hepatocytes/cholangiocytes and oval cells^[24,25]. However, some other researchers suggest that SHPCs may represent an intermediate cell type between mature hepatic parenchymal cells and oval cells rather than a distinct stem/progenitor cell population^[26,27]. Thus, further studies are required to better understand this phenomenon.

Extrahepatic LSCs

Extrahepatic LSCs comprise ES cells and bone marrow stem cells (BMSCs), which are usually present in small numbers but have a long-term proliferation capacity. These cells have been reported to be capable of self-renewal, giving rise to oval cells and mature, fully functioning liver cells both *in vitro* and *in vivo*^[22,28,29].

ES cells, continuously growing pluripotent stem cells

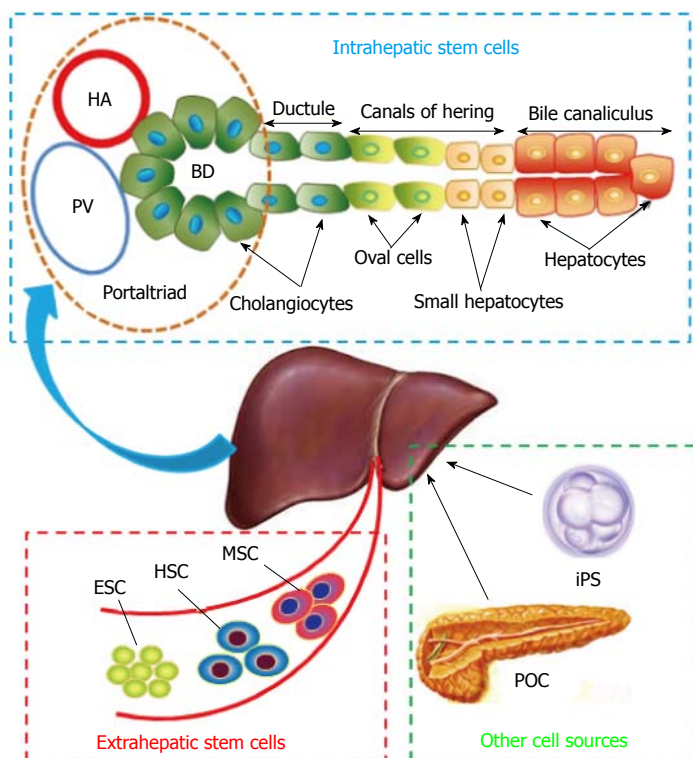


Figure 1 A schematic representation of various sources of liver stem cells^[13,16]. HA: Hepatic artery; PV: Portal vein; BD: Bile duct; ESC: Embryonic stem cell; HSC: Hematopoietic stem cell; MSC: Mesenchymal stem cell; POC: Pancreatic oval cell; iPS: Induced pluripotent stem cell.

derived from the inner cell mass of blastocysts, are capable of indefinite continuous culture and can generate all cell types in the body. Utilizing liver-specific marker staining and subsequent functional analysis, Jones *et al.*^[30] proved that murine ES cells can differentiate into hepatocytes. Using immunohistochemical assays and reverse transcription-polymerase chain reaction tests for hepatocyte-specific proteins and mRNAs, Kuai *et al.*^[31] confirmed that mouse ES cells can differentiate into functioning hepatocytes in the presence of hepatocyte growth factor and nerve growth factor- β . Similarly, increasing evidence shows that human ES cells can be progressively differentiated into definitive endoderm, LSCs, and hepatocytes/cholangiocytes^[32,33]. Recently, several newly developed techniques have been reported to facilitate the *in vitro* maturation of human ES cell-derived hepatocyte-like cells^[34-36].

BMSCs mainly contain two types of multipotent stem cells: hematopoietic stem cells (HSCs), which give rise to the three classes of mature blood cells; and mesenchymal stem cells (MSCs), which can differentiate into a variety of cell types such as osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells), and adipocytes (fat cells)^[37,38]. Both HSCs^[39] and MSCs^[40,41] have been shown to differentiate/transdifferentiate into oval cells and mature hepatic parenchymal cells, although these phenomena occur weakly and infrequently^[42]. In addition, MSCs can be found in nearly all tissues, and various lines of experimental evidence have shown that non-bone marrow-derived MSCs such as adipose-derived MSCs (AD-MSCs)^[43], umbilical cord-derived MSCs^[44,45], and peripheral blood-derived MSCs^[46] also can give rise to oval cells and mature liver parenchymal cells^[47].

Other cell sources

Strikingly, LSCs also can be transdifferentiated from non-hepatic sources such as pancreatic cells and induced pluripotent stem cells. Rao and Reddy first reported that massive depletion of the acinar cell pool causes a change in the oval and ductular cells that result in transdifferentiation into hepatocytes. Pancreatic hepatocytes exhibit all the morphological and functional properties of liver parenchymal cells. The cells that generate hepatocytes have been thought to be pancreatic oval cells^[48]. The results of the studies by Shen *et al.*^[49] and Marek *et al.*^[50] demonstrated that a rat pancreatic cell line, AR42J-B13, can be transdifferentiated into functional hepatocytes *in vitro*, expressing albumin and functional cytochrome P450s, in response to treatment with dexamethasone.

Induced pluripotent stem cells (also known as iPS cells or iPSCs) are a type of pluripotent stem cell that can be generated directly from adult cells^[51]. Yu *et al.*^[52] reported that liver organogenesis transcription factors (Hnf1 β and Foxa3) are sufficient to reprogram mouse embryonic fibroblasts into induced hepatic stem cells. These reprogrammed cells can be stably expanded *in vitro* and possess the potential for bidirectional differentiation into both hepatocyte and biliary lineages. However, pluripotent stem cells readily form a teratoma when injected into immunodeficient mice, which is considered a major obstacle to their clinical application^[53]. On this basis, Zhu *et al.*^[54] reported the generation of human fibroblast-derived hepatocytes that can proliferate extensively and function similarly to adult hepatocytes by cut short reprogramming to pluripotency to generate an induced multipotent progenitor cell from which hepatocytes can be efficiently differentiated.

THE STEM-CELL ORIGIN OF PLC

Several cell types in the liver, *i.e.*, hepatocytes, cholangiocytes, and LSCs, have the longevity that is needed to be the cellular origin of PLC^[19]. Determining the identity of the founder cells for PLC is more problematic and difficult. Therefore, unveiling the mechanisms by which these cells are activated to proliferate and differentiate during liver regeneration is important for the development of new therapies to treat liver diseases.

It is well known that different tumor cells can show distinct morphological and physiological features, such as cellular morphology, gene expression (including the expression of cell surface markers, growth factors and hormonal receptors), metabolism, proliferation, and immunogenic, angiogenic, and metastatic potential. This heterogeneity occurs both within tumors (intra-tumor heterogeneity) and between tumors (inter-tumor heterogeneity)^[55]. In 1937, Furth *et al*^[56] first demonstrated that a single malignant white blood cell is capable of producing leukemia. Afterwards, the cancer stem cell (CSC) hypothesis was proposed to explain the tumor heterogeneity phenomenon^[57,58]. This model postulates that most cancer cells have only a limited proliferative potential. However, a small subset of tumor cells has the ability to self-renew and is able to generate diverse tumor cells. These cells are defined as cancer stem cells (CSCs) to reflect their stem cell-like properties: indefinite potential for self-renewal and pluripotency. This theory assumes that only CSCs have the ability to initiate new tumors, both at primary and metastatic sites. Thus, this theory indicates that only elimination of all CSCs is fundamental to eradicate tumors^[57].

Over the past few years, there is a growing realization that many cancers contain a small population of CSCs. However, the cellular origin of PLC is controversial and whether PLC contains cells that possess properties of CSCs requires further exploration. Numerous observations indicate that any proliferative cell in the liver can be susceptible to neoplastic transformation. In the past, it has been considered that HCC is derived from dedifferentiation of hepatocytes and CCC originates from the dedifferentiation of intrahepatic biliary epithelial cells. In contrast, cHCC-CC is thought to be derived from transformed LSCs^[59,60]. More recently, due to the rapid progress of stem cell research, it is widely accepted that cancer is a disease of stem cells, as these are the only cells that persist in the tissue for a sufficient length of time to acquire the requisite number of genetic changes for neoplastic development^[61].

Previous studies reported by Steinberg *et al*^[62] have shown that transfection of an active Ha-ras proto-oncogene into oval cells can lead to their malignant transformation. By using hepatitis B virus X (HBx) transgenic mice and a drug-induced liver injury model, Wang *et al*^[63] found that HBx may enable malignant transformation and the acquisition of tumorigenic potential in LSCs, suggesting that liver cancer cells are of LSC origin. The results of Chiba *et al*^[64,65] implied that disruption of the

self-renewal of LSCs generates a CSC population and highlight the important role of LSCs in hepatocarcinogenesis. A study by You *et al*^[66] showed that inactivation of the tumor suppressor gene Tg737 results in the malignant transformation of fetal LSCs by promoting cell-cycle progression and differentiation arrest. In a clinical study, Ward *et al*^[67] concluded that PLC in children often arises from the malignant transformation of LSCs at an early stage. In a similar study, Ishikawa *et al*^[68] considered that CCC may derive from the oncogenic transformation of normal LSCs. Collectively, extensive animal modeling and clinical studies have demonstrated that PLC is a disease derived from maturation arrest of LSCs^[61].

This theory has been confirmed by the discovery of putative CSCs in the liver. Analysis of the cells in PLC supports the presence of cells with functional properties of somatic CSCs (*e.g.*, immortality, resistance to therapy, and efficient transplantability), which indicates that PLC derives from liver CSCs (LCSCs)^[61]. Suetsugu *et al*^[69] isolated CD133+ cells from human HCC cell lines and demonstrated that these cells possess cancer stem/progenitor cell-like properties. Ma *et al*^[70,71] and Yin *et al*^[72] also identified a CSC population in HCC characterized by a CD133 phenotype, suggesting that CD133 might be one of the markers for HCC cancer stem-like cells. Side population (SP) cells are a sub-population of cells that are distinct from the main population and exhibits distinguishing stem cell-like characteristics. In a study of SP cells in different hepatoma cell lines, Chiba *et al*^[73] concluded that SP cells in hepatoma cell lines possess extreme tumorigenic potential, which suggests that a minor population of liver cancer cells harbors LCSC-like properties. A variety of recent studies of hepatoma cell lines and clinical samples suggest that epithelial cell adhesion molecule (EpCAM)^[74-76], CD13^[77-80], CD24^[81-83], CD44^[84,85], CD90^[86,87], intercellular adhesion molecule-1 (ICAM-1)^[88], $\alpha 2\delta 1$ subunit of voltage-gated calcium channels^[89], and OV6^[90] may serve as putative LCSC markers. The CSC theory emphasizes the role of LSCs in the hepatocarcinogenesis of PLC. Although the aforementioned proteins and/or molecules have been postulated as putative LCSC markers, no definitive markers have yet been identified directly and widely recognized. Moreover, no LCSCs have been isolated^[61]. Therefore, additional studies are needed to obtain a definitive molecular marker of LCSCs and to isolate LCSCs from PLC cell lines, animal models, and clinical samples.

MOLECULAR MECHANISMS INVOLVED IN THE MALIGNANT TRANSFORMATION OF LSCS

Based on the studies mentioned above, we can scientifically conclude that PLC may derive from neoplastic transformation of LSCs. However, the underlying molecular mechanisms are poorly understood. Studies investigating cancer and CSCs show that several key genes and regulatory signaling pathways are oncogenic, such as

Bmi1, Wnt, Notch, Hedgehog, and transforming growth factor- β (TGF- β), and therefore are potentially involved in the malignant transformation of LSCs^[91]. Here, current knowledge of these pathways is discussed.

Polycomb group gene Bmi1

Polycomb group (PcG) proteins are a family of transcriptional repressors that epigenetically remodel chromatin and participate in the establishment and maintenance of cell fates. These proteins play a central role in hematopoiesis, stem cell self-renewal, cellular proliferation and neoplastic development. To date, four distinct PcG-encoded protein complexes have been purified from different species: Polycomb repressive complex 1 (PRC1), PRC2, Pho repressive complex (PhoRC), and Polycomb repressive deubiquitinase (PR-DUB)^[92].

Bmi1, encoded by the *BMI1* gene (B cell-specific Moloney murine leukemia virus integration site 1), is the most important core subunit of the PRC1 complex, which plays a pivotal role in the self-renewal of both normal stem cells and CSCs. Increasing evidence indicates that Bmi1 protein is elevated in many human malignancies including PLC and has a vital effect on tumorigenesis, cancer progression, and the malignant transformation of stem cells. Therefore, Bmi1 was identified as an important stem cell factor and a proto-oncogene^[93].

In PLC, a number of studies have shown that Bmi1 contributes to the maintenance of tumor-initiating SP cells^[94] and can cooperate with other oncogenic signals to promote hepatic carcinogenesis *in vivo*^[95]. Our empirical results suggest that Bmi1 is highly expressed in patients with PLC and correlates positively with the proliferation and invasiveness of human hepatoma cells^[96,97]. Furthermore, Chiba *et al.*^[64,65] observed that forced expression of Bmi1 promotes the self-renewal of LSCs, and the transplantation of such cells that have been clonally expanded from single LSC produces tumors that exhibit the histologic features of cHCC-CC. The above results indicate that Bmi1 plays a crucial role in the oncogenic transformation of LSCs and therefore drives cancer initiation.

Wnt signaling pathway

The Wnt signaling pathways are ancient and evolutionarily conserved pathways that transmit signals from outside of a cell through cell surface receptors to the inside of the cell and regulate cell-to-cell interactions. Wnt signaling is one of the most well studied molecular pathways during the human life span and involves a large number of proteins that are required for basic developmental processes such as embryonic development, cell fate determination, cell proliferation, cell migration, and cell polarity, in a variety of species and organs^[98].

Three major categories of Wnt signaling pathways are recognized: the canonical Wnt pathway in which the cytoplasmic protein β -catenin is a key mediator, the noncanonical planar cell polarity pathway (β -catenin independent), and the noncanonical Wnt/calcium pathway. Activation of the canonical Wnt/ β -catenin pathway

causes an accumulation of β -catenin in the cytoplasm and its eventual translocation into the nucleus to act as a transcriptional coactivator of transcription factors. Without Wnt signaling, β -catenin would not accumulate in the cytoplasm because it would be degraded by a destruction complex^[99]. Ever since its initial discovery, Wnt signaling has had an association with cancer^[100]. There is substantial evidence to suggest that dysregulation of Wnt signaling is critical for the initiation and progression of PLC^[101,102].

Wnt signaling pathways, particularly the canonical Wnt/ β -catenin pathway, are also involved in the self-renewal and maintenance of embryonic and adult stem cells, and as recent findings demonstrated, in CSCs. Functional characterization of LSCs has revealed that Wnt/ β -catenin pathways were critical for inducing the stem cell properties of hepatoma cells and in promoting self-renewal, tumorigenicity, and chemoresistance^[103]. In the aforementioned HBx-mediated tumorigenic effects, Wang *et al.*^[63] suggest that HBx may enable LSCs with tumorigenic potential *via* activation of the Wnt/ β -catenin signaling pathway. As shown in several *in vivo* and *in vitro* experiments, the Wnt/ β -catenin signaling pathway contributes to the activation of normal and tumorigenic LSCs^[104]. Moreover, Chiba *et al.*^[64] demonstrated that Wnt/ β -catenin signaling activation strongly enhances the self-renewal capability of LSCs and generates a CSC population as an early event, thereby contributing to the initiation of PLC.

Notch signaling pathway

Notch signaling is a complex, highly conserved signal transduction pathway in multicellular organisms. In mammalian cells, the pathway is initiated when Notch ligands (Jagged-1, Jagged-2, and Delta-like 1, 3, and 4) bind to the epidermal growth factor (EGF)-like receptors Notch1-4. Signaling is processed by the enzyme γ -secretase, which results in the subsequent activation of downstream target genes^[105,106]. The Notch signaling pathway functions as a major regulator of cell-fate decisions during embryonic development and adult life, and it is crucial for the regulation of self-renewing tissues. Accordingly, dysregulation of Notch signaling underlies a wide range of human disorders from developmental syndromes to adult-onset diseases and cancer^[105,107].

Like other solid tumors, misregulation of the Notch pathway in PLC has been described as both oncogenic and tumor suppressive, depending on the cellular context^[108]. Qi *et al.*^[109] reported that overexpression of Notch1 inhibits the growth of HCC cells by inducing cell cycle arrest and apoptosis. In 2009, the same authors showed that Notch1 signaling sensitizes tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in HCC cells^[110]. In addition, Viatour *et al.*^[111] demonstrated that activation of the Notch pathway serves as a negative feedback mechanism to slow HCC growth during tumor progression. At odds with these findings, however, some recent studies have provided strong evidence in favor of the pro-oncogenic activity of

Notch in PLC. For example, Wang *et al.*^[112] showed that aberrantly high expression of Notch1 is significantly associated with metastatic disease parameters in HCC patients, and shRNA-mediated silencing of Notch1 reverses HCC tumor metastasis in a mouse model. In human HCC cell lines, Gao *et al.*^[113] demonstrated that Notch1 activation contributes to tumor cell growth. In accordance, we have shown that Notch1 is overexpressed in human intrahepatic CCC and is associated with its proliferation, invasiveness and sensitivity to 5-fluorouracil *in vitro*^[114]. Taken together, these data highlight the concept that the Notch pathway plays an essential yet controversial role in PLC, presumably depending on the tumor cell type, local inflammatory microenvironment and the status of other signaling pathways^[115,116].

The aforementioned hypothesis was further supported by recent studies examining Notch signaling in the regulation of stem cell and in the development of LSC-driven PLC^[117,118]. Utilizing a genetically engineered mouse model and comparative functional genomics, Strazzabosco *et al.*^[115], Villanueva *et al.*^[119] and Razumilava *et al.*^[120] observed that liver-specific Notch activation in mice recapitulates different stages of human hepatocarcinogenesis and results in HCC, including histological features associated with stem cell expansion. They also confirmed that Notch1 is a bona fide oncogene in experimental liver cancer. Using a transgenic mouse model, Zender *et al.*^[116] proved that stable overexpression of Notch 1 in bipotential LSCs causes the formation of intrahepatic CCCs. Dill *et al.*^[121] and Cardinale *et al.*^[122] also reported that liver-specific expression of the intracellular domain of Notch2 (N2ICD) in mice is sufficient to induce HCC formation, while DEN^{N2ICD} (diethylnitrosamine-induced HCCs with constitutive Notch2 signaling) mice develop large hepatic cysts, dysplasia of the biliary epithelium, and eventually CCC. These studies also suggested that the LSC compartment is the most likely candidate for oncogenic events^[115,116,119-122].

Nevertheless, these newly published studies raise one question: how can one pathway, Notch signaling, contribute to two different subtypes of PLC: HCC and CCC? Of note, the balance between Notch/Wnt signaling has been proposed to be crucial for the determination of the LSC cell fate in liver disease. Activation of Notch signaling in LSCs leads to biliary specification; in contrast, Wnt signaling activation inhibits default-activated Notch signaling *via* Numb (a target of canonical Wnt signaling), allowing LSCs to escape the biliary cell fate and acquire a hepatocellular specification^[123-125]. Therefore, based on previous studies and to the best of our knowledge^[123-126], we propose that the balance between Notch/Wnt signaling pathways determines the oncogenic transformation of LSCs into HCC, CCC, or cHCC-CC phenotype. The predominance of Notch over the Wnt signaling in LSCs leads to the CCC phenotype, and activation of Wnt signaling likely prevents activation of the Notch pathway and thus leads to the HCC phenotype. When the comparison is balanced between the two signaling pathways,

the cell has a higher probability of entering the cHCC-CC phenotype. In summary, the role of such a pleiotropic pathway in liver regeneration and liver diseases seems to be highly context dependent. Additional research is required to clearly establish the effects of the Notch signaling pathway during hepatocarcinogenesis.

Hedgehog signaling pathway

The Hedgehog signaling pathway is one of the key regulators of embryonic development. Mammals have three Hedgehog homologues, Sonic (SHH), Indian (IHH), and Desert (DHH), of which Sonic is the best studied. Like the Wnt and Notch pathways, the Hedgehog signaling pathway also plays significant roles in stem cell self-renewal^[127] and cancer cell proliferation^[128,129].

Sicklick *et al.*^[130] showed that Hedgehog signaling is conserved in hepatic progenitors from fetal development through adulthood and is essential for the maintenance of LSC survival. In a study reported by Jeng *et al.*^[131], the SHH pathway is activated in CD133+ mouse liver cancer cells that harbor stem cell features. In human CCC tissues and cell lines, El Khatib and colleagues^[132] demonstrated that inhibition of Hedgehog signaling attenuates carcinogenesis *in vitro* and increases necrosis in CCC. Chen *et al.*^[133] showed that enhanced Hedgehog signaling activity may be responsible for the invasion and chemoresistance of hepatoma subpopulations. In a fibrosis-associated hepatocarcinogenesis model, Philips *et al.*^[134] further established that Hedgehog signaling pathway activation promotes hepatocarcinogenesis while inhibiting Hedgehog signaling safely reverses this process even in advanced HCC.

TGF- β signaling pathway

The TGF- β signaling pathway is involved in various cellular functions in both the developing embryo and the adult organism including cell growth, cell differentiation, apoptosis, and cellular homeostasis. The pathway is activated upon binding of TGF- β to its receptors, TGF- β receptor I (TGFR1) and TGFR2, resulting in the translocation of Smad proteins to the nucleus where they act as transcription factors and participate in the regulation of target gene expression^[135,136].

The role of TGF- β in tumors is rather complicated. In healthy tissue, it acts as a tumor suppressor controlling the cell cycle and inducing apoptosis. During carcinogenesis, TGF- β acts as a potent inducer of cell motility, invasion and metastasis. In liver cancer, TGF- β has been shown to have both tumor-promoting and tumor-suppressing effects, and its expression is decreased in early but increased in later stages of carcinogenesis. Although the underlying molecular mechanisms remain largely undefined, it had been speculated that the dual role of TGF- β signaling in liver cancer results from its effect on the tumor microenvironment^[135,136].

It has long been known that TGF- β signaling is vitally involved in stem cell renewal and lineage specification, including in LSCs^[137]. Recently, TGF- β signaling has also

been linked to the malignant transformation of LSCs in hepatocarcinogenesis. Nishimura *et al.*^[138] reported that TGF- β treatment increases the percentage of SP cells in a hepatoma cell line. Yuan *et al.*^[139] reported that HCC cells with aberrantly high expression of TGF- β signaling that are positive for Oct4 (octamer-binding transcription factor 4) are likely cancer progenitor cells with the potential to give rise to HCC. Using several experimental approaches, Wu *et al.*^[140] confirmed that long-term treatment of oval cells with TGF- β impaired their LSC potential but granted them tumor-initiating cell (TIC) properties including the expression of TIC markers, increased self-renewal capacity, stronger chemoresistance, and tumorigenicity in nude mice. In opposition to these findings, however, Tang *et al.*^[141,142] showed that activation of the interleukin-6 (IL-6) signaling pathway induces neoplastic transformation of LSCs along with inactivation of the TGF- β signaling pathway. Lin *et al.*^[143] suggested that disruption of TGF- β signaling is an important molecular event in the transformation of normal LSCs to cancer progenitor/stem cells. These data suggest an important but contradictory role for TGF- β signaling in LSC-driven hepatocarcinogenesis, potentially due to the interaction with other signaling pathways.

A NEW CONCEPT UNDERLYING THE LCSC LINEAGE: VASCULAR ENDOTHELIAL TRANSDIFFERENTIATION

Interestingly, CSCs can potentially transdifferentiate into cell types other than the original type from which the tumor arose. Several recent studies have shown that CSCs also can transdifferentiate into functional vascular endothelial cells that line the tumor vasculature, mediating tumor growth and metastasis^[144-146]. In 2010, Wang *et al.*^[147] and Ricci-Vitiani *et al.*^[148] provided strong evidence that a proportion of the endothelial cells that contribute to blood vessels in glioblastoma originate from the tumor itself, having differentiated from tumor stem-like cells. Wang *et al.*^[147] also demonstrated that blocking VEGF (vascular endothelial growth factor) or silencing VEGFR2 (VEGF receptor 2) inhibits the maturation of tumor endothelial progenitors into endothelium but not the transdifferentiation of tumor stem-like cells into endothelial progenitors, whereas γ -secretase inhibition or Notch1 silencing blocks the transition into endothelial progenitors. Subsequently, multiple studies have confirmed the presence of tumor-derived endothelial cells in several other malignancies, such as renal^[149,150], ovarian^[151], and breast cancers^[152,153], which suggests that this is a general phenomenon in CSCs.

Similarly, Marfels *et al.*^[154] found that chemoresistant hepatoma cells show increased pluripotent capacities and the ability to transdifferentiate into functional endothelial like cells both *in vitro* and *in vivo*. These tumor-derived endothelial cells possess increased angiogenesis and drug resistance capability (including chemotherapeutics and

angiogenesis inhibitors) compared with normal endothelial cells^[155,156]. Taken together, these data may provide new perspectives on the biology of CSCs and reveal new insights into the mechanisms of resistance to anti-angiogenesis therapy.

CONCLUSION

Our review of the literature shows that identification of the cellular origin and the signaling pathways involved is challenging issues in PLC with pivotal implications in the therapeutic perspectives. Although dedifferentiation of mature hepatocytes/cholangiocytes in hepatocarcinogenesis cannot be excluded, neoplastic transformation of a stem cell subpopulation more easily explains hepatocarcinogenesis. Elimination of LCSCs in PLC could result in the degeneration of downstream cells, making them potential targets for liver cancer therapies. Therefore, LSCs could represent a new target for therapeutic approaches to PLC in the near future. However, though LSCs have a bright future, their efficient therapeutic applications will demand further scientific advances.

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Roles of microRNA-140 in stem cell-associated early stage breast cancer

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Abstract

An increasing body of evidence supports a stepwise model for progression of breast cancer from ductal carcinoma *in situ* (DCIS) to invasive ductal carcinoma (IDC). Due to the high level of DCIS heterogeneity, we cannot currently predict which patients are at highest risk for disease recurrence or progression. The mechanisms of progression are still largely unknown, however cancer stem cell populations in DCIS lesions may serve as malignant precursor cells intimately involved in progression. While genetic and epigenetic alterations found in DCIS are often shared by IDC, mRNA and miRNA expression profiles are significantly altered. Therapeutic targeting of cancer stem cell pathways and differentially expressed miRNA could have significant clinical benefit. As tumor grade increases, miRNA-140 is progressively downregulated. miR-140 plays an important tumor suppressive role in the Wnt, SOX2 and SOX9 stem cell regulator pathways. Downregulation of miR-140 removes inhibition of these pathways, leading to higher cancer stem cell populations and breast cancer progression. miR-140 downregulation is mediated through both an estrogen response element in the miR-140 promoter region and differential methylation

of CpG islands. These mechanisms are novel targets for epigenetic therapy to activate tumor suppressor signaling *via* miR-140. Additionally, we briefly explored the emerging role of exosomes in mediating intercellular miR-140 signaling. The purpose of this review is to examine the cancer stem cell signaling pathways involved in breast cancer progression, and the role of dysregulation of miR-140 in regulating DCIS to IDC transition.

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Key words: Breast cancer; Ductal carcinoma *in situ*; Invasive ductal carcinoma; Cancer stem cells; MicroRNA-140

Core tip: MiR-140 is an important tumor suppressor. By inhibiting stem cell growth through interaction with the Wnt, SOX2 and SOX9 pathways, breast cancer initiation, progression and growth are reduced. miR-140 is progressively downregulated as breast cancer grade decreases, through both estrogen binding and differential methylation in the miR-140 promoter region. By targeting these mechanisms using epigenetic therapy miR-140 tumor suppressor signaling can be reactivated.

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INTRODUCTION

Breast cancer is a heterogeneous disease comprised of several histologic and molecular subtypes. Transformation from normal mammalian epithelial cells to aggressive malignancy is due to the accumulation of numerous genetic and epigenetic changes. Early breast cancer such as

ductal carcinoma *in situ* (DCIS) exhibit similar patterns of gene and protein expression to invasive ductal carcinoma (IDC), suggesting a stepwise model of non-obligate precursor^[1]. Following benign proliferative changes to the ductal lumen, atypical ductal hyperplasia (ADH), DCIS and IDC are more likely to occur^[2]. Molecular signatures for development and progression of breast cancer are poorly established, due to limited data for early lesions. Classification systems based on histological features and proliferation rate are useful in patient management to some extent, and are used to assign DCIS a grade of low, intermediate or high. The distinction between low grade DCIS and ADH is somewhat subjective, as they maintain many molecular and genetic similarities. High grade DCIS is much more likely to progress to IDC and is associated with increased likelihood of recurrence^[1]. Currently there is no way clinicians can predict if a DCIS lesion will progress to IDC, which would improve therapeutic management.

DCIS treatment is able to prevent progression from early stage breast cancer, but therapeutic options are lacking. DCIS lesions are heterogeneous with treatment success varying for the different molecular subtypes. Lumpectomy and radiation therapy remain the standard of care in most cases of DCIS. Estrogen receptor positive DCIS patients benefit from Tamoxifen treatment, but no molecularly targeted treatment is available for basal lesions^[2].

In contrast to the shared genetic and epigenetic alterations of IDC and DCIS, mRNA/miRNA expression profiles are significantly altered. Deep sequencing of DCIS and IDC lesions has identified differential miRNA signatures that may be involved in the acquisition of an invasive phenotype. miR-140-3p downregulation was observed for all investigated groups of IDC and DCIS patients, leading our lab to investigate potential tumor suppressive roles^[3].

Here we will review the underlying mechanisms behind microRNA-140 dysregulation in breast cancer. We will discuss the role of cancer stem cells in the DCIS to IDC transition and the importance of microRNAs in regulating breast cancer stem cells. Briefly, we will discuss the emerging role of exosomal miRNAs as intercellular signaling molecules. Finally, we will discuss possible therapeutic avenues for modulating miRNA signaling in breast cancer and highlight the potential for epigenetic therapies to activate tumor suppressor miRNAs.

MICRORNA BIOGENESIS

MiRNAs are short noncoding RNA molecules, approximately 22 nucleotides in length, which bind primarily to the 3' untranslated region (UTR) of messenger RNAs. The primary function of miRNAs is to regulate gene expression. miRNAs function through targeting mRNA for degradation or translational inhibition. mRNA is targeted through a semi-complementary seed sequence (6-9 bp) in miRNAs, which guides binding to the miRNA response

elements. Each seed sequence potentially matches hundreds of mRNA molecules, giving the miRNA many potential targets^[4]. Most mammalian miRNA genes are found in well-defined transcriptional units, and can be in either intronic or exonic regions in non-coding transcriptional regions, or as intronic miRNAs in coding regions^[5].

The primary miRNA transcript (pri-miRNA) genes are transcribed predominantly by RNA polymerase II, although other isoforms may be involved. Pri-miRNA is cleaved at the 5' and 3' ends by the Microprocessor complex, comprised of ribonuclease III Drosha and RNA-binding protein DGCR8, forming the pre-miRNA. The approximately 70 nucleotide stem-loop pre-miRNA is transported out of the nucleus by exportin-5 and Ran-GTP. In the cytosol the RISC loading complex, composed of RNase III Dicer, Argonaute-2, and double-stranded RNA-binding domain proteins Tar RNA binding protein (TRBP) and protein activator of PKR (PACT), facilitates pre-miRNA processing and RISC assembly^[6]. Dicer cleaves the pre-miRNA near the hairpin loop, forming a 20-23 nucleotide long miRNA duplex. The miRNA duplex incorporates into the RNA induced silencing complex (RISC), where it is unwound, isolating the guide strand while the complementary strand (miRNA*) is degraded by RISC^[6,7].

MiRNA dysregulation often occurs through modification of key enzymes associated with biogenesis. Specifically, loss of Dicer expression has been observed in many cancers, including breast cancer^[8]. This results in decreased miRNA expression, and is associated with breast cancer progression^[9]. Dysregulation occurs through a wide variety of genetic and epigenetic mechanisms, deletion or amplification of the miRNA genes, transcriptional activation and suppression, as well as epigenetic dysregulation, *i.e.*, methylation of CpG islands^[10].

MIR-140 IN CHONDROCYTES

MiR-140 was first identified as regulating cartilage development in chondrocytes^[11]. The primary transcript of miR-140 is found in intron 16 of the E3 ubiquitin protein ligase WWP2 gene on chromosome 16, and mature miR-140 is co-expressed with Wwp2-c. MiR-140 expression is induced by SOX9 binding to intron 10 of the WWP2 gene^[12], inhibition of SOX9 by Wnt/ β -catenin signaling has been demonstrated to suppress miR-140 in certain cell lines^[13].

MiR-140 promotes chondrocyte proliferation by targeting of transcription factor Sp1, leading to cell cycle inhibition^[12]. MiR-140 has also been found to suppress HDAC4, promoting cartilage differentiation^[14]. Additionally, miR-140 plays an important role in protecting against diseases of cartilage destruction through regulation of protease Adamts-5^[11]. MiR-140 has also been identified in other tissues, including breast, brain, lung, ovary and testis. A potential tumor-suppressive role has been identified, as miR-140 is down regulated in ovarian, lung, colon, osteosarcoma and breast carcinomas^[13].

In the majority of miRNA species, the 5-prime miRNA is annotated as the guide strand, while the complementary 3-prime miRNA* is degraded. Rakoczy *et al.*^[15] found that in testis and chondrocytes, miR-140-3p is more highly expressed than miR-140-5p, and likely has its own function. Our lab has observed this in breast tissue. MiR-140-3p and miR-140-5p have different seed sequences, and thus have a different set of target genes, many of which may not yet be known^[15]. The miRNA guide strand is chosen based on thermodynamic stability, with the strand that has relatively unstable base pairs at the 5' end remaining^[5]. Uracil-bias at the 5'-end of the highly expressed strand, cysteine-bias at the 3'-end of the low expressed strand and an excess of purines in the low expressed strand have also been identified as determinants of strand selection^[16]. However, the mechanism of strand selection is still unknown.

BREAST CANCER STEM CELLS

Cancer stem cells (CSCs) were first discovered in hematopoietic malignancies. They are believed to comprise a small subpopulation of cancer cells that have the ability to self renew and differentiate into heterogeneous tumor lineages. CSCs have an important role in resistance to chemotherapy and disease recurrence, a dangerous combination that allows them to survive treatment and regenerate the tumor leading to treatment failure^[17]. Overexpressed ABC transporters mediate the resistance of CSCs to most current chemotherapeutics^[18]. In order to cure cancer, therapeutics must be developed in conjunction with debulking therapies that can specifically eliminate cancer stem cells.

Isolation and characterization of CSC

There are a number of assays used to isolate and characterize cancer stem cells, the gold standard being the ability of a small number of cells obtained by serial dilution to initiate a tumor in NOD/SCID mice. Fluorescence-activated cell sorting (FACS) can be used to study cell surface markers associated with the cancer stem cell population. Further assays test common attributes of stem cells. Aldehyde Dehydrogenase 1 (ALDH1) activity is detectable by the Aldefluor assay. The presence of a "side-population" in FACs when cells are treated with Hoechst 33342 dye is an indicator of increased ABC transporters, which expel Hoechst 33342.

Stem cell surface markers were first identified in human acute myeloid leukemia. The CD34+/CD38- subpopulation is able to initiate tumors histologically similar to the parent tumor from a low cell count in NOD/SCID mice^[19]. Using a similar approach, cancer stem cells were identified in breast cancer as a CD44+/CD24- lineage. A small number of cells from this lineage are able to initiate xenografts and differentiate into heterogeneous tumors. This population also shares the extensive proliferative capacity and ability to self renew identified in hematopoietic cancer stem cell populations^[20].

DCIS stem cells

Previous studies have shown that cancer stem cells exist in DCIS lesions and may determine the malignant potential of the cancer. Unsorted cell populations from human DCIS lesions were able to form mammospheres under non-adherent conditions, as well as initiate tumors in NOD/SCID mice^[21]. We identified a cancer stem cell population within basal-like DCIS identified by ALDH1+ and CD49f+/CD24- cells. This group possesses enhanced migration and self-renewal capacity, and initiates fast growing tumors in nude mice. It is possible that this population is involved in progression of DCIS lesions to IDC and serves as a malignant precursor cell^[22]. We investigated stem cell signaling in both DCIS and triple negative invasive breast cancer models, focusing on stem cell regulators SOX2 and SOX9.

CSC signaling

There are a number of pathways associated with de-regulated self-renewal in cancer stem cells, including the Notch, Sonic hedgehog, Wnt, and Pluripotency factor pathways^[18]. Dysregulation in these signaling pathways is common in breast cancer. The Notch pathway is involved in breast development, and dysregulation is an early event in DCIS. Notch is up regulated in breast cancer stem cells^[23], and may be involved in DCIS stem cell mediated progression to IDC. The Wnt pathway is involved in regulation of stem cell proliferation. Deregulation of Wnt signaling and proliferation predisposes to cancer^[24]. Overexpression of Wnt is correlated with increased mammary tumor formation^[25], an event mediated by cancer stem cells. Sonic hedgehog is also involved in regulating self-renewal of mammary stem cells as well as inhibiting differentiation, potentially through the Notch signaling pathway^[26]. Hijacking of embryonic pluripotency factors (OCT4, SOX2, KLF4) has also been reported in cancer stem cells. Sry-related HMG box 2 (SOX2) has been reported to be an oncogene in early stage breast cancers^[27]. Furthermore, we have identified a critical role for the related HMG-box protein SOX9 in DCIS stem cells^[28].

SOX2 and SOX9

SOX9 transcription factor is an important stem cell regulator and works cooperatively with Slug to promote tumorigenesis and cancer initiation. Slug is an epithelial-mesenchymal transition transcription factor, upregulated in mammary stem cell populations. When coexpressed with SOX9, differentiated mammary epithelial cells are converted into mammary stem cells^[29]. SOX9 is overexpressed in a number of breast malignancies, and is necessary for mammosphere formation of basal DCIS cell lines. SOX9 expression increases with DCIS grade^[28]. In basal like, IDC cell lines, expression of both Slug and SOX9 is necessary for tumor initiation; SOX9 is also necessary for maintaining tumorigenicity^[29]. This may demonstrate a relationship between risk of progression from DCIS to IDC and an increase in cancer stem cell population.

SOX2, OCT4 and NANOG form a complex that binds promoters of numerous differentiation factors. Dysregulation of any member of this complex leads to aberrant self-renewal, a primary characteristic of cancer stem cells^[27]. Overexpression of SOX2 is a common mechanism of aberrant self-renewal signaling, and is required for tumor-initiation. Stable knockdown of SOX2 in MCF-7 breast cancer cells results in a significant decrease in the CD44 high/CD24 low stem cell population. SOX2 overexpression increased this population, as well as increasing mammosphere formation, the ability of breast cancer stem cells to grow in a non-adherent culture^[27].

A major risk factor for breast cancer is estrogen exposure. Mammary tumor formation is mediated through a combination of toxic estrogen metabolites and estrogen receptor signaling affecting survival and proliferation^[30]. Estrogen has been shown to increase the frequency of the CD44+/CD24- subpopulation through ER α association with the OCT4 promoter, potentially affecting self-renewal signaling through the OCT4/SOX2/NANOG complex^[27]. In ER positive breast cancer cells we have found that ER signaling can indirectly regulate SOX2 levels, one mechanism through which ER signaling may impact stem cell signaling in breast cancer.

MIR-140 IN THE DCIS TO IDC TRANSITION

To further interrogate the DCIS to IDC transition, we performed microarray profiling of DCIS lesions and matched normal tissue and compared our results to published deep sequencing datasets. We identified miR-140 loss as a reproducible marker of DCIS lesions. The level of miR-140 downregulation increases as DCIS grade increases and progresses to invasive ductal carcinoma (IDC), demonstrating a potential role in disease progression.

Role of miR-140 in DCIS stem cells

For patients with ER positive DCIS, adjuvant tamoxifen treatment significantly reduces recurrence and disease progression. However, for patients with basal like DCIS there are no available molecularly targeted therapeutics. In addition, basal like DCIS is a particularly aggressive form of DCIS (often also classified as comedo-type DCIS) frequently detected with concomitant IDC lesions. As such, we chose to continue our investigation into the tumor suppressor roles of miR-140 in a model of basal-like DCIS. Knockdown of miR-140 in 3D cell culture resulted in increased proliferation, as well as a decrease in acinar apoptosis, indicating a role for miR-140 in differentiation or stem cell signaling in mammary stem cells. Further investigation into the potential role of miR-140 in DCIS stem cells revealed dramatic loss of miR-140 in DCIS stem cells compared to normal mammary stem cells. We identified a CpG island in the miR-140 promoter with differential methylation, and validated its function using epigenetic therapy. This demonstrated that downregulation might be mediated through epigenetic mechanisms.

Predicted miR-140 targets SOX9 and ALDH1 are dramatically upregulated in DCIS stem cells compared to parental cell lines with miR-140 expression. Targeting of both by miR-140 was validated using luciferase reporters for either the SOX9 or ALDH1 3'-UTRs. Restoration of miR-140 in DCIS cells significantly reduced mammosphere formation, suggesting miR-140 negatively regulates DCIS stem cell renewal. Similarly, SOX9 overexpression/knockdown resulted in mammosphere formation suggesting that a miR-140/SOX9 pathway may be an important regulator of DCIS stem cells. DCIS tumor growth in nude mice was significantly reduced when miR-140 was overexpressed. When stem-like mammosphere cells were used to initiate xenografts, tumor growth and initiation was much faster than whole cell population. miR-140 overexpression was again able to almost completely eliminate growth of DCIS tumors^[28].

Role of miR-140 in IDC stem cells

In order to interrogate the role miR-140 plays in breast cancer, we investigated miR-140 expression in estrogen receptor positive invasive breast tumor cells. We found that miR-140 expression is inversely related with SOX2 expression. Tissue staining of ER α + IDC revealed a significant increase in SOX2 expression, and qRT-PCR revealed a dramatic downregulation in miR-140 expression. A luciferase reporter assay for the 3'-UTR of SOX2 showed that miR-140 directly targets and inhibits SOX2 expression, and mammosphere assays demonstrated that miR-140 targeting regulates stem cell signaling in tumors. While examining the molecular mechanisms regulating miR-140 expression we identified predicted estrogen response elements (ERE) in the miR-140 promoter region. Due to the previous reports linking ER α and self-renewal signaling, we investigated a potential ER α miR-140 relationship. In non-tumorigenic cells engineered to express ER α , E2 treatment significantly inhibited miR-140 expression, while also stimulating SOX2 expression. We examined the miR-140 promoter using a luciferase reporter and found that E2-mediated miR-140 downregulation was decreased when the ERE at -79/50 in the miR-140 promoter was mutated. Binding of ER α to the miR-140 promoter was validated using ChIP. In the absence of estrogen, miR-140 expression had very little effect on cancer stem cell frequency. There was a significant decrease in the CD44+/CD24- population when miR-140 was overexpressed following estrogen stimulation, indicating miR-140 plays an important role in the regulation of estrogen stimulated tumor-initiation cells, potentially through inhibition of SOX2^[27].

EXOSOMES

Exosomes are spherical membrane vesicles between 50-100 nm, secreted by the majority of cells. Multivesicular bodies fuse with the cellular membrane, releasing exosomes into the extracellular matrix^[31]. They contain a variety of protein, RNA, products of signaling pathways

and miRNAs, some common to all exosomes and some cell specific^[32]. The common set of proteins consists of the tetraspanin family (CD9, CD63, CD82), members of the endosomal sorting complexes required for transport (ESCRT) complex (TSG101, ALIX) and heat shock proteins (Hsp60, Hsp70, Hsp90)^[33]. Several of these proteins are used for exosome detection in Western blotting or FACS, including CD63 and CD9^[34,35].

Exosome function in tumorigenesis

There are three known functions of exosomes in tumorigenesis; restructuring of microenvironment, modulation of tumor immune response and direct modification of tumor cells *via* delivery of protein or genetic material^[31,36]. Tumor development is dependent on the relationship between cancer cells and the surrounding microenvironment^[37]. Secreted factors promote angiogenesis and invasion, aiding in tumor growth and progression. Communication between cancer cells and the microenvironment is likely mediated in part by exosomes, both secreted by cancer cells and the microenvironment itself. Stromal secreted exosomes promote breast cancer motility and metastasis^[38]. Tumor secreted exosomes can promote endothelial tubule formation^[39], as well as secrete matrix metalloproteinases, aiding in invasion^[40]. Molecular changes in tumor stroma are an important part of breast cancer initiation and progression^[37].

Exosomes can suppress immune response by promoting T regulatory cell expansion and inducing apoptosis of effector T cells^[41]. In tumor cells exosomes mediate upregulation of anti-apoptotic genes and anchorage independent growth^[42], and are believed to be involved in resistance to drug and radiation resistance^[32]. Exosomes transfer their contents to receiving cells *via* internalization of the exosome. Heparan sulfate proteoglycans are necessary receptors of cancer cell derived exosomes, and are necessary for exosome uptake and delivery of macromolecular contents^[43].

A precise method for identifying tumor secreted exosomes is not yet available. Tumor secreted exosomes are differentiated by analysis of their contents. Proteins and miRNA found in exosomes closely match those in the parent cell. In some cases, FACS can be conducted using antibody for tumor specific protein in exosomes, such as HER2/neu^[44]. Marker proteins that are often over-expressed in tumors are found in exosomes, including EpCAM, CD24, L1CAM, CD44 and EGFR. The utility of these markers for identification of tumor-secreted exosomes is under investigation^[45].

Exosomal miRNAs

Breast cancer heterogeneity is reflected in tumor-secreted exosomes. While miRNA sequencing of secreted breast cancer exosomes is still in its infancy, exosomal miRNA expression from other diseases exhibit a high level of correlation to parental cells^[46]. Exosomes have been successfully isolated from many sources in the body, including blood plasma, serum and urine^[32]. Due to their ubiquity

and disease specific expression, there is significant potential for exosomal use as biomarkers of disease state or progression^[36].

MiRNA array shows differential expression of miR-140 between DCIS stem-like and DCIS whole cell populations. Similarly, miR-140 is downregulated in exosomes derived from DCIS stem-like cells compared to exosomes derived from DCIS whole cell population. Exosomal levels of miR-140 from stem cell populations can be rescued by treatment with sulforaphane. Treatment of invasive basal like breast cancer cells and DCIS cells with miR-140 containing exosomes resulted in an increased level of miR-140 in both cell lines, demonstrating the potential of exosomal secretion to impact miR-140 signaling in nearby cells. Treatment with sulforaphane may block paracrine signaling by increasing miR-140 secretion in the tumor microenvironment^[22].

TRANSLATIONAL POTENTIAL

MiR-140 represents a potential target to prevent cancer initiation and progression. Promoter region hypermethylation is a common mechanism for miRNA dysregulation, and is also observed in early stage breast cancers. A CpG island exists within the miR-140 locus, and has a higher level of methylation in DCIS cells compared to nontumorigenic mammary epithelial cells. This methylation region is a potential therapeutic target to restore miR-140 expression^[28].

Targeting stem cells in ER α positive IDC

We demonstrated the presence of an ER α /miR-140/SOX2 signaling axis, through which ER α binds the miR-140 promoter region, halting transcription and preventing miR-140 targeting of SOX2 mRNA. Targeting ER α signaling may rescue miR-140 inhibition of SOX2, preventing stem cell signaling and promoting tumor cell differentiation. While this strategy could prove effective for ER α positive tumors, other avenues must be pursued to target miR-140 in basal-like breast cancers^[27].

Targeting DCIS stem cells

Treatment of DCIS cells with 5-aza-2-deoxycytidine (DNA methyltransferase inhibitor) or sulforaphane (inhibitor of histone deacetylase and DNA methyltransferase) restored miR-140 expression^[47,48]. Sulforaphane treatment significantly inhibited DCIS tumor growth *in vivo*, as well as restoring miR-140 expression and down regulating SOX9 and ALDH1. Treatment of triple negative, basal-like invasive breast cancer with sulforaphane had the same effect, upregulation of miR-140 and decreased cancer stem cell frequency. Cancer stem cell xenografts of MDA-MB-231 showed dramatically decreased growth when treated with sulforaphane^[28].

Targeting stem cell signaling in nearby cancer cells through exosomal miR-140

Sulforaphane treatment of DCIS stem-like cells resulted

in increased exosomal miR-140. This indicates that in addition to restoring miR-140 expression in treated stem cells, sulforaphane may block stem cell signaling in nearby cells through exosomal delivery of miR-140^[22].

CONCLUSION

Stem cells present in the DCIS population may serve a critical role in progression and recurrence of breast cancer. Through interaction with SOX2 and SOX9, miR-140 serves as a tumor suppressor in both DCIS and IDC, preventing stem cell signaling and tumor initiation. When miR-140 is downregulated there is an increase in stem cell populations and breast cancer progression, initiation and growth. We have identified two primary downregulation mechanisms. In IDC, we found estrogen binding in the miR-140 promoter, and epigenetic regulation through CpG island methylation in DCIS. By targeting these mechanisms, miR-140 signaling is recovered and the stem cell population decreased, reducing tumor growth and progression. Targeting of the DCIS stem cell population may be critical to preventing progression to invasive ductal carcinoma. Epigenetic therapy rescuing miR-140 suggests a novel therapeutic strategy for both DCIS and IDC lesions, and would be especially important for patients with tamoxifen insensitive ER α - DCIS lesions.

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Stem cells in gastrointestinal cancers: The road less travelled

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Abstract

Cancer stem cells (CSC) are thought to be malignant cells that have the capacity to initiate and maintain tumor growth and survival. Studies have described CSC in various gastrointestinal neoplasms such as colon, pancreas and liver and gastroesophageal tumors. The mechanism by which CSC develop remains unclear. Several studies have explored the role of dysregulation of the Wnt/ β -catenin, transformation growth factor-beta and hedgehog pathways in generation of CSC. In this review, we discuss the various molecular abnormalities that may be related to formation of CSC in gastrointestinal malignancies, strategies to identify CSC and therapeutic strategies that are based on these concepts. Identification and targeting CSC is an intriguing area and may provide a new therapeutic option for patients with cancer including gastrointestinal malignancies. Although great progress has been made, many issues need to be addressed. Precise targeting of CSC will require precise isolation and characterization of those cells. This field is also evolving but further research is needed to identify markers that are specific for CSC.

Although the application of this field has not entered the clinic yet, there continues to be significant optimism about its potential utility in overcoming cancer resistance and curing patients with cancer.

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Key words: Cancer stem cells; CD133+; WNT/ β -catenin; Transformation growth factor-beta; Hedgehog; Notch

Core tip: Cancer stem cells (CSC) are thought to be malignant cells that have the capacity to initiate and maintain tumor growth and survival. Several studies have explored the role of dysregulation of the Wnt/ β -catenin, transformation growth factor-beta and hedgehog pathways in generation of CSC. The exact mechanism of their development, however, remains unknown. Several investigators have researched modalities to identify and target CSC. In this review, we summarize the recent evidence exploring the mechanisms of development, identification and targeting of CSC in gastrointestinal malignancies.

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STEM CELLS IN GASTROINTESTINAL CANCERS: THE ROAD LESS TRAVELLED

Cancer is a disease of adult stem cells (SC). Adult SC are the only cells that persist in the tissue for a sufficient length of time to acquire the sufficient sequential genetic alterations for cancer development^[1]. Adult SC have been traditionally relatively quiescent, a feature thought to protect them from the accumulation of DNA errors that may

lead to carcinogenesis^[1]. In the gastrointestinal tract, the immediate stem cell progeny, however, proliferate rapidly to allow for tissue repopulation^[1]. Their limited life span restricts the impact of any replication errors. It is worth noting that this concept has been challenged by recent studies that suggest that adult stem cells are in fact capable of rapid self-renewal^[2]. Similarly, cancer stem cells (CSC) have the capacity to initiate and maintain tumor growth and survival^[3]. Studies have described CSC in gastrointestinal neoplasms such as colon, pancreas and liver^[4-6]. The mechanism by which CSC develop remains unclear^[1]. Several studies have explored the role of dysregulation of the Wnt/ β -catenin, transformation growth factor-beta (TGF- β) and hedgehog pathways in generation of CSC^[7-9]. In this review, we discuss the various molecular abnormalities that may be related to formation of CSC in gastrointestinal malignancies, strategies to identify CSC and therapeutic strategies that are based on these concepts.

MOLECULAR PATHWAYS ASSOCIATED WITH CSCS IN GASTROINTESTINAL MALIGNANCIES

Notch signaling pathway

The Notch signaling pathway plays an important role in embryogenesis, cellular homeostasis-, differentiation and apoptosis^[10-12]. While Notch mediates a number of biological processes through the “canonical” Notch signaling pathway, it also mediates a ligand- or transcription independent function known as the “non-canonical” pathway^[12,13]. The canonical Notch pathway includes at least four Notch receptors (Notch 1-4) and five Notch ligands Delta-like 1,3 and 4 and Jagged 1 and 2^[14]. When Notch ligand binds to a Notch receptor, Notch will be cleaved through a series of proteolytic cleavages by multiple enzymes leading to release of the active Notch fragment and activation of Notch target genes^[15]. Notch target genes include Akt, mTOR (mammalian target of rapamycin, NF- κ B, c-Myc and VEGF (vascular endothelial growth factor) and cyclin D1^[16,17]. Activation of the Notch pathway can have tumor suppressor function in HCC but may play an oncogenic role in colon and pancreatic cancers^[14]. Notch signaling has been found to play a pivotal role in CSC. Overexpression of Notch-1 and -2 was observed in pancreatic CSC and was associated with increased expression of CSC surface markers such as CD44 and EpCAM^[15,17-19]. This observation suggests that Notch signaling may be involved in pancreatic CSC self-renewal but will need further confirmation.

WNT/ β -catenin pathway

Notch signaling also performs a “non-canonical role” through antagonizing Wnt/ β -catenin signaling^[12,13]. Disrupted Wnt signaling is observed in a variety of gastrointestinal cancers which underscores its importance in carcinogenesis^[20]. The Wnt pathway plays a crucial role in embryogenesis with signaling effects that regulate proliferation and apoptosis in developing cells^[21].

Wnt pathway activation plays a fundamental role in maintenance of SC compartment and regulation of cellular differentiation^[22]. The “canonical” Wnt pathway plays a crucial role in modulating the balance between self-renewal and differentiation in several adult CSC^[21]. The “canonical” Wnt pathway describes a sequence of events beginning with the translocation of β -catenin from the cell membrane into the nucleus, where β -catenin then acts as a co-activator of the TCF/LEF family of transcription factors^[23,24]. The signaling cascade is typically initiated when Wnt ligand binds to Frizzled (FZD), a transmembrane receptor^[23]. The transcription factors activated by β -catenin subsequently regulate specific target genes including c-myc, cyclin D1 and survivin. FZD binding to Wnt ligand also promotes the escape of β -catenin from its association with E-cadherin^[23,25]. The cytoplasmic elements of the activated Wnt pathway prevent β -catenin from being phosphorylated by degradation complex composed of a serine-threonine kinase, glycogen synthase kinase-3 β (GSK3 β), protein scaffolds, AXIN and adenomatous polyposis coli (APC)^[25]. Mutations of these proteins allow β -catenin to accumulate in the nucleus to enhance the transcription of its target genes which are found in many cancers^[9]. For example, in hepatocellular carcinoma (HCC), mutations of β -catenin are located in exon 3 of CTNNB1 gene which is the phosphorylation site for GSK3 β , AXIN1 and AXIN2 mutation^[26]. It is worth noting that 20%-40% of human HCC exhibit abnormal cytoplasmic and nuclear accumulation of β -catenin by immunohistochemistry (IHC)^[27]. B-Catenin can also undergo downregulation *via* the non-canonical Notch pathway. In this case, membrane-bound Notch forms a complex with active B-Catenin in the presence of Wnts. This action degrades active B-Catenin and thus inhibits its pathway. This process allows for regulation of SC and its dysfunction could lead to expansion of CSC^[13]. Markers for elevated expression of Wnt include CD133+ and EpCAM+^[28]. The knockdown of expression of EpCAM, in HCC stem cells resulted in decreased proliferation, colony formation, migration and drug resistance which highlight the role and Wnt signaling in tumor survival^[28,29]. Additionally, knockdown of β -catenin resulted in inhibition of CSC^[30]. Similarly mutations in APC gene acts to suppress Wnt signaling and result in familial adenomatous polyposis (FAP) syndrome^[31]. In the majority of sporadic colorectal cancers, loss of APC or β -catenin mutations seems to be early events in carcinogenesis^[32]. Of note, Apc 1638N has been shown to result in multiple intestinal tumors in mice^[32].

TGF- β pathway

TGF- β signaling is crucial for self-renewal and maintenance of SC and in the formation of gastrointestinal cancers^[8,33]. TGF- β forms a complex with the serine-threonine kinase receptor type I and II^[34]. The receptors are activated sequentially and subsequently phosphorylate one of the receptor-activated R-mads^[35]. The activated R-mad will heterodimerize with Smad4 and then trans-

Table 1 Markers used in gastrointestinal cancer stem cell identification

Markers	Ref.
CD133+	[34]
CD44+	[55]
CD24+	[84]
Lgr5	[53]
mTert	[85]
Olfm4	[86]
Ascl2	[87]
ALDH	[79]
Sox9	[88]
Msi 1	[89]
Dcamk1l	[90]

CD: Cluster of differentiation; Ascl2: Achaete Scute-like 2; ALDH: Aldehyde dehydrogenase; Msi 1: Musashi1; Dcamk1l: Doublecortin and CaM kinase-like-1.

locate to the nuclear to regulate gene transcription^[36]. Disruption of TGF- β signaling results in dysregulated gene expression and hence gastrointestinal malignancies are associated with suppressed activity of different members of TGF- β pathway^[37,38]. For example, inactivation of Smad4 is seen in approximately 50% of patients with pancreatic cancer^[39]. Similarly, reduced Smad4 expression and loss of ELF, a modulator of activity of Smad3, are observed in human colon and gastric cancer tissue^[40,41]. Additionally, inactivating mutation of TGF- β II receptor was described in colon cancer^[37].

Hedgehog pathway

The Hedgehog signaling pathway consists of a complex of molecules which regulate cell differentiation, regeneration and stem cell biology^[9]. The pathway plays a central role in the development and homeostasis of the gut tissue^[9]. The Hedgehog pathway is deregulated in gastrointestinal cancers^[42]. Up to 60% of HCC samples express Sonic, the predominant ligand of the hedgehog pathway^[42]. Additionally, genes involved in the hedgehog pathway are highly expressed in CD133+ liver cancer SC^[43]. It is worth noting that suppression of Hedgehog pathway decreased HCC cell proliferation and sensitized HCC cells to treatment with 5-fluorouracil^[44]. Hedgehog signaling has been shown to be essential for proliferation and survival of human colon cancers^[45]. It is thought to affect both tumor growth and CD133+ CSC^[43]. Similarly, HH signaling has been associated with pancreas cancer invasion and metastasis. Conversely inhibition of HH signaling inhibited pancreatic metastatic spread^[46].

PTEN pathway

PTEN is a phosphatase that antagonizes PI3 kinase activity^[47]. PTEN helps control the proliferative rate and the number of intestinal stem cells and its loss is associated with an increase in intestinal SC^[47]. It is also thought that PTEN pathway controls SC activation *via* interaction with the Wnt pathway^[48]. It is also proposed that PTEN pathway interacts with the TGF- β pathway described

above^[48]. Mutations in PTEN, result in a cancer syndrome (Cowden's syndrome) characterized by hamartomas in the gastrointestinal tract, central nervous system and skin in addition to tumors in the breast and thyroid gland^[49]. PTEN deficient mice exhibit increase in intestinal SC which results in excess crypt formation^[47].

Identification of CSCs

Eradication of CSC stems is an intriguing concept that provides hope in the possibility of finding a cure for cancer. Any therapeutic modality that targets CSC will require accurate identification and characterization of the CSC and differentiating them from normal SC. Isolation of cancer cells through the identification of pathognomonic surface markers has recently gained popularity and is an area of active investigation^[50,51]. CD133⁺ emerged as a promising surface marker for CSC^[50]. Singh *et al.*^[51] used flow cytometry to successfully isolate CD133⁺ CSC in human brain tumors and implanted them into forebrain of immunodeficient mice. Transplantation of as few as 100 cells produced tumors that were phenotypically similar to original tumors. Similar findings were reported in colorectal cancer. Several groups isolated subpopulations of cells, accounting for approximately 1% of total number of cells within a tumor, that were CD133⁺ and we capable initiating cancer when transplanted in immunodeficient mice^[5,52,53]. Other studies have identified new CSC markers (Table 1) that may be promising in isolation of CSC such as Lgr5, CD44, CD24 and epithelial specific antigen^[54-57]. These markers were isolated in HCC and pancreatic cancer. This field is currently in evolution. Efforts have been made to identify surface marker “signatures” that are specific for each type of cancer (Table 2) It is worth noting that isolation of cancer cells is far from perfect and remains an area of controversy. Not all CSC express SC markers and some tumor cells that are not SC may also express those markers^[1]. Great progress has been already made in this area but this more works remains to be done.

Resistance of CSCs to anticancer therapy

Several studies demonstrated that CSC exhibit resistance to chemotherapy agent^[2,58]. One of the widely accepted theories is that the elevated levels of ATP-binding cassette (ABC) transporters mediate resistance to chemotherapy^[2,3,58,59]. ATP transporters are membrane transporters that can pump small molecules including cytotoxic drugs out of cells in exchange for ATP hydrolysis^[59]. CSC as well as normal SC appear to express high levels of ABC transporters^[60]. This characteristic can lead to multidrug resistance and enhanced tumorigenesis. Evolving evidence suggests that numerous cell lines and tumors contain CSC, referred to as side population (SP) cells that possess a differentially greater capacity to resist chemotherapeutic agents and invade surrounding tissues^[2,61-63]. This phenomenon, however, may allow for development of therapies that could target ATP transporters in CSC.

Table 2 Surface markers of gastrointestinal cancer stem cell

Tumor type	Phenotype of CSC markers	Ref.
Liver	CD133+, CD49f+, CD90+	[1,6,91]
Colon	CD133+, CD44+, CD166+, EpCAM+, CD24+	[5,45,52]
Pancreatic	CD133+, CD44+, EpCAM+, CD24+	[57]
Stomach	CD44+, CD133+, NESTIN, CD90+, CD54+, ALDH1	[79]

CSC: Cancer stem cell.

Targeting CSCs

Targeting CSC is an intriguing concept that may offer several therapeutic advantages. Targeting the inherently resistant CSC may overcome resistant to chemotherapeutic agents. Most patients with metastatic gastrointestinal cancers tend to experience treatment failure and resistance to palliative chemotherapy^[64-66]. Additionally, targeting CSC may, not only improve efficacy of treatment but may also reduce therapy-related toxicity through developing treatment that are selective for CSC and not toxic to healthy tissues. Novel treatment strategies are, therefore, being developed that target surface markers on CSC, ATP-binding cassettes, key signaling pathways or their tumor microenvironment^[1].

Targeting surface markers: Since CD133⁺ is expressed in CSC in gastrointestinal cancer, it represents an interesting target to selectively inhibit CSC. A recent study demonstrated that carbon nanotubes conjugated with CD133⁺ monoclonal antibodies caused photothermolysis of CD133⁺ glioblastoma cells when affixed to an anti-CD133 antibody that selectively targeted those cells^[67]. This study represents an encouraging proof of concept that gastrointestinal CSC can be possibly targeted with similar strategies.

Targeting cancer stem cell pathways: Targeting signaling pathways that are thought to be active in CSC is an ongoing area of active research. Lin *et al* demonstrated that a curcumin analogue, GO Y030, may have clinical activity against colorectal cancer SC *in vitro* and *in vivo*^[68]. They identified aldehydehydrogenase (ALDH) positive and CD133⁺ colorectal CSC using flow cytometry. The demonstrated that isolated CSC exhibited STAT-3 (signal transducers and activators of transcription-3) activation and treated them with GO-Y030. GO-Y030 inhibited STAT3 phosphorylation and reduced STAT3 downstream target gene expression resulting in induction of apoptosis in colon CSC. Additionally, GO-Y030 suppressed tumor and CSC growth of SW480 and HCT-116 colon cancer cell lines *in vivo* in mouse models. Interestingly, Curcumin has been shown to also inhibit cell growth and apoptosis in pancreatic cancer cells. Its effect was associated with down-regulation of Notch-1 expression, which suggests that Curcumin may be associated with potential advantageous activity against pathways that are upregulated in CSC^[18]. Other attempts to target Notch signaling in gastrointestinal CSC have, however, not been very successful. Gamma-secretase inhibitors (GSI) are thought to antago-

nize Notch signaling through blocking of Notch receptor cleavage^[69]. Evaluation of the effect of GSI in two gastric cancer cell lines did not result in any appreciable antitumor effects^[70]. These results were surprising since GSI have shown promising antitumor potential in leukemia, breast and glioblastoma multiformes models^[71-73].

Evolving evidence suggests that targeting the Hedgehog pathway may be a feasible strategy to inhibit CSC. Cyclopamine, a naturally occurring hedgehog inhibitor has shown promising potential^[46]. As a single agent cyclopamine suppressed the invasion of pancreatic cancer cells^[4]. Cyclopamine reduced the percentage of cells expressing the pancreatic CSC markers such as ALDH^[74]. In combination with gemcitabine, cyclopamine resulted in reduction of metastasis in an orthotopic xenograft model^[74]. To further clarify this observation, Yao *et al*^[74] demonstrated that cyclopamine downregulated the expression of CD44 and CD133⁺ in gemcitabine-resistant pancreatic cancer cells indicating that it may be an effective modality for reversing gemcitabine resistance in pancreatic CSC. A similar observation was made in gastric CSC where blocking of Hedgehog pathway with cyclopamine decreased self-renewing properties and enhanced sensitivity of gastric cancer cells to chemotherapeutic agents^[75]. Additionally, Feldmann *et al*^[76] demonstrated that IPI-269609, a novel Hedgehog inhibitor, inhibited growth and metastasis of pancreatic cancer mostly through targeting of the CSC.

Since the Wnt pathway is also deregulated in CSC, it represents an intriguing target for cancer treatment. Anti-Wnt therapy is in early stages of clinical development^[77]. He *et al*^[77] demonstrated that a monoclonal antibody against Wnt-1 induced apoptosis in human cancer cells. Also, Salinomycin, an antibiotic commonly used in poultry firmly, is thought to suppress Wnt/ β -catenin signal transduction^[78]. In gastric cancer, salinomycin, selectively inhibited gastric CSC *in vitro*^[79]. Wnt inhibitors also are being investigated in phase I clinical trials. Oral LGK974^[80] is a potent and specific inhibitor of O-acyltransferase Porcupine (Pocn) that acetylates Wnt proteins required for their biological activities is being investigated in a phase I clinical trial in patients with malignancies dependent on Wnt ligands. This trial is enrolling patients with pancreatic and colon adenocarcinoma.

Targeting ATP-driven efflux transporters has been explored in preclinical and early phase clinical trials. The first drug efflux pump inhibitor is verapamil. Simultaneous treatment with verapamil and chemotherapy resulted in promising antitumor activity. Other agents such as Dofequidar Fumarate (MS-209), Biricolar (VX-710), and tariquidar are in various stages of clinical development^[81-83]. Most of the experience with these agents is derived from lung and breast cancer trials but these agents, to our knowledge, have not been investigated in gastrointestinal cancers.

CONCLUSION

Identification and targeting CSC is an intriguing area and

may provide a new therapeutic option for patients with cancer including gastrointestinal malignancies. It is a rapidly evolving area in the treatment of gastrointestinal and other tumors. Although great progress has been made, many issues need to be addressed. The CSC model does not fully explain the observed genetic heterogeneity of many tumors. This criticism may however be explained by the fact that even CSC may evolve over time and give rise to cells that are both genetically and functionally heterogeneous^[1]. Furthermore, accurate targeting of CSC will require precise isolation and characterization of those cells. This field is also evolving but further research is needed to identify markers that are specific for CSC. Nevertheless, there continues to be significant excitement about this field and hope that it may represent a new treatment modality in patients with cancer.

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Stem cell biology in thyroid cancer: Insights for novel therapies

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have been discussed. The review also outlines various setbacks that still remain to target these tumor initiating cells. Future perspectives of therapeutic strategies and their potential to treat advanced stages of thyroid cancer are also disclosed in this review.

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Key words: Stem cells; Cancer Stem cells; Thyroid Neoplasms; Carcinogenesis; Cell Differentiation; Epithelial-Mesenchymal transition

Core tip: The concept of cancer stem cells in thyroid gland tumors has recently evolved. Since this sub-population of cells appear to have a potential for self-renewal and cell differentiation, their role envisions newer ideas in the field of anti-cancer therapy and regenerative medicine. The controversies have been raised for their origin in different cell lines and effectiveness in thyroid pathologies including chemo- and radio-resistant thyroid cancer. Newer concepts like epithelial-mesenchymal transition have been investigated to define its role in metastatic activity. Literature discusses various methods to target these cells by interfering signaling pathways, destruction of niche and other factors which facilitate and sustain tumor growth.

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INTRODUCTION

The incidence of thyroid cancer is rapidly rising in the US accounting for 62980 cases with 1890 deaths every

Abstract

Currently, thyroid cancer is one of the most common endocrine cancer in the United States. A recent involvement of sub-population of stem cells, cancer stem cells, has been proposed in different histological types of thyroid cancer. Because of their ability of self-renewal and differentiation into various specialized cells in the body, these putative cells drive tumor genesis, metastatic activity and are responsible to provide chemo- and radioresistant nature to the cancer cells in the thyroid gland. Our Review was conducted from previously published literature to provide latest appraises to investigate the role of embryonic, somatic and cancer stem cells, and discusses the hypothesis of epithelial-mesenchymal transition. Different methods for their identification and isolation through stemness markers using various *in vivo* and *in vitro* methods such as flow cytometry, thyrosphere formation assay, aldehyde dehydrogenase activity and ATP-binding cassette sub-family G member 2 efflux-pump mediated Hoechst 33342 dye exclusion

year^[1]. It is the seventh most common cancer diagnosed in women and peaks earlier than in men. Despite its high prevalence, death rate from thyroid cancer is fairly stable from past many years. In general, thyroid cancer offers a good prognosis with an overall survival rate of approximately 90%^[2]. Papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) termed as differentiated thyroid cancer (DTC) contribute to majority of thyroid cancers sharing a superior prognosis. Medullary thyroid carcinoma (MTC), mostly acquired as a part of familial syndromes, display only modest cure rates. While surgical resection followed by radioiodine therapy remains the treatment of choice for localized thyroid cancer, it fails to eradicate tumors with aggressive behavior. In marked contrast to DTC, anaplastic carcinoma (ATC), an undifferentiated sub-type of thyroid cancer, has a higher propensity to locally invade nearby structures and metastasize rapidly. It approaches to almost 50% of all thyroid cancer-related deaths, the median survival being only six months^[3]. The grim prognosis of ATC is due to the fact that it is diagnosed at an advanced stage which offers palliative treatment as the only option for patients suffering from the disease. Because of the chemo- and radio-resistant nature observed in aggressive thyroid cancers, many researchers have been continuously attempting to create new treatment strategies that are aimed at eradication of cancer cells. These trials led to a phenomenal breakthrough that the acquired resistance of thyroid cancer cells which initially were responding to conventional therapies may harbor heterogeneous cell types. Interestingly, these cells were hypothesized to be acquired with stem cell-like properties and were labelled a distinct entity called as cancer stem cells (CSCs)^[3-5]. Recent advances in the stem cell technology have made it possible to understand diverse biological and molecular mechanisms that control the disease process; however, the validity of the origin of CSCs and their distinct role in thyroid cancer still uphold a great interest.

Stem cells are the population of cells that have a tremendous potential for self-renewal and can differentiate into various specialized cells in the body. These are distinguished from other cell types by two important properties. Firstly, they have the ability for self-renewal through continuous cell division and secondly, under specialized circumstances, they can be induced to become tissue/organ specific cells carrying their designated functions. Among these cells, of particular importance are (1) Embryonic stem cells (ESCs) - which are pluripotent cells that divide infinitely and give rise to ectodermal, endodermal and mesodermal cells; and (2) Somatic stem cells (SSC) - also known as adult stem cells, are tissue specific cells with limited life-span that give rise to all cells in a particular lineage, for instance thyroid follicular cells or hematopoietic cells. However, the putative role of ESC and SSC in adult thyroid pathophysiology still remains to be proven.

A sub-type of cancer cells that has recently gained much recognition are CSCs, also referred to as Tumor-initiating cells (TICs)^[6-8]. These cells possess characteris-

tics associated with normal stem cells with a remarkable potential to reconstitute and sustain tumor growth. However, it does not infer their origin from a normal stem cell. It has been reported that basal-like epithelial cells can de-differentiate into stem-like cell^[9]. Moreover, existing literature illustrates that CSCs may depend on a specific microenvironment or the niche for sustained stem-cell like properties^[6,10]. One such example of CSCs niche is hypoxia of cancer where these cells undergo continued proliferation on exposure to increased free radical generation within the tumor. Therefore, several studies have attempted to identify the niche that necessitates these cells to sustain and promote tumor growth.

In 1997, Bonnet *et al*^[11] were the first to provide conclusive evidence of CSCs in leukemia. The isolated leukemic cells expressed cell surface markers CD34 but lacked CD38. On injection into an immunodeficient mice, these cells initiated tumor with similar histological features of the parental tumor^[11]. In 2002, Ignatova *et al*^[12] were the first to isolate CSCs from human brain gliomas which were described to be clonogenic with special sphere-forming property. Since then there have been many published clinical researches that have successfully identified CSCs in solid cancers of breast, colon, pancreas, prostate and ovary^[13-15]. Most recently, existence of stem cells in thyroid cancer with an understanding of its developmental biology, especially from the perspective of innovation of newer cell-replacement therapies for aggressive thyroid cancer, has been laid much importance in the field of regenerative medicine and tissue engineering^[3-8].

Organogenesis of thyroid gland is dependent on specific transcription factors which are responsible for differentiation of progenitor cells. Certain cell-specific transcription factors namely thyroid transcription factor (TTF) 1, TTF 2, Hhex factor, pax 8, fgfr-2, and Eya1 possess distinctive roles in thyroid development^[10,16]. However, their combined expression through a controlled regulation is essential to carry out cellular differentiation and expression of thyroid specific genes. Mature thyroid cells display a number of markers of differentiation such as thyroglobulin (Tg), thyroid peroxidase (TPO), and thyroid stimulating hormone receptor.

The origin of cancer cells in thyroid has been described in various literatures; however, it still maintains its avenue for debate. The basic concept of multistep carcinogenesis considers transformation of well differentiated thyroid cancer cells of follicular origin into undifferentiated cells through sequential events which occur during maturation of thyroid epithelial cells^[6]. On the contrary, others propose that these well-differentiated follicular cells rarely proliferate and thus carry limited accumulated mutations in the cells. Also, the genetic mutations that are seen in well-differentiated cancers are not evident in anaplastic cancers^[17]. Some authors favor the notion of fetal carcinogenesis which postulates that thyroid CSCs originate from abnormal transformation of fetal cells: (1) fetal thyroid stem cells, the primitive cells that express onco-fetal protein responsible for the origin of ATC; (2)

Thyroblasts, which express fetal protein and Tg give rise to PTC; and (3) Prothyrocytes, which are differentiated cells responsible for FTC/follicular adenoma^[8,18]. Once they follow aberrant pathways of malignant transformation, these cells lose their ability to differentiate further and become a potential source of CSCs.

Another concept of CSC theory, which has been proposed previously, suggests that these cells originate either from stem cells, progenitor cells or from de-differentiated mature thyroid cells^[19]. Because of a shorter lifespan of somatic cells, researchers claim that stem cells or progenitor cells represent their most likely the source. Much evidence exists on the fact that cancer comprises of heterogeneous cells out of which only a sub-population with stem-cell like characteristics are tumorigenic^[6,10,19]. However, the concept of CSC in the cellular origin for thyroid tumors, in particular, cannot be clearly demonstrated using this model. Because CSC are isolated at an advanced-stage of the tumor, these cells, though, capable of initiating new tumor formation, are not described for cellular-origin by some authors^[20]. This contemplates the use of term TICs or tumor-propagating cells by some authors.

According to Zhang *et al.*^[21] because of loss of specific markers that govern degree of differentiation, thyroid CSCs undertake aberrant differentiation pathways and suffer maturation arrest. If this arrest is seen late in the differentiation process, they give rise to well differentiated carcinoma^[21], when encountered early in the process, poorly differentiated carcinoma results. Therefore, different oncological pathways are responsible for providing diverse histological and morphological patterns to thyroid cancer.

Other studies demonstrate that stem cells can be recruited to the site of tumor and probably can acquire tumor-like properties and acting as parental tumor cells. Moreover, these cells have internal driven-force for supporting tumor progression and metastasis and they have the power to communicate with other cells through exosomes^[22,23].

ISOLATION AND IDENTIFICATION OF CANCER STEM CELLS

Various pre-clinical *in vivo* and *in vitro* models have been designed by the researchers to determine thyroid cancer progression and their response to treatment. According to the American Association for Cancer Research, 'cancer stem cell can only be defined experimentally by their ability to recapitulate the generation of a continuously growing tumor', proving the term TIC's^[6,24]. The commonest and most definite way to confirm their presence is by isolating cells and then serially injecting them into immuno-deficient, for example non-obese diabetic mice or severe combined immunodeficiency (SCID) mice, to identify tumor initiation. CSCs isolated by flowcytometry are sorted according to CSC-specific surface markers, thyrosphere formation assay, aldehyde dehydrogenase

activity (ALDH) and ATP-binding cassette sub-family G member 2 (ABCG2) efflux-pump mediated Hoechst 33342 dye exclusion^[6,9,10]. The sphere-forming assays are the best *in vitro* strategy to study clonal behavior and multi-potential of thyroid stem cells. There are different CSC-specific markers proposed by different authors such as side population (SP), CD-133+, CD-44, POU5F1, ALDH, insulin and insulin-like factor (IGF). The existence of embryonic remnants with stem-cell properties in mature thyroid gland has already been hypothesized using Oct-4, ABCG-2, GATA-4, HNF-4 α , α -fetoprotein and p63 markers^[10,25,26]. Malgounera *et al.*^[25] demonstrated expression of various stemness markers (Oct-4, NANOG, Sox-2, CD44, and CD133) in follicular thyrospheres. However, the sphere cultures displayed very low levels of thyroid differentiation markers (Tg and TPO). Additionally, their findings also displayed higher expression of IGF components in the stem cells suggesting their important role in the regulation of precursor cells in follicular cancer^[25]. Specific genetic alterations such as RET/PTC and PAX8/PPAR γ rearrangements play a crucial role in thyroid carcinogenesis. These alterations prevent differentiation of thyroid fetal cells, leading to their uncontrolled proliferation and malignant transformation within the gland. Moreover, dysregulation in signaling pathways of stem cell renewal (Wnt/ β -catenin, Hedgehog and Notch pathways) may contribute to malignant transformation of normal thyroid resident cells.

The existence of CSCs has been considered in several thyroid cell lines. Mistutake *et al.*^[26] reported ability of SP cells to efflux Hoechst 33342, a DNA-binding dye. They demonstrated that SP cells were enriched with stem-cell like characteristics. These cells were clonogenic that could give rise to both SP and non-SP cells. Additionally, SP cells showed up-regulation of "stemness" genes including those found in Notch and Wnt signaling pathways. However, both sub-population of cells (SP and non-SP cells) were tumorigenic on injection in a nude mice^[26]. A research demonstrated a function role of CSCs in human ATC cell line (THJ-11T, THJ-16T, THJ-21T, THJ-29T). In their study, 3%-9% of cells formed thyrospheres expressing NANOG and Oct4 markers, which possessed the ability to self-renew. On orthotopic thyroid transplantation of thyrospheres in NOD/SCID *Il2rg*^{-/-} mice, aggressive and metastatic tumors were generated depicting that thyroid provides the niche for these thyrospheres derived cells^[3]. Another such results were recently displayed by Todaro *et al.*^[8] using 3 histological variants (PTC, FTC, ATC). They demonstrated that only a small population of cells (1.2-3.5%) retains tumorigenic potential in thyroid cancer. Cells with ALDH^(high) expression were associated with unlimited replication potential and self-renewing property in serum-free media with highest percentage in ATC tissues. On orthotopic thyroid injection of thyrospheres in immunodeficient mice, these cells were able to reproduce similar phenotypic characteristics of parental tumor cells with ALDH^(high) UTC spheres exhibiting cervical nodal and distant metastasis^[8]. Accordingly, these re-

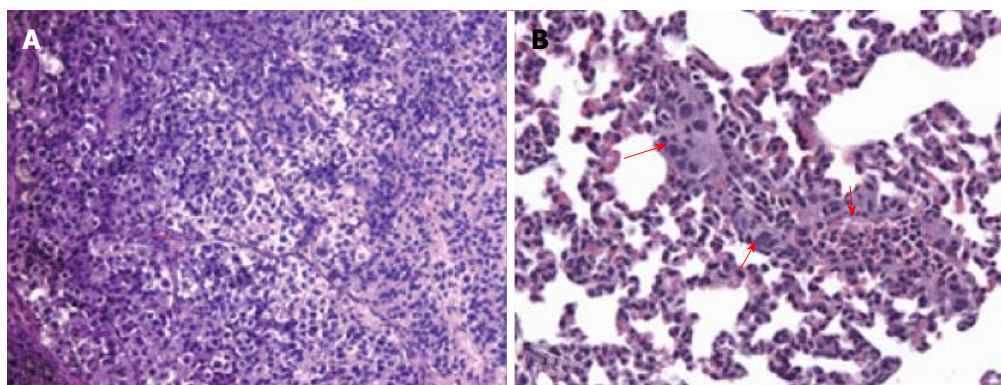


Figure 1 Histological images showing subcutaneous thyroid cancer mouse model. A: Hematoxylin and eosin stained microphotograph of tumor xenografts engrafted human thyroid cancer cell line (K1) with adipose-derived stem cells (ASCs) ($\times 100$); B: HE microphotograph of lung metastasis (red arrows) in the group transplanted with K1 cells and ASCs ($\times 200$). Methods of image acquisition: Tumor and organs removed from mouse, photographed, and stored in 10% neutral buffered formalin for paraffin sectioning and HE staining. Tumor tissue were sectioned and stained with HE^[28].

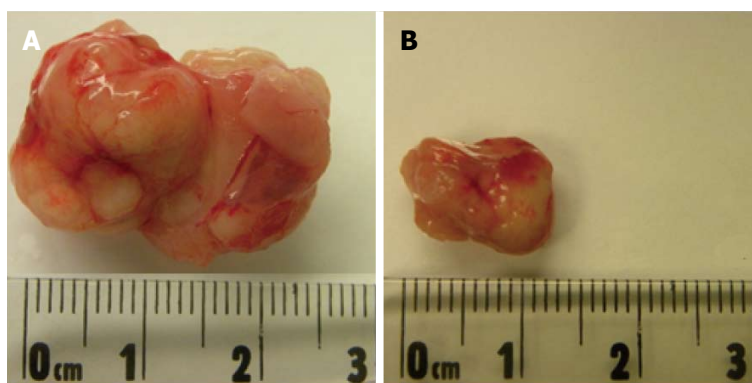


Figure 2 Representative tumor from severe combined immunodeficiency mice injected with: (A) K1 + ASCs and (B) K1 alone. ASCs: Adipose-derived stem cells.

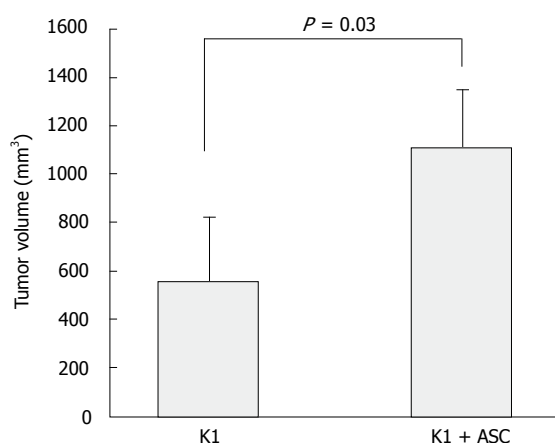


Figure 3 Adipose-derived stem cells promote tumor growth of papillary thyroid cancer cells. K1 cells alone and K1 cells with ASCs (5×10^5 cells each) were injected subcutaneously into nude mice ($n = 5$, each group)^[28]. ASC: Adipose-derived stem cell.

sults were also reported by Shimamura where their results displayed higher sphere forming ability with ALDH^{pos} in FRO, KTC3 and ACT1 and CD326^{high} in FRO cell lines^[27].

Although PTC accounts for majority of thyroid cancers, the data on CSCs existence in PTC cell lines is currently limited. A recent *in vivo* model has been designed by our group, where we described a subcutaneous mouse

model of metastatic human thyroid cancer by combining human adipose-derived stromal/stem cells (ASCs) with the human mutant BRAF V600E PTC cell line K1 (Figure 1A). Over a period of six weeks, we observed development of large tumors with distant metastasis in mice that were concomitantly injected with ASCs (5×10^5 cells) and K1 cells (5×10^5 cells). About 100% of lung metastasis was identified in ASCs + K1 group (Figure 1B) compared to 40% in mice receiving only K1 cells. Tumors in ASCs + K1 were significantly larger ($P < 0.05$) (Figure 2A) and developed earlier than the group of K1 alone (Figure 2B) demonstrating the role of ASCs in promoting dramatic tumor growth and seeding within the metastatic organs (Figure 3). To date, our model is the first model to display the use of ASCs to produce metastatic thyroid cancer^[28]. Zhu *et al.*^[29] demonstrated the existence of CSCs in MTC cell lines. These cells showed positivity for CD133 and displayed that RET proto-oncogene with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) favor self-renewal in MTC cells. Additionally, these cells also expressed neuron specific markers namely β -tubulin isotype III and glial fibrillar acidic protein^[29]. The purpose of demonstration of stemness markers using different cell lines in the models suggests that these markers should be targeted with an intent to develop new efficacious treatment for refractory tumors.

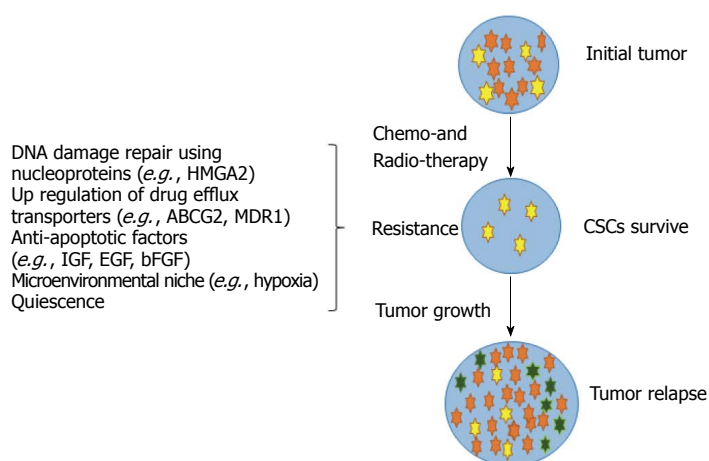


Figure 4 A schematic representation showing cancer stem cells resistance to chemo- and radio-therapy causing tumor to relapse. Cancer stem cells (CSCs) (yellow) with differentiated cells committed to a particular lineage (red). Ability of CSCs to resist anti-cancer therapy due to various mechanisms and ability to proliferate into heterogeneous group of cells cause tumor to relapse. HMG2: High mobility group A2; ABCG2: ATP-binding cassette sub-family G member 2; MDR1: Multi-drug resistance protein-1; IGF: Insulin-derived growth factor; EGF: Epidermal growth factor; bFGF: Basic fibroblast growth factor.

FUTURE PERSPECTIVES

Various genetic alterations defining oncogenic pathways in aggressive thyroid cancer have been recognized, yet, the ability to decode these mutations into novel anti-cancer therapies is limited. This recent discovery of thyroid CSCs marks an imperative stage for innovation of efficient anti-cancer treatment for resistant tumors. It is a well-known fact that conventional anti-cancer therapies target differentiating/differentiated cells, which form the bulk of the tumor but are unable to generate new cells. If CSCs remain in the quiescent stage (dormant cells), they resist the therapy targeted for dividing cells. CSCs self-renewal and ability to constitute a very small proportion of the tumor, they might develop resistance to chemo- and radiotherapy, ultimately causing the disease to relapse. It is possible that these cells may repair DNA damage more rapidly than normal cells^[30]. A newer concept has been postulated by various authors that points out the metastatic potential of CSCs secondary to epithelial-mesenchymal transition (EMT) and the inverse [mesenchymal-epithelial transition (MET)] at an advanced stage of the disease^[6,10,31,32]. A strong association between EMT/MET and CSCs has been highlighted recently, suggesting that EMT increases epithelial plasticity, confers tumor progression and therapeutic resistance to cancer cells. These transformed cells, then, behave like stem-cells similar to those seen in normal thyroid tissue. In a study done by Vasko *et al*^[31] PTC was associated with EMT due to overexpression of vimentin which led to regional lymph node invasion by the tumor^[31]. EMT is also associated with loss of E-cadherin, SNAIL, Twist and activation of β -catenin gene expression which make cells lose their adhesion and facilitate metastasis^[32]. However, some studies claim that the process of EMT is only observed in ATC. Authors have scripted the role of microRNAs in this transition process which makes CSCs undergo unlimited proliferation and capable of initiating tumor growth at metastatic sites^[10,25,31]. However, this area needs to be further explored before designing therapies aimed at the eradication of transformed cells.

In an attempt to develop targeted therapeutic strategies to eradicate this subset of CSCs, it becomes essen-

tial to determine their origin and whether they differ in various sub-types of thyroid cancer. Studies have shown overexpressed multi-drug resistance protein 1 (MDR 1) and ABCG2 transporters to cause resistance to cytotoxic drugs. With this concept, Zheng *et al*^[32] displayed how doxorubicin becomes ineffective and fail to eradicate CSC population. Because these drugs can specifically kill cancer cells, it provides a major space for CSCs to proliferate making the tumor resistant to chemotherapy, thus causing the disease to relapse^[32] (Figure 4).

In conclusion, therapeutic rationale should be laid, specifically, on destruction of CSCs by abruption of self-renewal signaling pathways, induction of differentiation of cancer cells and inhibition of survival-related mechanisms. Another venue to develop specific targeted therapies is by identification and destruction of the niche that nourishes CSCs for tumor growth. Because CSCs are heterogeneous and the cell-specific markers vary enormously amongst different tumor-types, there is an urgent need to identify further specific markers to support their existence. Further advancements in stem cell technology should focus on conglomerated existence of factors responsible for failure of current therapies in eradicating thyroid CSCs with the aim to target specific subpopulation of cells in the patients with refractory thyroid cancers.

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Kallikrein-kinin in stem cell therapy

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Abstract

The tissue kallikrein-kinin system exerts a wide spectrum of biological activities in the cardiovascular, renal and central nervous systems. Tissue kallikrein-kinin modulates the proliferation, viability, mobility and functional activity of certain stem cell populations, namely mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), mononuclear cell subsets and neural stem cells. Stimulation of these stem cells by tissue kallikrein-kinin may lead to protection against renal, cardiovascular and neural damage by inhibiting apoptosis, inflammation, fibrosis and oxidative stress and promoting neovascularization. Moreover, MSCs and EPCs genetically modified with tissue kallikrein are resistant to hypoxia- and oxidative stress-induced apoptosis, and offer enhanced protective actions in animal models of heart and kidney injury and hindlimb ischemia. In addition, activation of the plasma kallikrein-kinin system promotes EPC recruitment to the inflamed synovium of arthritic rats. Conversely, cleaved high molecular weight kininogen, a product of plasma kallikrein, reduces the viability and vasculogenic activity of EPCs. Therefore, kallikrein-kinin provides a new approach in enhancing the efficacy of stem cell therapy for human diseases.

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Key words: Tissue kallikrein; Plasma kallikrein; Kinin; Mesenchymal stem cells; Endothelial progenitor cells; Neural stem cells; Heart; Kidney; Brain

Core tip: Tissue kallikrein-kinin exerts beneficial actions in the cardiovascular, renal and central nervous systems. Recent studies demonstrated that genetic modification of mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs) by tissue kallikrein provides enhanced protection against renal ischemia/reperfusion, lupus nephritis, myocardial infarction and hindlimb ischemia. Tissue kallikrein stimulates the proliferation, viability, migration and functional activity of cultured MSCs, EPCs and neural stem cells. Moreover, plasma kallikrein-kinin augments EPC mobility and function in arthritis, whereas the cleaved kininogen product of plasma kallikrein inhibits EPC viability and tube formation. Thus, kallikrein-kinin may enhance the efficacy of stem cell therapy for human diseases.

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INTRODUCTION

Tissue kallikrein (*KLK1*) and plasma kallikrein (*KLKB1*) are serine proteinases encoded by distinct genes, and thus differ in molecular weight, amino acid sequence and immunogenicity^[1-3]. Human tissue kallikrein cleaves low molecular weight (LMW) kininogen to produce Lys-bradykinin (Lys-BK), which is subsequently converted to BK by aminopeptidase^[2]. Plasma kallikrein processes high molecular weight (HMW) kininogen substrate to form BK^[2]. Both kinin peptides bind to the kinin B2 receptor to elicit a diverse array of biological effects^[2-5], including enhancing stem cell function (Figure 1). The kinin B2 re-

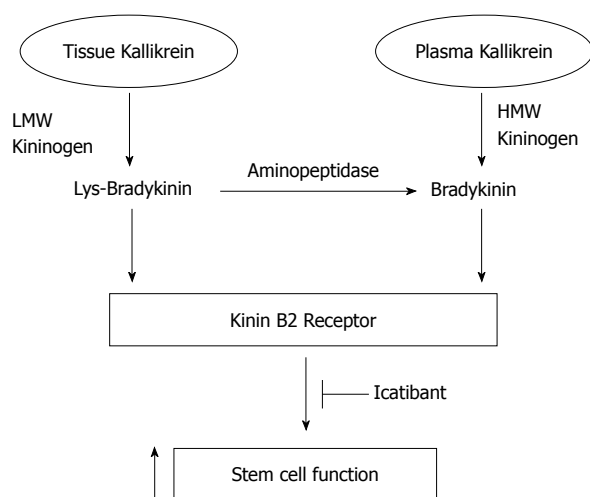


Figure 1 Tissue kallikrein-kinin and plasma kallikrein-kinin systems on stem cell function. LMW: Low molecular weight; HMW: High molecular weight.

ceptor is constitutively expressed with a wide tissue distribution, but can be blocked by the specific antagonist icatibant (Hoe140)^[5]. Kinin metabolites of kininase I, such as des-Arg⁹-BK and des-Arg¹⁰-Lys-BK, bind to the kinin B1 receptor, which is expressed at very low levels under normal conditions but is induced by inflammation^[5]. The tissue kallikrein-kinin system triggers a broad spectrum of biological activities, including stimulation of angiogenesis and reduction of hypertension, cardiac and renal damage, ischemic stroke, restenosis, diabetes and skin wound injury^[6]. Plasma kallikrein circulates in the blood as a proenzyme and, upon its activation, functions to produce BK to increase vascular permeability and stimulate vasodilation and inflammation^[7,8]. Activated plasma kallikrein also initiates the intrinsic pathway of coagulation and the fibrinolytic system^[7,8]. In this review, we discuss the involvement of tissue kallikrein, plasma kallikrein and kinin peptides in promoting the mobility and functional capacity of stem cells, which may lead to enhanced protection against organ injury in human diseases.

TISSUE KALLIKREIN-KININ IN RENAL INJURY

Tissue kallikrein was first discovered in human urine as a hypotensive substance^[9]. Urinary (tissue) kallikrein excretion is significantly reduced in patients with mild kidney disease and severe renal failure^[10,11]. Tissue kallikrein gene transfer or protein infusion in hypertensive Dahl salt-sensitive (DSS) rats has been observed to decrease kidney injury, improve renal function, and stimulate nitric oxide (NO) generation *via* the kinin B2 receptor^[12-15]. Moreover, tissue kallikrein or kinin administration not only attenuated but also reversed renal inflammation, apoptosis and fibrosis in conjunction with reduced oxidative stress and increased NO production in hypertensive DSS and deoxycorticosterone acetate (DOCA)-salt rats^[15-18]. The renal protective effects of tissue kallikrein in DSS rats

were abolished by icatibant, indicating a kinin B2 receptor-mediated event^[15]. Conversely, endogenous tissue kallikrein depletion in DOCA-salt rats augmented renal injury, inflammation and fibrosis in association with increased expression of pro-inflammatory and pro-fibrotic genes, oxidative stress, and reduced NO levels^[19]. Moreover, double knockout of the kinin B1 and B2 receptors in mice demonstrated that these receptors protect against ischemia/reperfusion (I/R)-induced renal damage, apoptosis and mortality^[20]. In a unilateral ureteral obstruction model, interstitial collagen content in the kidney was increased in kinin B2 receptor deficient mice, whereas transgenic rats expressing human tissue kallikrein displayed reduced renal fibrosis^[21]. Therefore, endogenous tissue kallikrein-kinin *via* kinin B2 receptor signaling can prevent and reverse renal injury by inhibiting oxidative stress, apoptosis, inflammation and fibrosis.

TISSUE KALLIKREIN-KININ IN CARDIAC INJURY

Tissue kallikrein-kinin components have been localized in the heart and blood vessels, indicating their involvement in cardiovascular function^[22-24]. Indeed, both tissue kallikrein and kinin B2 receptor knockout mice develop dilated cardiomyopathy, and mice with kinin B2 receptor genetic ablation exhibit cardiac fibrosis^[25,26]. However, expression of tissue kallikrein in transgenic rats reduces isoproterenol-induced cardiac hypertrophy and fibrosis^[27]. Likewise, tissue kallikrein gene delivery protects against cardiac remodeling as well as neovascularization in spontaneously hypertensive rats (SHR) and salt- and pressure-induced hypertensive rats^[28-31]. Tissue kallikrein infusion or gene transfer also improved impaired cardiac function and reduced heart remodeling, apoptosis and inflammation in animal models of myocardial infarction (MI), myocardial I/R and streptozotocin-induced diabetes^[32-36]. The cardioprotective effects of tissue kallikrein on apoptosis and inflammation were blocked by icatibant and a NO synthase (NOS) inhibitor, indicating a kinin B2 receptor-NO-mediated event^[35,36]. Furthermore, tissue kallikrein gene delivery to the peri-infarct myocardium increased cardiac progenitor cell (CPC) levels and promoted cardiac neovascularization and function in rats with post-MI heart failure^[37]. Although tissue kallikrein increased CPC density, their levels were low compared to other cardiac cells. Thus, the regenerative capacity of CPCs in the adult heart appears to be limited and requires further investigation. Taken together, tissue kallikrein-kinin elicits cardiac protection by inhibiting apoptosis, inflammation and myocardial remodeling, and increasing angiogenesis through kinin B2 receptor-NO signaling.

TISSUE KALLIKREIN-KININ IN VASCULAR INJURY

Endothelial cell loss leads to vascular dysfunction and

vascular-related diseases. Tissue kallikrein levels in the circulation are significantly higher in patients with coronary artery disease (CAD) compared to non-CAD patients, and increase with disease severity, from moderate CAD to multi-vessel CAD with acute obstruction^[38]. This suggests that circulating tissue kallikrein levels may be used as a predictive tool to assess the presence and extent of CAD. Tissue kallikrein gene transfer into rat left common carotid artery after balloon angioplasty was shown to cause a marked reduction in neointima formation at the injured vessel, and this effect was mediated by a kinin B2 receptor-NO pathway^[39,40]. In addition, endothelium-dependent relaxation was improved in tissue kallikrein transgenic rats with diabetic cardiomyopathy, but significantly reduced in kinin B1 and B2 receptor knockout mice in association with a decrease in NO production^[41,42]. Moreover, kinin B2 receptor-deficient mice exhibit myocardial capillary rarefaction^[43]. Conversely, tissue kallikrein gene delivery promoted neovascularization and attenuated cardiac remodeling in animal models of hypertension and MI^[31,32]. Tissue kallikrein is capable of accelerating spontaneous angiogenesis in a mouse model of hindlimb ischemia by activating Akt and endothelial NOS (eNOS) signaling pathways^[44,45]. Tissue kallikrein also enhanced the migration and tube formation of cultured endothelial cells, but these effects were blocked by icatibant, constitutively active glycogen synthase kinase (GSK)-3 β , vascular endothelial growth factor (VEGF) antibody and VEGF receptor inhibitor^[46]. Furthermore, kinin stimulated the proliferation and capillary tube formation of endothelial cells *via* transactivation of VEGF receptor-2 through the kinin B2 receptor^[47,48]. These findings indicate that tissue kallikrein-kinin attenuates vascular injury by preventing neointima formation and promoting angiogenesis through Akt-eNOS and Akt-GSK-3 β -VEGF mediated signaling pathways.

TISSUE KALLIKREIN-KININ IN ISCHEMIC STROKE

The time window for treatment of stroke patients is limited, as the clinically accepted treatment regimen with tissue plasminogen activator (tPA) requires initiation within 3 h of symptom onset^[49]. Tissue kallikrein has a superior advantage over tPA with a wide time window after stroke. In a double-blinded clinical trial, human tissue kallikrein was shown to be effective in the treatment of patients with acute brain infarction when infused within 48 h of established stroke^[50]. These findings indicate that tissue kallikrein therapy is a promising regimen in the treatment of ischemic stroke in humans. Moreover, tissue kallikrein-kinin therapy has been shown to be an effective approach in the treatment of stroke-induced brain injury in animal models^[51,52]. Neuroprotective effects were observed upon local injection of the human tissue kallikrein gene into rat brain immediately after cerebral I/R injury, or by systemic delivery of the tissue kallikrein gene at 8 h after ischemic stroke onset^[51,52].

Tissue kallikrein administration reduced I/R-induced cerebral infarction and promoted the survival and migration of glial cells from penumbra to the ischemic core up to two weeks^[51,52]. Tissue kallikrein also decreased I/R-induced apoptosis of neuronal cells and inhibited inflammatory cell accumulation in the ischemic brain, but these effects were blocked by icatibant^[52]. Furthermore, tissue kallikrein gene transfer enhanced neurogenesis and angiogenesis in rats after cerebral I/R^[52]. Tissue kallikrein's effects occurred in association with increased NO levels and reduced oxidative stress *via* activation of the kinin B2 receptor^[51,52]. In contrast, ischemic brain injury is exacerbated in kinin B2 receptor knockout mice^[53]. Thus, tissue kallikrein-kinin therapy may serve as a valuable approach in the treatment of stroke-induced brain injury, especially if treatment is delayed.

MESENCHYMAL STEM CELLS IN RENAL AND CARDIAC DISEASES

Mesenchymal stem cells (MSCs) are heterogeneous, multi-potent stromal cells that possess non-immunogenic and immunosuppressive properties^[54]. MSCs have been documented to reside in bone marrow, adipose tissue, umbilical cord blood, placenta, amniotic fluid and amniotic membrane^[55]. MSCs can be characterized by three main criteria: (1) adherent to plastic culture dishes; (2) expression of the cell surface markers CD73, CD90, CD105, and CD271; and (3) differentiation into lineages of osteoblasts, adipocytes, and chondroblasts *in vitro*^[55]. MSCs have the ability to migrate to sites of organ injury and participate in tissue repair by exerting paracrine actions to produce therapeutic effects, such as neovascularization and organ regeneration^[54,56-59]. Clinical trials using human bone marrow-derived MSCs are currently underway to treat diseases such as renal, cardiovascular, and cerebrovascular disorders (<http://clinicaltrials.gov>). Efficacy can be maximized by pre-treatment of MSCs with drugs, cytokines, and growth factors, and by genetically modifying MSCs^[60]. Indeed, enhancing stem cell therapy by genetic modification has been shown provide advanced benefits in the treatment of various diseases^[61]. For example, MSCs genetically modified with hepatocyte growth factor or VEGF ameliorated I/R- or cisplatin-induced renal damage, inflammation and apoptosis^[62,63]. Moreover, modification of MSCs with the anti-apoptotic *Akt* gene or the anti-oxidant heme oxygenase-1 gene was observed to augment ischemic cardiac function and stem cell viability, and decrease ventricular remodeling and apoptosis compared to control MSCs^[64,65]. Thus, modification of MSCs with a gene that suppresses inflammation, apoptosis and oxidative stress would be highly desirable in the treatment of renal and cardiovascular dysfunction. Tissue kallikrein fits this profile, and MSCs modified with tissue kallikrein have been shown to exert enhanced protective actions in the heart and kidney as well as *in vitro*^[66,67].

STUDIES OF CULTURED MSCs MODIFIED WITH TISSUE KALLIKREIN GENE

Bone marrow-derived rat MSCs transduced with adenovirus harboring the human tissue kallikrein gene (*TK-MSCs*) secrete tissue kallikrein along with elevated VEGF levels in culture medium^[66,67]. *TK-MSCs* were also found to be more resistant to hypoxia- and H₂O₂-induced apoptosis, and exhibited less caspase-3 activity compared to control MSCs. In addition, *TK-MSC* conditioned medium stimulated the proliferation, migration and tube formation of cultured human endothelial cells, most likely *via* VEGF^[67]. In cultured cardiomyocytes, conditioned medium from *TK-MSCs* suppressed hypoxia-induced apoptosis and caspase-3 activity, and increased Akt phosphorylation^[67]. Moreover, human MSCs possess kinin B2 receptors, as kinin stimulation increased intracellular calcium levels in MSCs, but this effect was blocked by icatibant^[68]. This suggests that *TK-MSCs* exert their effects *via* autocrine and paracrine mechanisms. Furthermore, these results demonstrate that culture medium of MSCs genetically modified with the tissue kallikrein gene promotes the function, migration and viability of cultured endothelial and cardiac cells.

TISSUE KALLIKREIN-MODIFIED MSCs PROVIDE ENHANCED PROTECTION IN KIDNEY INJURY

Acute renal failure is a common disease with high morbidity and mortality^[69]. In kidney transplants, ischemia can lead to long-term renal dysfunction^[69,70]. However, implantation of bone marrow-derived MSCs after acute I/R resulted in renal function and morphological recovery, implicating the high therapeutic potential of MSCs in healing damaged kidney^[56,71]. Indeed, *TK-MSC* administration in rats subjected to I/R injury was shown to be protective against kidney damage^[66]. After systemic injection of *TK-MSCs*, human tissue kallikrein expression was identified in rat glomeruli. Rats receiving *TK-MSCs* exhibited an improvement in renal function after I/R. *TK-MSC* implantation in the kidney also markedly reduced tubular injury, renal cell apoptosis, and interstitial inflammatory cell accumulation. The protective effects of *TK-MSCs* occurred in conjunction with decreased myeloperoxidase activity, superoxide formation, and pro-inflammatory gene expression. Therefore, MSCs incorporating the human tissue kallikrein gene have advanced benefits in protection against ischemia-induced renal injury by suppression of oxidative stress, apoptosis and inflammation.

TISSUE KALLIKREIN-MODIFIED MSCs IN LUPUS NEPHRITIS PROTECTION

Tissue kallikrein has been identified as a lupus nephritis-

susceptibility gene and is associated with anti-glomerular basement membrane (GBM) antibody-induced nephritis^[72,73]. *TK-MSCs* were shown to exert beneficial effects in mice receiving anti-GBM antibody injection and in a murine model of lupus nephritis by suppressing inflammation and oxidative stress^[74]. *TK-MSC* administration to mice subjected to anti-GBM antibody injection resulted in the expression of human tissue kallikrein in the kidney as well as a significant reduction in proteinuria, blood urea nitrogen levels and renal pathology, compared to mice injected with control MSCs. Similarly, *TK-MSC* implantation in lupus-prone bicongenic mice improved kidney function and attenuated renal inflammatory cell infiltration and apoptosis in conjunction with reduced expression of numerous inflammatory cytokines and apoptotic factors in both kidney and serum. These novel findings indicate that tissue kallikrein-modified MSCs may serve as a targeted therapeutic agent in lupus nephritis.

TISSUE KALLIKREIN-MODIFIED MSCs IN CARDIAC PROTECTION

Chronic heart failure induced by MI leads to a loss of cardiac tissue and impairs left ventricular function^[58]. MSCs are a promising strategy for the repair and regeneration of heart cells as well as the restoration of cardiac function after an ischemic insult. However, a major limitation to the efficacy of stem cell therapy is the poor viability of implanted cells. Thus, genetic modification of MSCs to promote their viability may further aid in the treatment of cardiac damage. Cell culture studies showed that *TK-MSCs* display decreased apoptosis induced by hypoxia or oxidative stress^[66,67]. In rats with acute and chronic MI, myocardial injection of *TK-MSCs* resulted in enhanced cardiac protection compared to control MSC treatment^[67]. One day after MI, rats receiving *TK-MSC* administration were shown to have improved cardiac function and decreased apoptosis, inflammatory cell accumulation, and expression of pro-inflammatory genes. At two weeks after MI, *TK-MSC* implantation enhanced cardiac function, decreased infarct size, and attenuated cardiac hypertrophy and fibrosis. Furthermore, *TK-MSC* injection increased capillary and arteriole density in the peri-infarct area. These results indicate that *TK-MSC* treatment after acute and chronic MI provides significant protection against heart damage by promoting neovascularization and preventing apoptosis and inflammation.

ENDOTHELIAL PROGENITOR CELLS IN CARDIOVASCULAR DISEASES

Endothelial injury is a critical factor for complications associated with cardiovascular disease^[75]. Endothelial progenitor cells (EPCs) are a continuous endogenous source of replenishment for damaged vessels, and thus serve to maintain vascular integrity in response to endothelial injury^[75,76]. Bone marrow-derived EPCs are considered to be adult stem cells due to their participation in postnatal

angiogenesis^[77]. EPCs contribute to vasculogenesis by incorporating into the vasculature, thereby implicating their therapeutic potential in endothelial repair^[78]. Decreased numbers of circulating EPCs have been observed in patients with hypertension, chronic renal failure, CAD, and rheumatoid arthritis^[78-81]. Moreover, EPCs isolated from patients with hypertension and CAD displayed an impaired migratory response^[79]. However, the correlation of circulating EPC number and outcome of stroke patients is inconsistent. Lower EPC numbers were found to be associated with acute ischemic stroke^[82], whereas higher EPC levels were reported in hemorrhagic stroke patients^[83]. Reduced EPC numbers may be attributed not only to defective mobility and proliferation, but also to accelerated apoptosis or senescence. Therefore, augmented viability and mobilization of EPCs from bone marrow may be an alternative means to promote vascular repair. Furthermore, EPCs may serve as a vehicle for gene transfer approaches in the treatment of cardiovascular diseases. The tissue kallikrein-kinin system has been shown to be involved in cardiovascular remodeling, vascular function and angiogenesis^[6], making tissue kallikrein an ideal candidate for EPC genetic modification.

KININ B2 RECEPTOR ACTIVATION PROMOTES EPC RECRUITMENT

Healthy human subjects express high levels of kinin B2 receptor in CD133⁺CD34⁺ peripheral blood-mononuclear cell (PB-MNC) subsets and EPCs; kinin B1 receptor expression, however, is barely detectable in these cells^[84]. Kinin administration exerted a potent chemoattractant activity on EPCs *via* a kinin B2 receptor-phosphoinositide 3-kinase (PI3K)-eNOS-mediated mechanism. The role of the kinin B2 receptor in kinin-induced migration was verified using EPCs derived from kinin B2 receptor knockout mice. Kinin-responsive human PB-MNCs exhibited a pronounced pro-angiogenic activity, whereas EPCs from kinin B2 receptor-deficient mice were unable to sufficiently stimulate neovascularization in a mouse model of hindlimb ischemia. In addition, circulating CD133⁺CD34⁺ progenitor cells from patients with acute MI or stable angina expressed low levels of kinin B2 receptor, which corresponded to diminished migratory capacity toward kinin. Moreover, human circulating CD34⁺CXCR4⁺ MNCs expressing high levels of kinin B2 receptor adhered to cultured endothelial cells upon kinin treatment, and these kinin-stimulated mononuclear subsets were recruited to injured arterial wall *in vivo via* the kinin B2 receptor^[85]. Conversely, CD34⁺CXCR4⁺ MNCs from CAD patients exhibited low kinin B2 receptor expression levels. Furthermore, kinin administration had no effect on cellular recruitment upon icatibant treatment or in monocytes with low kinin B2 receptor expression. These studies indicate a novel mechanism of kinin B2 receptor activation in endothelial repair through recruitment of circulating EPCs and MNC subsets.

TISSUE KALLIKREIN-MODIFIED EPCs ENHANCE CARDIAC PROTECTION BY PROMOTING EPC MOBILIZATION AND FUNCTION

Tissue kallikrein was recently demonstrated to promote vasculogenesis and improve cardiac function after MI by enhancing peripheral EPC functional capacity^[86,87]. Human tissue kallikrein gene delivery significantly increased the number of circulating CD34⁺Flk-1⁺ EPCs as well as the growth of capillaries and arterioles in the peri-infarct myocardium in a mouse model of MI^[86]. In cultured EPCs, tissue kallikrein treatment stimulated cell migration and tube formation, and decreased hypoxia-induced apoptosis^[86]. Tissue kallikrein's effects were blocked by icatibant and a PI3K inhibitor, indicating a kinin B2 receptor-Akt signaling event. Moreover, adenovirus-mediated transduction of cultured EPCs with tissue kallikrein (TK-EPCs) resulted in the secretion of tissue kallikrein and VEGF into culture medium^[86,87]. TK-EPCs were also resistant to oxidative stress- and hypoxia-induced apoptosis in association with increased Akt phosphorylation and decreased caspase activity. Furthermore, mice receiving intra-myocardial injection of TK-EPCs after MI exhibited advanced protection against ischemic damage, as indicated by improved cardiac function and reduced infarct size^[87]. TK-EPC engraftment significantly decreased cardiomyocyte apoptosis and increased the retention of transplanted EPCs in the myocardium. The effects of TK-EPC administration were accompanied by increased capillary and arteriole density in the infarct border zone. These results show that implantation of tissue kallikrein-modified EPCs in the heart augments protection against cardiac injury by reducing apoptosis and promoting angiogenesis.

TISSUE KALLIKREIN-MODIFIED EPCs INDUCE ANGIOGENESIS IN THE ISCHEMIC HINDLIMB

Tissue kallikrein's pro-angiogenic activity has been clearly established^[6,44,45,86], and genetic modification of EPCs with tissue kallikrein was shown to promote neovascularization and cardiac function in an MI mouse model^[87]. Moreover, the effect of TK-EPC administration on spontaneous angiogenesis was identified in a rat model of hindlimb ischemia^[88]. Compared to control EPCs, TK-EPC injection *via* the caudal vein markedly increased muscular capillary density, blood flow and myofiber number at 7, 14 and 21 d after femoral artery ligation. The angiogenic effect of TK-EPCs correlated with elevated expression of eNOS and integrin α 3 on the surface of EPCs. Moreover, cultured TK-EPCs exhibited higher proliferative, migratory and adhesive activity than control EPCs^[88]. Inhibition of integrin α 3 blocked TK-EPC

Table 1 Enhanced protection by stem cells genetically modified with tissue kallikrein

	Apoptosis	Inflammation	Oxidative stress	Tissue remodeling	Angiogenesis
TK-MSCs					
Renal I/R ^[66]	↓	↓	↓	--	--
Lupus nephritis ^[74]	↓	↓	↓	--	--
MI ^[67]	↓	↓	--	↓	↑
TK-EPCs					
MI ^[87]	↓	--	↓	--	↑
Limb ischemia ^[88]	--	--	--	--	↑

TK-MSCs: Tissue kallikrein-modified mesenchymal stem cells; I/R: Ischemia/reperfusion; MI: Myocardial infarction; TK-EPCs: Tissue kallikrein-modified endothelial progenitor cells.

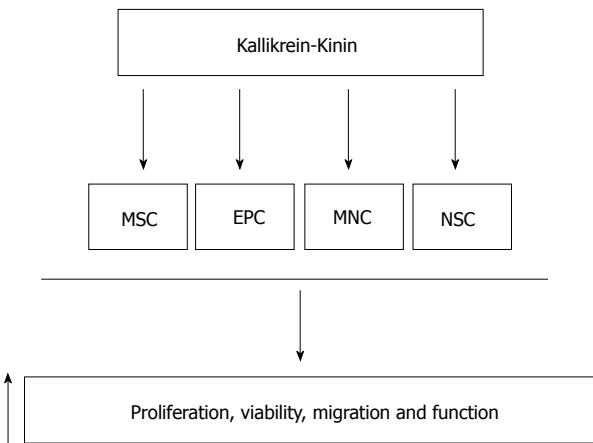


Figure 2 Kallikrein-kinin enhances the proliferation, viability, migration and function of stem cells. MSC: Mesenchymal stem cell; EPC: Endothelial progenitor cell; MNC: Mononuclear cell; NSC: Neural stem cell.

migration and adhesion, but had no effect on the proliferative activity of TK-EPCs. This suggests that EPCs genetically modified with tissue kallikrein enhance neovascularization and blood perfusion recovery after hindlimb ischemia.

TISSUE KALLIKREIN STIMULATES NEURAL STEM CELL GROWTH

Tissue kallikrein-kinin treatment has been shown to be effective in preventing stroke-induced ischemic brain injury by promoting neurogenesis and angiogenesis in animal models and cultured cells^[51,52]. In addition, tissue kallikrein was observed to stimulate the growth of rat neural stem cells independent of kinin formation, as icatibant had no effect on tissue kallikrein's actions^[89,90]. However, tissue kallikrein did not induce the differentiation of neural stem cells to neurons or glial cells^[90]. The proliferation of neural stem cells by tissue kallikrein is quite specific, with no detectable effect on other cell types, such as glial, pheochromocytoma, pituitary tumor, and cervical cancer cells^[90]. Thus, stimulation of neural stem cell proliferation by tissue kallikrein administration may lead to the generation of new neurons in the ischemic brain. Importantly, this stimulating effect of tissue kallikrein on neural stem cells may have significant value in the treatment of isch-

emic stroke.

PLASMA KALLIKREIN-KININ SYSTEM IN RECRUITMENT OF EPCs TO INFLAMED SYNOVIUM

Plasma kallikrein has been demonstrated to play a role in the pathogenesis of arthritis^[91,92]. As kinins are known to promote EPC mobilization and functional activity^[84,85], the involvement of the plasma kallikrein-kinin system in EPC mobilization was examined in a Lewis rat model of arthritis^[93]. The Lewis rat strain possesses a mutation in HMW kininogen (HK), resulting in accelerated HK cleavage and increased susceptibility to chronic inflammation^[94]. In arthritic Lewis rats, EPCs were recruited to the synovium at the acute phase of arthritis, and then differentiated into endothelial cells to form new blood vessels^[93]. Inhibition of plasma kallikrein by a specific inhibitor or anti-plasma kallikrein antibody dramatically suppressed synovial recruitment of EPCs and the proliferation of synovial cells. Moreover, EPCs isolated from bone marrow of Lewis rats were observed to have higher expression levels of kinin B2 receptor compared to control rat lung microvessel endothelial cells^[93]. In addition, kinin stimulated EPC migration and up-regulated expression of the homing receptor CXCR4 *in vitro* via the kinin B2 receptor. These results demonstrate a potential role of plasma kallikrein-kinin, *via* a kinin B2 receptor-dependent mechanism, in the recruitment of EPCs to inflamed synovium in arthritis.

CLEAVED HIGH MOLECULAR WEIGHT KININOGEN INHIBITS EPC FUNCTION

Cleaved HMW kininogen (HKa), a product of plasma kallikrein, has been shown to reduce the angiogenic function of endothelial cells as well as to stimulate their apoptosis^[95,96]. In cultured EPCs, HKa significantly inhibited VEGF-mediated tube formation and cellular differentiation into capillary-like networks^[97]. VEGF stimulated the secretion and activation of matrix metalloproteinase-2 (MMP-2), but not MMP-9, in the conditioned medium of EPCs. Inhibition or gene knockdown of MMP-2 indicated that this enzyme is required for EPC vasculo-

genesis. Although HKa prevented the conversion of pro-MMP-2 to MMP-2, it had no effect on MMP-2 activity. Furthermore, HKa was demonstrated to accelerate EPC senescence by increasing oxidative stress, leading to activation of the p38MAPK-p16^{INK4a} signaling cascade^[98]. These results indicate that HKa inhibits the vasculogenic capacity of EPCs by suppressing MMP-2 activation and promoting EPC senescence *via* oxidative stress-p38MAPK signaling, thus providing a link between the plasma kallikrein product HKa and EPC function.

CONCLUSION

The tissue kallikrein-kinin system plays an important role in the cardiovascular, renal and central nervous systems by inhibiting apoptosis, inflammation, fibrosis and oxidative stress. Tissue kallikrein-kinin may also enhance stem cell number and function. Indeed, tissue kallikrein-kinin increases the mobility, viability and functional capacity of stem cells, such as MSCs, EPCs, and MNC subsets, leading to protection against multi-organ injury and stimulating neovascularization. Tissue kallikrein may also exert a protective effect against cerebral ischemic damage in stroke patients by promoting neural stem cell growth. Moreover, studies showed that tissue kallikrein-modified MSC or EPC engraftment into injured tissues provided advanced protection against vascular and organ damage (Table 1). Thus, transplantation of tissue kallikrein-modified stem cells may be used for the treatment of patients with renal, cardiovascular, and cerebrovascular diseases. Furthermore, plasma kallikrein-kinin was observed to enhance EPC mobility and functional capacity in arthritis, while the cleaved kininogen product HKa inhibited EPC tube formation and viability. Collectively, these studies show that kallikrein-kinin stimulates the proliferation, viability, migration and function of various types of stem cells (Figure 2), and implicate the potential role of kallikrein-kinin in stem cell-based therapy for numerous human diseases.

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Sox2 transcription network acts as a molecular switch to regulate properties of neural stem cells

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Abstract

Neural stem cells (NSCs) contribute to ontogeny by producing neurons at the appropriate time and location. Neurogenesis from NSCs is also involved in various biological functions in adults. Thus, NSCs continue to exert their effects throughout the lifespan of the organism. The mechanism regulating the core functional properties of NSCs is governed by intra- and extracellular signals. Among the transcription factors that serve as molecular switches, Sox2 is considered a key factor in NSCs. Sox2 forms a core network with partner factors, thereby functioning as a molecular switch. This review discusses how the network of Sox2 partner and target genes illustrates the molecular characteristics of the mechanism underlying the self-renewal and multipotency of NSCs.

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Key words: Neural stem cells; Self-renewal; Multipotency; Sox2; Transcriptional network

Core tip: Neural stem cells (NSCs) are cells that are capable of both self-renewal and multipotency. In these two processes, the transcription factor Sox2 serves as a switch for the central molecular mechanism. Sox2

forms complexes with its partner factors to perform its transcription-related functions. This partner switching presumably serves as an important key to the intrinsic functions of NSCs. A detailed understanding of these molecular mechanisms will advance our understanding of basic neuroscience and increase the feasibility of employing cell reprogramming technology in regenerative medicine.

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INTRODUCTION

Neural stem cells (NSCs) are cells that are capable of self-renewal and maintaining multipotency^[1,2]. NSCs differentiate into neurons, astrocytes, and oligodendrocytes. The cellular origin of mouse NSCs dates back to the initial stage of ontogeny. A blastocyst generates the primitive ectoderm, further differentiating into the neuroectoderm, which serves as a source of primitive NSCs^[3,4]. The neuroectoderm then develops and differentiates into the neuroepithelium^[3,4]. Primitive NSCs exhibit self-renewal with a rather limited multipotency^[5]. On embryonic day 11.5 (E11.5) in the murine fetal period, differentiation into neurons dominates while differentiation into the astrocyte lineage is suppressed by DNA methylation. Then at E14.5, NSCs begin to produce neurons and astrocytes^[6-8]. After birth, NSCs manifest their ability to produce oligodendrocytes^[8]. NSCs also actively undergo repeated self-renewal in the region of the central nervous system after birth to generate neurons, astrocytes, and oligodendrocytes in a region-dependent manner to build the brain as an organ. It was previously believed that neurons

do not regenerate once the brain organogenesis is complete in an adult organism. However, the study^[9-13] revised this dogma, and it is now known that neurogenesis takes place even in the adult brain. This process has been best studied in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) lining the hippocampal region, where NSCs are located and produce new nerve cells through self-renewal^[1,14-20]. Understanding the molecular biology underlying the capacity of NSCs to exhibit self-renewal and multipotency is expected to stimulate our exploration of basic neuroscience and lead to practical applications in regenerative medicine, allowing lost neurons to be regenerated as desired. Although some NSCs can be cultured from body tissues as the monolayer^[21-23], there are technical challenges as well as issues of productivity and quality related to the practical use of such cultured NSCs in regenerative medicine. However, a new technology was recently developed to reprogram somatic cells through the gene transfer. Using this technology, combinational transfection of the *Oct4*, *Sox2*, *Klf4*, and *c-Myc* genes into various cells can establish a type of multipotent stem cells, called induced pluripotent stem (iPS) cells^[24,25]. By changing the culture conditions under which iPS cells are established, we can artificially induce differentiation into NSCs^[26]. This technology has also been utilized to develop induced neuronal (iN) cells, which directly induce differentiation into neurons^[27]. iN cells are obtained by transfecting the *Ascl1*, *Brn2*, and *Myt1l* genes into fibroblasts. The gene cluster serving as the switch to precisely regulate cell fate mainly includes transcription factors. One key factor that plays an important role in NSCs is the transcription factor Sox2. Transcription factors bind to response regions in the genome to initiate or terminate the expression of target genes. Concomitantly, they interact with a group of chromatin-regulating factors other than transcription factors to perform various regulatory functions. In this review, I focus on the transcription regulatory network centered around Sox2 to shed light on the molecular regulatory mechanism underlying the biology of NSCs.

NEURAL STEM CELLS AND SOX2

Sox2 belongs to the *Sry* gene family and contains a DNA-binding domain referred to as a high-mobility group (HMG) domain, which is highly conserved across the family. To date, more than 20 genes have been identified in the *Sox* gene family^[28,29]. Sox2 is a maternal factor that is specifically expressed in the inner cell mass (ICM) and primitive ectoderm^[30]. Sox2 expression is widely observed among the cells within the neural tube at early stages of neurodevelopment^[31]. Its expression is subsequently localized to the ventricular layer in the neuronal cortex, where NSCs and their precursor cells are present after the mid-fetal period. During this period, Sox2 is not expressed in layers where terminally differentiated neurons are present^[32]. In the adult brain, NSCs are localized to the SVZ of the lateral ventricle and the SGZ lining the hip-

poampal region, where they undergo self-renewal and perform neurogenesis^[1,14]. All of such self-renewing cells express Sox2. Sox2 plays an important role in maintaining the functions of NSCs^[32-35]. It has been reported that SoxB1 family members, Sox1 and Sox3, which show high sequence homology to Sox2, exhibit similar functions^[36]. Sox2 functions as a maternal factor in pre-implantation embryos^[30]. Zygotic knock-down of Sox2 using a specific siRNA resulted in an incomplete trophectoderm (TE) in fertilized embryos, which failed to progress beyond the morula stage^[30]. Sox2 expression is detected in both the ICM and TE, and its expression becomes restricted to the ICM^[29]. During embryogenesis, the ICM becomes the embryo, and the TE forms the placenta. A high level of *Sox2* gene expression has been confirmed in the neuroectoderm that gives rise to NSCs^[31]. During embryogenesis, Sox2 promotes neuroectoderm cell fate by suppressing the mesodermal cell fate^[37]. Moreover, Sox2 plays important roles in the differentiation of the central nervous system and peripheral nervous system during embryogenesis by controlling the proliferation and differentiation of neural stem/progenitor cells^[32]. Sox2 deficiency is embryonically lethal in mice because the fetus fails to form embryonic stem (ES) cells from the ICM or produce trophoblast stem cells^[30,38]. Sox2 conditional knock out (KO) mice have been reported to undergo neurodegeneration leading to dysfunctional neuronal differentiation in the adult brain^[35,39]. Various research approaches have been employed to demonstrate that Sox2 expression is localized to NSCs and that its function is essential for these cells.

SOX2 AND ITS PARTNERS

Sox2 collaborates with other transcription factors^[40,41]. In ES cells and NSCs, Sox2 regulates the self-renewal mechanism and suppresses differentiation in a dosage-sensitive manner^[42,43]. Sox2 and a POU factor known as Oct4 form a specific partnership to coordinately regulate the mechanism that maintains undifferentiated ES cells^[44,45]. The target genes of this partnership include *Nanog*, *Ulf1*, and *Fgf4*^[4]. Sox family members form partner complexes with POU factors, but the partnership assumes various forms depending on the cell type^[41]. In NSCs, Sox2 interacts with POU factors such as Pax6, Brn1, and Brn2, where Pax6 forms complexes with Sox2 to regulate the differentiation of cells of the optic nerve and lens^[46-49]. Pax6 is coexpressed in Sox2-positive cells and reportedly regulates the self-renewal and neurogenesis of NSCs in the hippocampus in the adult brain^[50]. The expression of Nestin, a marker for NSCs, is coordinately regulated by Sox2 and POU factors^[47,51]. Sox2 and the partner code of Brn1 and Brn2 bind to the regulatory region of the Nestin and Sox2 genes to perform an important function in the regulation of gene expression^[47,51-53]. Furthermore, Sox2 can bind to Prx1 (MHox1/Prrx1) and function as its partner^[54]. Because Prx1 and Sox2 are coexpressed in certain cells in the NSC region, they are expected to

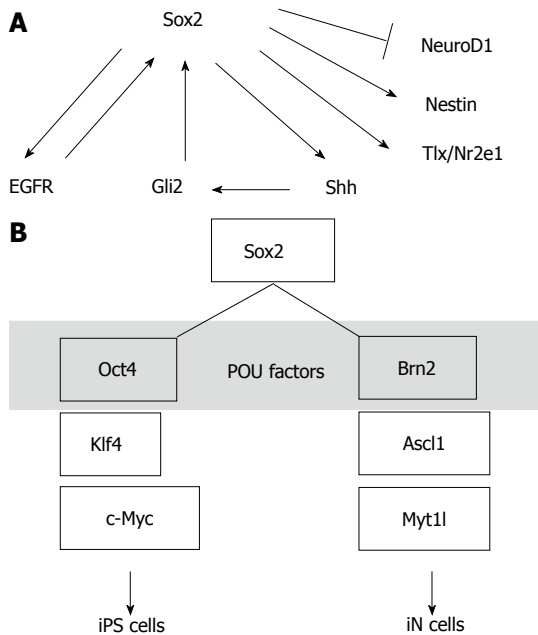


Figure 1 Diagrams of the Sox2 transcription network and reprogramming. A: Examples from the Sox2 transcription network. Sox2 activates *Egfr* transcription, and EGFR signaling activates Sox2 transcription. Sox2 also activates *Shh* transcription, and the *Shh* signaling downstream factor *Gli2* activates Sox2 transcription. Sox2 activates the *Nestin* and *Tlx/Nr2e1* genes but represses *NeuroD1* transcription; B: Sox2 and POU factors are assumed to function as a core-partner unit in gene-induced cell reprogramming. iPS: Induced pluripotent stem; iN: Induced neuronal.

coordinately activate the target genes, and they have been suggested to be involved in the regulatory mechanism that maintains the undifferentiated state of NSCs.

SOX2 TARGET GENES AND STEM CELL FUNCTION

Sox2 is a transcription factor, and many reports have been published describing analyses of its target genes. Sox2 regulates the expression of its target gene called *Sonic hedgehog* (*Shh*) to regulate NSCs in the hippocampus^[39,55,56]. *Shh* is a humoral factor that transmits outside signals from outside into the cell via its receptor Patched, and induces Smo/Gli signal activation^[57-59]. Another transcription factor, *Gli2*, is a downstream target of *Shh* and regulates *Sox2* gene expression^[60]. Therefore, these factors may constitute a positive feedback loop. Additionally, Notch and the epidermal growth factor receptor (EGFR) pathway regulate the number of NSCs and their self-renewal^[61]. EGF stimulation can turn neural progenitors into multipotent NSCs through the receptor, EGFR^[62]. Whereas EGFR signaling increases *Sox2* expression, Sox2 enhances *Egfr* expression, which suggests a positive feedback mechanism^[63] (Figure 1A). The nuclear receptor, *Tlx* (*Nr2e1*), is an essential factor in the mechanism that maintains undifferentiated NSCs^[64-66]. A possible negative feedback model of *Tlx* gene expression has been reported, in which Sox2 binds to *Tlx* to regulate its transcription^[67]. Based on these findings, it is conceivable that the

Sox2-centered feedback loop mechanism involving Sox2 target genes serves as an important system for the self-renewal mechanisms of NSCs.

It was recently reported that the crosstalk between Sox2 and Wnt signaling regulates the switching during the differentiation of NSCs to neurons^[68]. Sox2 and Tcf act as molecular switches thus interacting with the overlap sequence^[68], and this process, in turn, regulates *NeuroD1* expression^[68]. Although the mechanisms underlying the molecular switching of numerous genes are being increasingly revealed, it remains unknown how such mechanisms activate differentiation switches at the appropriate times and locations in response to intra- and extracellular changes, while suppressing the expression of genes other than those involved in neuronal differentiation.

STEM CELL REPROGRAMMING AND THE SOX2 GENE NETWORK

Combined transfection of the *Oct4*, *Sox2*, *Klf4*, and *c-Myc* genes transforms somatic cells into pluripotent stem cells^[24,25]. In this process, the transcriptional network is switched on to generate multipotent stem cells. It is likely that the partnership between Sox2 and Oct4 functions as the core switch^[4]. The addition of *Klf4* to the partner complexes presumably allows for multidimensional regulation of various modes of switching. In the multipotency induction process, the use of serum-free culture medium with EGF actively induces the formation of NSCs^[26]. Conversely, induction of the iN cell phenotype is conducted using a cell engineering technology that directly transdifferentiates somatic cells into neurons^[27]. Forced expression of the *Ascl1*, *Brn2*, and *Myt1l* genes can induce neuronal differentiation. However, this method is not intended for the maintenance of NSCs. *Brn2* is a partner factor of Sox2^[51,53]. When Sox2 is added to the group of iN-factors and cells are cultured in EGF- or bFGF-containing medium, combinations other than Oct4, *Klf4*, and *c-Myc* may be able to produce artificial NSCs. Moreover, based on the concept of the Sox2 partner code^[41], the establishment of neuronal subtype-specific NSCs also seems possible, using combinations of Pax6 and Prx1 or other POU factors (Figure 1B).

CONCLUSION

I have reviewed the link between the molecular mechanisms at work in NSCs and properties of stem cells, with a focus on the network involving the Sox2-centered partner code and its target genes. The localized expression of Sox2 in NSCs/neural progenitors enhances its molecular specificity. By forming complexes with its partner factors, Sox2 exerts its transcriptional-regulation function. The partner factors involved vary depending on the molecular context of the stem cell lineage. Sox2 target genes include molecular switches controlling the *NeuroD1* gene (which is capable of inducing neuronal differentiation) as well as the feedback loop with the factors involved in self-renew-

al such as members of the EGFR signaling pathway. By manipulating Sox2 and its partner factors, researchers can now artificially induce differentiation into pluripotent, or multipotent stem cells and into neurons. Nevertheless, many questions remain unanswered regarding the Sox2-based self-renewal mechanism and the regulatory mechanism underlying multipotency. Further research using conditional KO mice is needed to explore functions of Sox2, its partner factors, and chromatin-regulating factors that interact with Sox2 and its partner factors as well as to identify the entire panel of Sox2 target genes.

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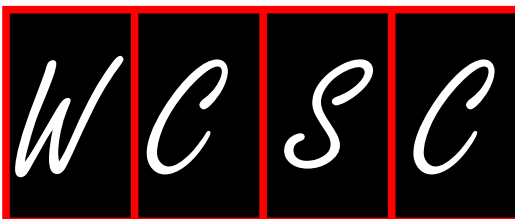
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Brain stem cells as the cell of origin in glioma

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Abstract

Glioma incidence rates in the United States are near 20000 new cases per year, with a median survival time of 14.6 mo for high-grade gliomas due to limited therapeutic options. The origins of these tumors and their many subtypes remain a matter of investigation. Evidence from mouse models of glioma and human clinical data have provided clues about the cell types and initiating oncogenic mutations that drive gliomagenesis, a topic we review here. There has been mixed evidence as to whether or not the cells of origin are neural stem cells, progenitor cells or differentiated progeny. Many of the existing murine models target cell populations de-

fined by lineage-specific promoters or employ lineage-tracing methods to track the potential cells of origin. Our ability to target specific cell populations will likely increase concurrently with the knowledge gleaned from an understanding of neurogenesis in the adult brain. The cell of origin is one variable in tumorigenesis, as oncogenes or tumor suppressor genes may differentially transform the neuroglial cell types. Knowledge of key driver mutations and susceptible cell types will allow us to understand cancer biology from a developmental standpoint and enable early interventional strategies and biomarker discovery.

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Key words: Glioma; Cell of origin; Cancer stem cells; Genetic models; Gliomagenesis; Neurogenesis

Core tip: The origins of glioma are not well understood. We approach the topic by review of our knowledge concerning the different cell types found in the mammalian brain, we describe mouse models aiming to model gliomagenesis and highlight relevant clinical data. Our aim is to integrate these three areas to provide a comprehensive snapshot of progress made towards the discovery of the process driving glioma formation.

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INTRODUCTION

Gliomas can be classified as many different genetically-driven diseases that manifest under the guise of only a few histological variations^[1-3]. Our understanding of glioma biology has grown immensely with the advent of cancer genetics and molecular characterization. Large-scale

multi-platform characterization of gliomas has revealed strong relationships that tie certain combinations of genetic changes with characteristic epigenetic modifications, transcriptome alterations and clinical presentations to define subtypes^[4-7]. Ultimately these findings suggest that the cancer biology in each molecular subclass varies to an extent that remains to be seen. Among the different genetic subclasses of gliomas there is reason to believe that the process of gliomagenesis may also vary. There are many aspects of gliomagenesis to consider: what cell type gives rise to the tumor? What genetic changes are compatible with initiating gliomagenesis? Are there non-cell autonomous factors that play a role in gliomagenesis, such as microenvironment changes? Understanding these tumor-initiating events will allow insight into the spatio-temporal progression of gliomas, the identification of key driver mutations and discovery of early biomarkers.

The cell of origin is the cell type that initiates tumor formation. This differs from the cell of mutation, which is the cell type that acquires oncogenic changes but may not necessarily proliferate until it moves to another point in its respective cellular hierarchy. It is thought that the cell of mutation may either differentiate or de-differentiate to a different cell type, which may then act as the cell of origin *via* uncontrolled growth^[8]. It is unclear if more than one cell of origin or cell of mutation may exist for a single type of tumor. Furthermore, the cells of origin of the different genetic subtypes of glioma are still either a matter of debate or left unexplored. Most of what we know about the potential cells of origin as a function of different combinations of oncogenic mutations in glioma comes from a variety of mouse models. This review will focus on the cell of origin in gliomas by reviewing the different cell types of the neuroglial lineage, exploring cell of origin glioma models and discussing clinical data that suggest differing cells of origin per glioma subtype.

Before proceeding, it is important to recognize the difference between the stem-like cells in a mature tumor and the cell of origin. These stem-like cells are commonly referred to as cancer stem cells (CSCs), brain tumor stem cells (BTSCs), or tumor-initiating cells. For the purposes of this review, the term “tumor-initiating cells” will not be used, as it does not distinguish between the re-initiation of a mature tumor and the initiation of a tumor from its cell of origin. For clarity, we will refer to these cancer stem-like cells as BTSCs or CSCs in this text. In addition, it is also necessary to consider the different context in which we discuss a “stem cell” and “differentiated cell”. When discussing normal human cellular biology, a stem cell is capable of self-renewal and asymmetric differentiation. Progenitors downstream of stem cells may symmetrically differentiate following proliferation. When a fully differentiated stage is reached, the cell typically has limited proliferation potential. Within a tumor, CSCs carry over the same definitions as normal stem cells. It is still a matter of debate as to whether or not the more differentiated cancer cells have limited or unlimited proliferation potential.

There are two prevalent models for the propaga-

tion of tumors: the clonal model and cancer stem cell model^[9,10]. In the clonal model, single cells within a tumor progressively acquire competitively advantageous genetic changes, accounting for the cellular and genetic heterogeneity observed in tumors. In the cancer stem cell model, there are thought to be CSCs within the tumor that have the ability to self-renew and differentiate. By definition, CSCs can be seeded into another organism and give rise to the tumor it was isolated from, while the non-CSCs either cannot do so, or can do so only with much lower efficiency. In the CSC model, CSCs are thought to give rise to a cellular hierarchy *via* their differentiation and self-renewal abilities. Both CSCs and non-CSCs acquire genetic mutations, leading to the observed cellular and genetic heterogeneity. BTSCs identified in gliomas are thought to play a key role in the maintenance and virulence of the tumor. How and when the BTSCs arise in the tumor remains a mystery, although at least two possibilities exist. We can hypothesize that differentiated cells in the early tumor eventually de-differentiated to form BTSCs. Conversely, the other possibility is that BTSCs are derivatives of a cell of origin that was once a normal stem cell or progenitor cell. The missing links between cell types in the early tumor and mature tumor are yet to be uncovered. Cell of origin models must be used to explore the developmental arc of a mature tumor that contains a complex cellular hierarchy from a single clone. As was previously mentioned, two major variables are at play in these modeling efforts: the oncogenic mutations and the plethora of cell types found in the brain. In this review we begin with an overview of neurogenesis in the adult brain and follow with a discussion of glioma genetics, glioma cell of origin models and clinical evidence for stem cells as the cells of origin in glioma.

NEUROGENESIS IN THE ADULT BRAIN

Neural stem cells and their progeny have become candidates for the cell of origin of glioma since the discovery of neurogenesis in the adult brain. It is necessary to recognize the variety of cell types in the brain, when they are present and how they arise when discussing the cell of origin of gliomas. Neurogenesis in adults is thought to be responsible for the replacement of neurons and glia for the purposes of cellular replenishment, remodeling and response to injury^[11]. We know that adult gliomas arise from the neuroglial lineage during post-natal life due primarily to strong evidence from the histological characteristics of glioma, their molecular signature and mouse glioma models that target the neuroglial lineage. Accordingly, this introduction is mostly limited to adult neurogenesis (*vs* embryonic or pre-natal neurogenesis) and excludes extensive discussion of other central nervous system (CNS) and non-CNS cell types found in the brain (such as the meninges, endothelium, ependyma and microglia).

There are two identified neurogenic niches in the adult mammalian brain: the subventricular zone (SVZ) and the subgranular zone (see review by Alvarez-Buy-

la^[11]). Ciliated ependymal cells that encase the cerebrospinal fluid line the lateral ventricles and this monolayer of cells is contained within the ventricular zone^[12,13]. On the lateral surfaces of the ventricles, the ependymal cells are laterally lined by neural stem cells (NSCs), or type B NSCs, in a second layer of cellular stratification within the SVZ^[13-15]. These type B NSCs arise from neuroepithelium-derived radial glia that are responsible for the stratified organization of the cortex^[16-18]. During the transition to post-natal life, radial glia differentiate into type B NSCs that extend a small process to make contact with the cerebrospinal fluid in the ventricular zone. Their cell bodies are mostly confined in the SVZ, with an apical process that extends laterally to contact blood vessels. The type B NSCs in the SVZ are capable of asymmetric division leading to the production of glia or neurons (Figure 1). To produce neurons, the type B cells give rise to transit amplifying cells, or type C cells, which proliferate and progress to type A cells, or neuroblasts. These neuronal precursors are known to migrate through the rostral migratory stream (RMS) in the frontal cortex to replenish interneurons in the olfactory bulb, becoming granule or periglomerular neurons^[19-22]. Depending on the regulatory signals in the SVZ niche, type B cells may also generate cortical astrocytes or oligodendrocyte precursors cells (OPCs), which mature to oligodendrocytes^[11,23,24].

In the hippocampal formation, radial astrocytes (type 1 cells) serve as stem cells^[25]. Type 1 NSCs differentiate into intermediate progenitor cells (type 2 cells), which form immature granule cells (type 3 cells). Subsequently, type 3 cells will mature into the granule neurons found in the hippocampus^[26].

Because most of what we know about post-natal neurogenesis and its cellular hierarchy in the brain comes from the study of rodents, there has been intense speculation as to whether human brains harbor active NSCs that generate progenitors and what their subsequent roles are during adult life. The implication of active neurogenesis in adult humans suggests that a decline or defect in the process may play a role in neurodegenerative disorders or glioma formation, respectively. The quest for uncovering neurogenesis in higher organisms consisted mostly of labeling studies in post-mortem brains of monkeys and human patients. Through these studies we have gained substantial evidence for the presence of post-natal human neurogenesis, although their roles in maintaining the human brain's function remain matters of ongoing study.

Mounting evidence for two neurogenic regions in the rodent brain led to the search for their human homologues. Explant culture and labeling experiments of human brain surgical specimens generated new neurons and glia^[27,28]. This was the first direct observation and *in vitro* generation of human neuronal cell types. Shortly thereafter, many others demonstrated that multipotent or neurosphere-forming cells could be isolated and cultured from the human SVZ and subgranular zone (SGZ). Such cultures were extremely heterogeneous, but they were shown to be capable of directed differentiation *in vitro*

to both glia and neurons, indicating that they contained either undifferentiated precursors or NSCs^[29-34]. In a rare form of scientific inquiry, human cancer patients were injected with Bromodeoxyuridine (BrdU), a mitotic marker, as a part of a diagnostic procedure. Post-mortem examination of their hippocampi revealed BrdU-labeled neural and glial cell types, and a small population of BrdU-positive cells that did not co-stain for differentiation markers. These unidentifiable cell types were presumed to be the undifferentiated stem cells or progenitors^[35]. Interestingly, BrdU-positive cells in the SVZ were also noted in all five patients examined, who were between the ages of 58 and 72 years old at time of death, indicating that neurogenesis may continue late into adult life.

The evidence supporting neurogenic activity in the human brain raises other important questions: where do stem cells reside? How does the cellular hierarchy operate in the primate brain? The first identification of neurogenesis in monkeys was made in the hippocampus structure. Kornack *et al.*^[36] and Gould *et al.*^[37] observed that the rate of formation of new granule neurons in the SGZ could be modulated by stress and that the primate brain was also capable of generating astrocytes and oligodendrocytes, a process that continued even as the monkeys increased in age. Neuroblast (type A cell) formation was also observed in the adult forebrain of monkeys, lending further evidence for adult SVZ neurogenesis in primates^[38]. These neuroblasts were also found to travel along the RMS^[39], as observed in their mammalian rodent counterparts^[19,40]. The first evidence for the existence of human neuroblasts (type A cells) in the olfactory bulb came from examination of post-mortem brains, which showed immuno-positivity for neuroblast markers^[21]. Following this study, three separate groups provided evidence, once again through immunostaining and ultrastructural studies of post-mortem human tissue, for neuroblast chain migration through the RMS^[22,41-43]. In addition, Alvarez-Buylla and colleagues claim to have identified the Medial Migratory Stream, an additional migratory pathway for neuroblasts that extends medially to the pre-frontal cortex^[42]. They indicated, however, that chain migration through this region ends after approximately 18 mo of age. The direct identification of multipotent NSCs (type B cells) in the adult human SVZ has provided us with evidence that humans do harbor NSCs and that they are capable of producing both glia and neurons in a fashion similar to other mammals^[12,44]. Given the hypothesis that tumorigenesis is more likely to occur in mitotically active cells rather than in quiescent cell types, it will be interesting to explore if tumor incidence, and type, vary with neuronal developmental stages in a child or adolescent, or with stress, injury and increased age.

GLIOMA MODELS AND THE GLIOMA CELL OF ORIGIN

The discovery of human NSCs and their progeny has

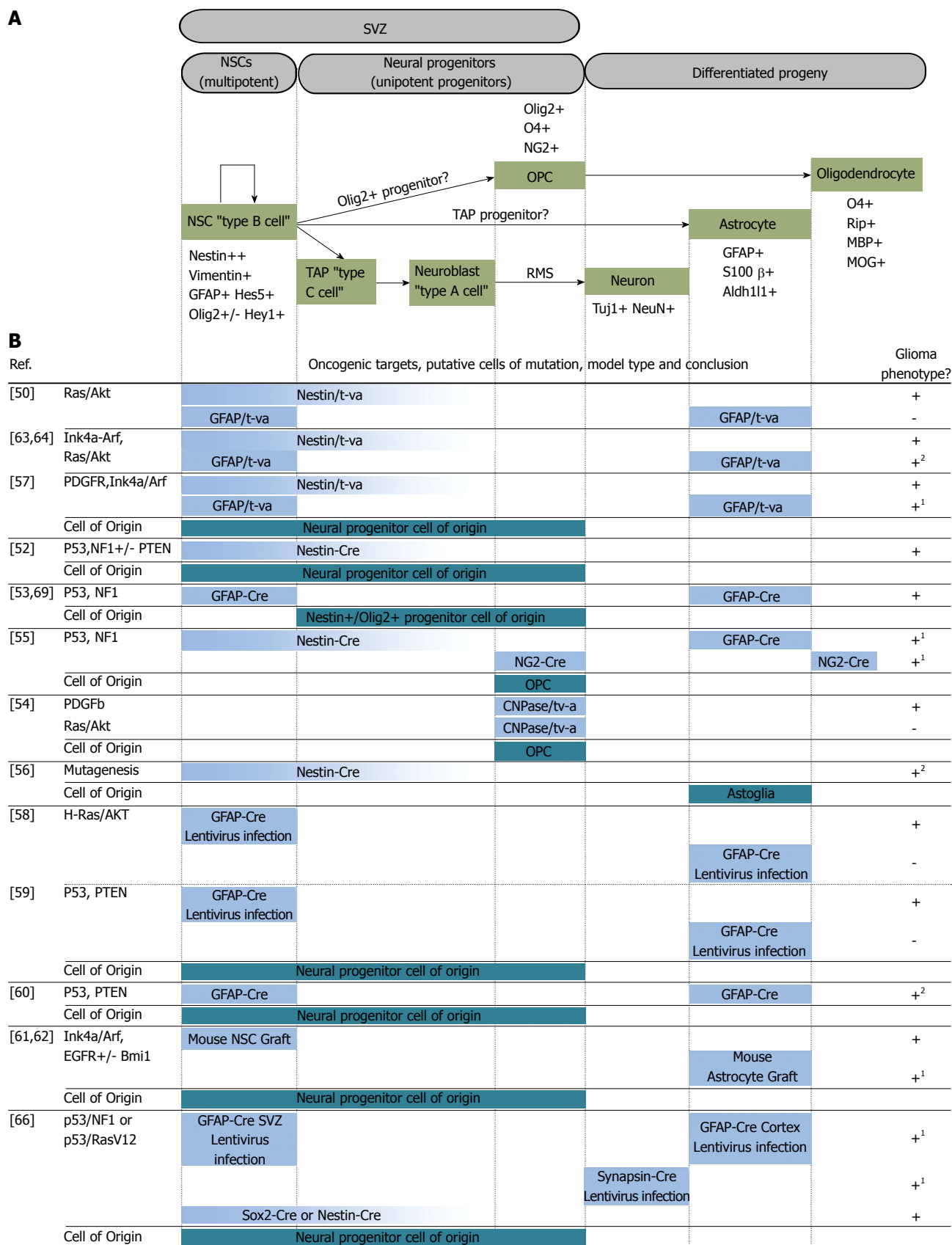


Figure 1 Targeted oncogenesis in the neuroglial cellular hierarchy. (A) Neurogenesis in the adult mammalian brain; (B) Summary of murine models that targeted selected cell types for the purposes of discovering the glioma cell of origin. Horizontal bars in (B) correlate to the representative cell types in (A). ¹Experimental results by authors suggest differentiated progeny acted as the cell of mutation and de-differentiated before forming a tumor; ²Lack of lineage trace, distinguishing cell of mutation from cell of origin is inconclusive despite tumor forming capacity of differentiated progeny. Partially adapted from Ref. [8,48]. SVZ: Subventricular zone; NSCs: Neural stem cells; TAP: Transit amplifying progenitors.

led to the question of whether or not they act as the cell of origin in glioma. A number of mouse models have been developed to explore this topic. Mouse models recapitulate a small number of genetic mutations found in human glioma by functionally expressing an oncogene or inactivating a tumor suppressor. Genetically engineered mice or targeted lentiviral transduction systems are used for the purposes of modeling gliomagenesis. The genetic targets in these models, although found to be mutated in human gliomas, are not necessarily driver mutations in glioma development, but we are limited in our ability to identify driver mutations from human gliomas. This is also evident by the fact that some mutations, in the context of mouse models, do not produce tumors or fail to produce appropriate phenotypes when mutated alone^[45]. There are very limited mechanisms by which we can infer or identify driver mutations from human cancers. One way is to see which types of mutations occur at highest frequencies within a subclass of tumor; another method is to determine what percentage of the cell population carries the mutation. When a mutation is found in nearly every tumor cell, it implies that a disproportionate growth advantage is conferred or that a particular mutation occurred very early in tumor development. Regardless of what we can infer from clinical data, we do have a good understanding of the most common genetic lesions found in gliomas and modeling efforts have focused on dissecting the role of these common culprits.

There are a number of ways to model gliomagenesis. Some model systems aim to create a “mature” glioma, while others aim to identify how limited and defined oncogenic mutations drive initial glioma formation, or gliomagenesis (see review^[45]). The most genetically faithful models of glioma are xenografts of human brain tumors in the mouse brain. Xenografts of primary tumors have been used successfully to study glioma biology and genetics because they are very close representations of the mature tumor that is removed during surgery^[46]. The drawbacks of such systems are the selection process during cell line derivation, the need to culture these cells *ex vivo* (which over time leads to epigenetic and genetic alteration), and the need to grow tumors in immune deficient mice. Although human glioma xenografts replicate human pathology, they do not represent the earliest stages of glioma formation. For example, glioma xenografts do not recapitulate the transformation of a normal endogenous neuroglial cell type to neoplastic stages and beyond. Furthermore, glioma xenografts cannot be used to identify the cell of origin or cell of mutation. Explants of glioma have also been used to study glioma biology, although such systems are technically challenging and are limited to the tissue obtained after surgery^[47]. Since human tumors cannot be used to understand the beginning stages of gliomagenesis, approaches that involve selective mutation of tumor suppressor genes or induction of oncogenes in model organisms are used to dissect oncogenic transformation.

The most commonly used model system to study gliomagenesis has been genetically engineered mice that

form tumors either spontaneously or after induction. One of the main advantages in using mice is the scale and reproducibility in which genetic alterations can be studied, which has proved to be a powerful tool in understanding cancer genetics. The disadvantage of using mice is their species difference from humans, which obviously translates to differing genetics, physiology and anatomy, as well as the failure of some of these models to capture the molecular diversity and heterogeneity of human tumors.

Virally mediated oncogenic transduction is also used to target specific areas and cell types in the mouse brain for gliomagenesis. Such an approach allows the localization of genetic alterations to specific areas within the brain and selective targeting of cell types within that region depending on the type of model used. The drawback of this system is the need for invasive injection of viruses or virus producing cells. Nevertheless, functional mutations in these model systems have provided the platform to study the cell of origin in cancers (see reviews^[8,48,49]).

The genetic targets used for these studies are primarily those that are mutated in Glioblastoma Multiforme (GBM), or World Health Organization (WHO) grade IV gliomas. Gliomas are graded based on histological characteristics on a WHO grading scale of I-IV^[3]. In GBM, the most common and deadly of the glioma subtypes, a number of high frequency alterations have been found most commonly in the tumor suppressors p53, PTEN, CDK2A/p16^{INK4A}/p14^{ARF}, CDK4, RB and in proto-oncogenes EGFR, PDGFR, PIK3CA, PIKR1, Kras and IDH1^[4-7]. The models discussed here have dually aimed to recreate functional recapitulations of genetic alterations to these genes and to understand in what cell type they initiate gliomagenesis.

One of the landmark papers in modeling the cell of origin in glioma came from Holland *et al*^[50]. This unique mouse model employed a genetically engineered strain that expressed a receptor for a retrovirus that harbored either a mutant form of Kras or Akt. Retroviruses were produced by xenografts of chicken cell lines harboring Replication-Competent ALV Splice-acceptor (RCAS) viral vectors^[51]. The receptor for these retroviruses is expressed under the control of tissue-specific promoters, such as glial fibrillary acidic protein (GFAP) (expressed primarily in glia, but also NSCs) or nestin (expressed in NSCs and early progenitors). The novelty of this approach lied in targeting of two different cell populations in the neural lineage that were either neural progenitors (using the nestin promoter), or differentiated astrocytes (using the GFAP promoter). When Kras and Akt were targeted to nestin-expressing cells, high-grade glioma formation was observed. Conversely, targeting GFAP-expressing cell types did not yield tumors. This was the first example of a glioma model that differentiated between the oncogenic potential of two different populations of cells along the same neuroglial axis. One weakness of this model was that, by virtue of the nestin promoter being active in both NSCs and lineage-restricted progenitor cells, the exact cell of origin could not be pinpointed still.

Many mouse models followed in dissecting the rela-

tionships between genetic lesions, cell types targeted and tumor phenotype produced. Tumor suppressor models produced by Alcantara Llaguno *et al.*^[52] aimed to recreate some of the most common genetic lesions in GBM using combinations of p53, phosphatase and tensin homolog (PTEN) and neurofibromin 1 (NF1) knockout in mice. With their models, they concluded that nestin-positive NSCs or their progenitor cells found in the SVZ harbored the ability to initiate high-grade glioma^[52]. Using a mutated p53 model that allowed the tracking of p53 mutant cells, Wang *et al.*^[53] observed that type B, C and A cells were capable of accumulating mutant p53. However, it was a nestin/olig2-positive population that resembled type C cells, which was thought to initiate the high-grade glioma. Interestingly, they noted that some SVZ type A neuroblasts that harbored the p53 mutation traveled to the olfactory bulb, but no glioma formation was observed^[53]. Additionally, two separate groups generated p53 and NF1 knockout mouse models of glioma and also claimed that it was the OPCs that served as the cell of origin in the production of high-grade tumors^[54,55]. Koso *et al.*^[56] used a transposon-mediated mutagenesis approach in isolated mouse NSCs. Their model revealed dozens of mutations in combination that could sensitize NSCs to immortalization and tumor formation. Interestingly their mutagenized NSCs were most sensitive to oncogenic transformation after differentiation to the astroglial lineage. Other models, such as PDGFR activation *via* RCAS-tVA^[57], lentiviral delivery of Kras/Akt oncogenes^[58], and PTEN/p53 inactivation^[59,60] suggested multipotent progenitors found in the SVZ as potential cells of origin for glioblastoma (Figure 1).

Cell types found outside of the neurogenic niches were also found to harbor tumor-initiating potential in mouse models. The demarcation between cell of mutation and cell of origin is less commonly explored due to lack of lineage tracing in many of these models however. In cases where lineage tracing has been used, differentiated progeny were found to de-differentiate to a stem cell state preceding tumor growth. These mouse models include Ink4a-ARF knockout^[61], Bmi knockout^[62], combined Ink4a-ARF knockout and Kras activation^[63,64] and aberrant platelet-derived growth factor signaling^[57,65], all of which initiated tumors in areas and cell types both outside and inside the neurogenic regions.

Interestingly, there is also evidence that neurons can act as the cell of mutation in a mouse GBM model when they acquire p53/NF1 mutations after undergoing de-differentiation^[66]. The implication for this is that non-neurogenic regions of the brain containing quiescent neurons may be capable of gliomagenesis as well. As mouse modeling continues, emphasis will likely be placed on lineage tracing of defined cell types to understand the plasticity of the cell of mutation and the relationship, if any, between genetic lesions and cell of origin. In addition, the field is faced with the challenge of correlating the mouse glioma cells of origin to the likely cell of origin in human glioma. By drawing parallels between the cell of origin and the restricted number of genetic events

that must occur in early tumorigenesis we may one day be able to discover early tumor biomarkers, target tumors when they are exponentially more sensitive to therapy and develop therapies that target the unique stem cell biology of tumor formation and propagation.

CLINICAL EVIDENCE FOR STEM CELLS AS THE CELL OF ORIGIN

The presence of CSCs in human glioma specimens and other tumors from the clinic raises the question of why they are present in the tumors to begin with. The interesting aspect of the presence of CSCs in glioma, as it relates to the cell of origin, is that CSCs are thought to be multipotent and capable of self-renewal, as well as express markers of NSCs. CSCs were identified in human GBM and have been shown to re-initiate mature tumors when seeded into the mouse brain at a much higher efficiency than their non-stem counterparts in the tumor. Did CSCs come from a stem cell of origin? Did CSCs de-differentiate from differentiated cell types in the tumor? At what point during gliomagenesis do CSCs appear? These questions remain entirely unanswered as the continuum between the origination of a tumor and its mature form has not been explored. Here, we will present epidemiological and radiographic evidence that cell types with stem cell properties in the tumor may have originated from NSCs or lineage-restricted progenitors.

A number of radiographic studies have shown that there is a tendency for glioma formation to occur near the periventricular area of the brain^[67-69], although not all gliomas are necessarily in contact with the ventricles. GBM (WHO grade IV) appears to occur mostly supratentorially, with tumor epicenters around the ventricles and frontal lobe propensity before the age of 65 and temporal lobe propensity after the age of 65^[67,70-72]. Once again, this discrepancy can be accounted by two possibilities: gliomas may initiate from many different cell types found in all areas of the cortex, or gliomagenesis may be preceded by migration of the cell of mutation/cell of origin. Correlating radiographic evidence to molecular subtypes of GBM has yielded interesting patterns in anatomical distribution, but most of these imaging studies are conducted after the tumor has had months or even years to grow. In such cases, the large size of tumors precludes the exact localization of its epicenter.

WHO grade II and III gliomas have a very different anatomical distribution than their grade IV counterparts and a much more “compact” set of associated genetic lesions. The exact reason for this is unknown, but one logical possibility is that these lower grade gliomas have different cells of origin. Up to 80% of the low-grade gliomas are mutated in Isocitrate Dehydrogenase (IDH) with an accompanying p53 mutation or 1p19q deletion^[73]. IDHs normally convert isocitrate to α -ketoglutarate and produce a nicotinamide adenine dinucleotide phosphate (or NADH) molecule, an essential metabolic process that occurs in the mitochondrial Krebs (tricarboxylic acid

cycle) cycle, cytosol and peroxisomes^[74,75]. The mutated form found in glioma and leukemia is a gain-of-function mutation that causes the conversion of α -ketoglutarate to 2-Hydroxyglutarate, a so called “oncometabolite” due to its ability to cause epigenetic reprogramming that is thought to drive tumorigenesis^[76-79]. The low-grade IDH mutated gliomas are often found supratentorially in the frontal lobe in young adults^[67,80]. Interestingly, this area overlaps with the SVZ in the frontal lobe that generates neuroblasts destined for the olfactory bulb and possibly the medial pre-frontal cortex. However the significance of this finding remains to be understood as the only known mouse models of IDH have failed to produce appropriate human tumor phenotypes in both brain and myeloid neoplasm contexts^[81-83].

CONCLUSION

How glial tumors form and develop into their lethal variety remains a standing question in glioma biology. Our understanding of the molecular events, niche changes and cell types involved has brought us closer to this goal. Since the discovery of neurogenesis in the adult brain and the growing body of work on cancer genetics, we have both cellular and genetic candidates to pursue to this end. Many have employed murine models that target the neuroglial cell population with genetic changes akin to those found in human glioma in search of the cell of origin and to understand key initiating genetic changes. These advances have produced mixed results, with the majority of models pointing to the neuronal progenitors or NSCs as the most likely cell of origin. However, other models have found that differentiated cell types may be capable of tumor initiation as well. The models described varied significantly in the type of genetic mutation made, cell population targeted and lineage tracing technique, if any. Furthermore, it is difficult to infer which of the many genetic mutations identified in glioma act as the initiating event. It is quite possible that more than one cell of origin may exist for the various subtypes of glioma and that more than one genetic change is capable of sending a cell down the path to carcinogenesis. As we dissect the roles of these oncogenes and tumor suppressor genes in glioma initiation, we will gain a broader understanding of gliomagenesis as a process rather than a random event. Through these ongoing efforts it is possible that we will identify very early biomarkers and develop an understanding of what type of restricted changes a young tumor must make to progress to a more malignant state, presumably at a stage where these pre-malignant cells are most susceptible to therapies.

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Sox2, a key factor in the regulation of pluripotency and neural differentiation

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Abstract

Sex determining region Y-box 2 (Sox2), a member of the SoxB1 transcription factor family, is an important transcriptional regulator in pluripotent stem cells (PSCs). Together with octamer-binding transcription factor 4 and Nanog, they co-operatively control gene expression in PSCs and maintain their pluripotency. Furthermore, Sox2 plays an essential role in somatic cell reprogramming, reversing the epigenetic configuration of differentiated cells back to a pluripotent embryonic state. In addition to its role in regulation of pluripotency, Sox2 is also a critical factor for directing the differentiation of PSCs to neural progenitors and for maintaining the properties of neural progenitor stem cells. Here, we review recent findings concerning the involvement of Sox2 in pluripotency, somatic cell reprogramming and neural differentiation as well as the molecular mechanisms underlying these roles.

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Key words: Sex determining region Y-box 2; Pluripotent stem cells; Pluripotency; Neural differentiation; Reprogramming

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Core tip: Sex determining region Y-box 2 (Sox2) plays important roles in pluripotent stem cells, not only for maintaining their pluripotency but also for directing their neural differentiation. There have been many intensive studies in the last decade, which serve to ascertain the function of Sox2 in these processes. In this review, we have summarized the recent progress made regarding the involvement of Sox2 in pluripotency, somatic cell reprogramming and neural differentiation as well as the molecular mechanisms underlying these roles.

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INTRODUCTION

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), possess two important properties: indefinite self-renewal in culture and the ability to generate most, if not all, cell types in the human body *via* differentiation into one of the three embryonic germ layers^[1,2]. These unique properties of hPSCs make them an invaluable cell resource not only for regenerative medicine, disease modelling and drug development, but also for the study of early human development, serving as a cell model to elucidate the molecular mechanisms regulating embryonic cell proliferation and differentiation. Understanding the mechanisms underlying the self-renewal and differentiation of hPSCs is fundamentally important for the subsequent utilization of these cells. In the past

decade, increasing evidence has shown that cell fate determination of a pluripotent stem cell, either maintaining pluripotency or differentiating into one of the three germ layers, is controlled by both extrinsic and intrinsic factors^[5]. Intrinsic factors refer mainly to transcription factors that play an essential role in the direct control of gene expression in cells, while extrinsic factors, including growth factors, extracellular matrices and cytokines, have considerable effects on expression levels of intrinsic transcription factors through various signalling pathways. The core intrinsic factors for regulating pluripotency have been identified as octamer-binding transcription factor 4 (Oct4), sex determining region Y-box 2 (Sox2) and Nanog^[4,5], while Oct4 and Sox2 are also proposed as lineage specifiers to regulate mesendoderm and ectoderm differentiation, respectively^[6,7]. Thus, Sox2 is one of the critical factors that control both pluripotency and neural differentiation of hPSCs.

In this review, we place particular emphasis on the biological functions of Sox2 in regulating pluripotency and early neural differentiation of ESCs and summarize the recent findings on the role that Sox2 plays in the regulation of PSC fate.

SOX2 IS INDISPENSIBLE DURING EARLY EMBRYONIC DEVELOPMENT

Sox2 is a member of the Sox family of transcription factors. The Sox gene family was first defined by the discovery of the mammalian testis-determining factor, Sry^[8,9]. Proteins of the Sox family all share a highly conserved high-mobility-group (HMG) DNA binding domain. To date, 20 different Sox genes have been identified in mouse and human^[10], which are divided into subgroups, according to the degree of homology within the HMG domain and other structural motifs. Sox2 is classified as a member of SoxB1 group, which also includes Sox1 and Sox3. Although Sox1, Sox2 and Sox3 share more than 80% sequence similarity and are functionally redundant, Sox2 can exert distinct functions in a biologically context-dependent manner and is indispensable for embryonic development. Many factors have been shown to influence binding of Sox proteins to their target genes, leading to diverse functional effects. One such factor is the interaction between Sox proteins and various cofactors. Interaction with various cofactors confers upon Sox2 greater functional versatility during developmental processes^[11].

During mouse embryogenesis, a totipotent zygote undergoes cleavage to increase the cell number and the resulting multi-cellular morula further develops to form the blastocyst, in which the cells, for the first time, appear to acquire spatially derived identities, segregating into the inner cell mass (ICM) and trophoctoderm. Cells in the ICM give rise to the embryo proper, differentiating into all cell types found within the body and are thus classified as pluripotent. Conversely, trophoblast cells develop into placental tissues, assisting with implantation and nourishment of the embryo during development. Sox2

expression is initially detected in cells at the morula stage, becoming more specifically located in the ICM of blastocyst and epiblast^[12] during the latter stages. This implies that Sox2 may have important roles in the formation of early pluripotent embryonic cells. Indeed, zygotic deletion of Sox2 is embryonically lethal due to the failure to form pluripotent epiblast whilst the absence of Sox2 has little effect on the formation of trophoctoderm^[12]. Therefore, Sox2 is an essential factor in the formation of pluripotent cells in early embryos and ultimately an critical factor for embryonic development.

CRITICAL ROLE OF SOX2 IN MAINTAINING PLURIPOTENCY OF ESCS AND GENERATION OF IPSCS

ESCs, derived from the ICM of preimplantation embryos, share many characteristics with the ICM cells. One major similarity is their pluripotent capability, being able to give rise to all cell types of the adult body. However, ESCs are not identical to the cells in the ICM as ESCs are able to amplify themselves during extended culture without compromising their pluripotency. Consistent with the data in pre-implantation embryos, Sox2 is highly expressed in ESCs. Depletion of Sox2 by either gene-knockout or RNA interference considerably compromises the pluripotent state of both mouse and human ESCs as shown by the changes in cell morphology, loss of pluripotent marker expression and their differentiation primarily into trophoctoderm^[2,13]. However, forced expression of Oct4 in Sox2-null mouse ESCs (mESCs) can rescue the pluripotency of these cells, indicating that the role of Sox2 in maintaining the pluripotent state of ESCs is primarily to sustain a sufficient level of Oct4 expression^[2,13]. Collectively, these results demonstrate that Sox2 is crucial in the maintenance of pluripotent ESCs, possibly through promoting and maintaining Oct4 expression.

Interestingly, to maintain pluripotency of stem cells, levels of Sox2 expression need to be stringently regulated, with either higher or lower Sox2 expression leading to the loss of pluripotency in ESCs. This could be attributed to the fact that both low and high levels of Sox2 reduce the promoter/enhancer activity of Sox2-Oct4 target genes^[14,15]. The expression level of Sox2 needs to be retained in a dynamic equilibrium with other synergistic factors in order to maintain pluripotency. This concept is also supported by the finding that Sox2 cooperates with other highly dose-dependent transcription factors, such as Oct4 and Nanog, in the regulation of pluripotency^[4]. In human and mouse ESCs, Oct4, Sox2 and Nanog form a core transcriptional regulatory circuitry in pluripotent stem cells to maintain their self-renewal. Oct4 and Sox2 co-occupy a large number of enhancers/promoters and regulate the expression levels of their target genes (Figure 1). They activate the expression of pluripotent genes, including Nanog and themselves, whilst repressing the ex-

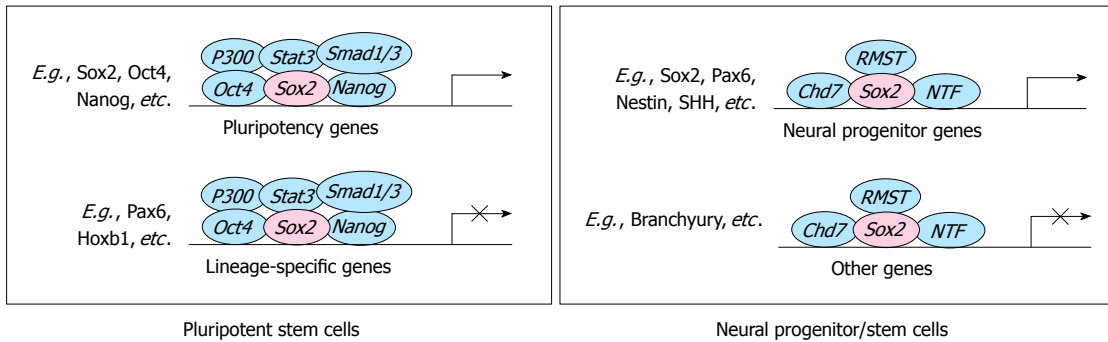


Figure 1 Sex determining region Y-box 2 regulates gene expression in pluripotent stem cells and neural progenitor/stem cells. Sex determining region Y-box 2 (Sox2) interacts with Oct4 and other nuclear factors to activate pluripotent gene expression and repress differentiation gene expression in pluripotent stem cells. In neural progenitor/stem cells, Sox2 interacts with neural transcription factors, such as Brn2 and Pax6, to activate neural progenitor gene expression. Oct4: Octamer-binding transcription factor 4; Pax6: Paired-box protein 6.

pression of key genes that are responsible for the *in vitro* differentiation and *in vivo* developmental processes, such as paired-box protein 6 (Pax6) and gastrulation brain homeobox 2^[4,5]. These transcriptional regulatory activities of Sox2 and Oct4 have been shown to require their direct interaction^[16]. The cooperative interaction between HMG of Sox2 and pit-oct-unc (POU) homeodomain of Oct4 is thought to be critical in regulation of ESC pluripotency^[17].

IPSCs are generated from various somatic cell types by ectopically expressing transcription factors that are important for ESC pluripotency. The most commonly used factors are Oct4, Sox2, Kruppel-like factor 4 (Klf4) and c-Myc^[18]. These factors are able to reprogram the somatic cells back to their embryonic state, making them share the dual properties of pluripotency and long-term self-renewal much like their ESC counterparts. Given that Sox2 is essential in the maintenance of pluripotency in ESCs, it is conceivable that Sox2 is one of the key factors for the generation of iPSCs^[18]. In fact, by analysing gene expression profile on a single cell level during reprogramming, it has been found that the activation of endogenous Sox2 is a relative early event, which initiates a cascade of transcriptional changes, leading to the formation of iPSCs^[19]. Interestingly, based on the shared biological properties between *SoxB1* genes, Sox2 can be replaced by closely related Sox family members, Sox1 and Sox3, in the generation of iPSCs, but not by more distant members, like Sox7 and Sox15^[20]. However, it has been reported that Sox17 is able to replace Sox2 in the successful generation of iPSCs after it is genetically modified in which two amino acids in the Sox17 HMG domain are converted to those of Sox2. This conversion does not alter Sox17 HMG DNA binding motif but confers its ability to interact with Oct4. The modified Sox17 is able to interact with Oct4 and the resulting Sox17-Oct4 complex can cooperatively bind to the canonical subset of Sox-Oct motifs and successfully reprogram somatic cells^[21]. Taken together, Sox2 is therefore important for the successful reprogramming of somatic cells to iPSCs. As such, the physical interaction between Sox2 and Oct4 is likely to be critical in induction and maintenance of

pluripotency.

SOX2 IN NEURAL DIFFERENTIATION AND MAINTENANCE OF NEURAL PROGENITOR/STEM CELLS

During embryonic development, Sox2 is persistently expressed, initially in the epiblast of preimplantation embryos, then more predominantly in the central nervous system after gastrulation, hinting at a possible function for Sox2 in neural commitment^[22]. Recently, it has been suggested that the three core pluripotent transcription factors Sox2, Oct4 and Nanog not only play an important role in the induction and maintenance of pluripotency, but also in functioning as lineage specifiers, regulating the differentiation of ESCs to specific lineages^[6,23]. Sox2 governs ESC specification to neuroectoderm while Oct4 and Nanog promote their differentiation to mesendoderm, a common precursor of mesoderm and definitive endoderm^[7]. Sox2 induces neural induction and enhances neural differentiation by repressing key regulators of other lineage fates, for example brachyury^[7,23,24]. Therefore, Sox2 appears to be an important regulator in controlling PSC neural initiation and differentiation.

In addition to its role in regulating neural induction, Sox2 also functions to maintain the self-renewal of neural progenitor stem cells *in vitro* as well as *in vivo*. Sox2 is highly expressed in proliferating neural progenitor cells (NPCs) and is downregulated upon differentiation to post-mitotic neuronal and glial cells. Reduction of Sox2 in neural progenitor stem cells hinders their self-renewal and proliferation, promoting their earlier exit from cell cycle and terminal differentiation; whereas ectopic expression of Sox2 inhibits the differentiation of NPCs into neurons and glia^[25,26]. The fact that ectopic expression of Sox2 alone or in combination with other neural transcription factors can directly reprogram fibroblasts to multipotent neural progenitor stem cells further highlights the essential role Sox2 plays in these cells^[27,28]. Taken together, Sox2 is therefore a key factor in both the establishment and maintenance of neural progenitor

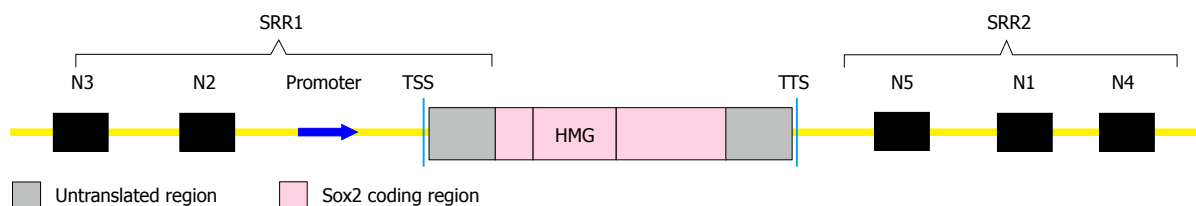


Figure 2 Transcriptional expression of sex determining region Y-box 2 is regulated by multiple enhancers located in the sex determining region Y-box 2 locus. The sex determining region Y-box 2 (*Sox2*) gene locus is illustrated in yellow box, in which *Sox2* exon and location of the N1 to N5 enhancers are indicated. TSS and TTS represent transcription starting and termination sites, respectively. SRR1 and 2 are *Sox2* regulatory regions 1 and 2.

properties.

MOLECULAR MECHANISMS UNDERLYING THE ROLES OF SOX2 IN PLURIPOTENCY AND NEURAL DIFFERENTIATION

In the last decade, intensive studies have been carried out in order to elucidate the molecular mechanisms that control pluripotency and lineage specification. Although considerable progress has been made, the mechanisms are still not fully understood. Given that *Sox2* functions in both PSCs and NPCs, it is thought that stringently regulated *Sox2* expression is necessary to govern both pluripotency and initiation of neural differentiation in PSCs. Furthermore, differentially orchestrated mechanisms are required to control distinct functions of *Sox2* in self-renewal of PSCs and during their neural differentiation.

Like other transcription factors, the expression of *Sox2* is regulated by both intrinsic factors and extrinsic signalling pathways. It has been identified that several regulatory regions in the *Sox2* locus are responsible for controlling *Sox2* expression, which include *Sox2* core promoter^[29] and a number of enhancers located both upstream and downstream of the *Sox2* gene (Figure 2)^[30-33]. All of these regulatory regions are highly conserved across species, responding to different factors and signalling pathways^[30]. In ESCs, several laboratories have clearly demonstrated that *Sox2* interacts with Oct4 to form a regulatory complex, which binds to *Sox2* regulatory region 2 to activate *Sox2* transcription^[4,5,34,35], indicating that *Sox2* is positively auto-regulated by the *Sox2*-Oct4 complex in ESCs. In addition to Oct4, several other transcriptional factors, including Nanog, mothers against decapentaplegic homolog 1 (*Smad1*) and signal transducer and activator of transcription 3 (*Stat3*), are also identified to be involved in the formation of the autoregulatory complex in mESCs, which activate *Sox2* as well as other pluripotent genes^[5]. In this complex, *Stat3* and *Smad1*, which are the key components of the bone morphogenetic protein and leukemia inhibitory factor signalling pathways of mESCs, allow the core transcriptional network integrated into external signalling pathways of mESCs. In hESCs, *Sox2*-Oct4 complex co-occupies their target genes with mothers against decapentaplegic homolog 3 (*Smad3*) protein, a downstream effector of the

transforming growth factor beta signalling pathway which is required for hESC maintenance^[36]. In NPCs, *Sox2* expression is promoted by transcriptional factors that are highly expressed during neural development and differentiation, such as activating protein 2, prospero homeobox protein 1 and *Pax6*^[29]. Signalling pathways, such as phosphatidylinositol-4,5-bisphosphate 3-kinase/*Akt* and *Stat3*, also function to regulate *Sox2* expression^[37,38]. Recently, cell cycle regulators *E2f3a* and *E2f3b* have been reported to regulate *Sox2* expression and control neural progenitor cell proliferation in adult brain^[39,40]. *E2f3a* and *E2f3b* are shown to differentially regulate *Sox2* expression in neural progenitor cells, thus affecting adult neurogenesis^[39]. *E2f3a* cooperates with the pRb family member p107 to repress *Sox2* expression, reducing neural progenitor self-renewal and promoting terminal differentiation, whereas *E2f3b* activates *Sox2* expression by recruiting RNA polymerase II to its promoter, leading to increased self-renewal and neural progenitor/stem cell expansion. Cyclin-dependent kinase inhibitor P21 has also been found to directly bind to a *Sox2* enhancer and repress *Sox2* expression in NPCs^[40]. The various enhancers/regulatory regions of *Sox2* work together to stringently regulate the expression of *Sox2* from early preimplantation embryos to various neural progenitor cells^[30,35,41].

Other than transcriptional regulation, *Sox2* expression and activity are also regulated by post-transcriptional and translational mechanisms. MicroRNA-145 has been demonstrated to negatively affect the expression of pluripotent transcription factors, including Oct4, *Sox2* and *Klf4*^[42]. *Sox2* protein can be modulated by methylation, acetylation, sumoylation and phosphorylation, which subsequently affect its activities as a transcriptional regulator. Three phosphorylation sites, S249, S250 and S251, have been identified in *Sox2*, the phosphorylation of which promotes sumoylation of *Sox2*, subsequently inhibiting the binding of *Sox2* to DNA motifs^[43,44]. Acetylation of *Sox2* by a histone acetyltransferase, p300, induces its nuclear export in ESCs, leading to increased ubiquitination and proteasomal degradation of *Sox2* protein^[45].

Sox2 regulates distinct target genes in pluripotent ESCs and during neural differentiation. Although the exact mechanisms that govern its selection on target genes are not fully elucidated, transcription factors that function as *Sox2* interacting partners may play an important role in this selection. *Sox2* is similar to all *Sox* family members, in that achieving their regulatory functions requires

Table 1 Sex determining region Y-box 2-pairing partners and their functions

Sox2 binding partner	Species	Function	Target genes	Ref.
Oct3/4	Human, mouse	Maintain pluripotency in ES and repress genes involved in developmental process	<i>Sox2, Oct3/4, Nanog, Fgf4, Utlf1, FbX15</i>	[4,34,35]
Pax6	Chicken, mouse	Initiate lens development	<i>Sox2, Pax6, delta crystallin</i>	[42,47]
Brn2	Mouse	Regulate <i>Nestin</i> gene in neural primordial cells	<i>Sox2, Nestin</i>	[48]
Oct1	Mouse	Regulate Pax6 expression which is required for the lens and olfactory placode development	<i>Sox2, Pax6</i>	[49]
Chd7	Mouse	Involved in the regulation of neural stem cells	<i>Jag1, Gli3, Mycn</i>	[50]

Sox2: Sex determining region Y-box 2; Oct3/4: Octamer-binding transcription factor 3/4; Pax6: Paired-box protein 6.

pairing and coordination with other transcription factors to form complexes^[46]. The Sox transcription factor and its interacting partner bind to adjacent DNA sequences in promoter/enhancer of target genes to regulate their expression. Several Sox2 partners have been identified in various cell types (Table 1)^[4,34,35,41,47-50]. The most studied Sox2 partner is Oct4 in PSCs. As discussed earlier, Sox2 has been shown to interact directly with Oct4 in PSCs and the Sox2-Oct4 complex binds to adjacent DNA motifs located in the enhancer/promoter regions of thousands of genes genome-wide to either activate or repress the expression of these genes. They cooperatively activate pluripotent genes whilst repressing lineage-specific ones, hence maintaining pluripotency in these cells (Figure 1)^[4,34,35]. However, it is less clear which transcription factors serve as the Sox2 binding partners during the neural differentiation of PSCs. In early murine neural progenitors, Sox2 is shown to interact with the brain-specific POU domain-containing transcription factor Brn2 to activate the NPC-associated *Nestin* gene expression^[48]. During lens development, Sox2 and Pax6 form a complex which binds to lens-specific enhancer elements to initiate lens development^[41]. Recently, it is also reported that Sox2 is able to interact with long non-coding RNA rhabdomyosarcoma 2 associated transcript to activate the expression of their neural target genes and to promote neural differentiation^[51]. It is possible that Sox2 requires different partners in different neural progenitor cells, which regulate expression of different gene sets, leading to the formation of different neural cell types.

CONCLUSION

Sox2 is one of the key transcription factors that play an essential role in maintaining pluripotency of stem cells. Sox2 interacts with Oct4 to form a binary complex, which then recruits other nuclear factors to activate pluripotent gene expression and repress genes involved in differentiation. Furthermore, Sox2 is also a critical factor for initiating the neural induction and maintaining neural progenitor stem cell properties throughout neural differentiation. Recently, it has been reported that Sox2 is expressed in adult stem cells of several epithelial tissues and regulates trophoblast stem cell differentiation^[47,52]. However, how Sox2 achieves these pleiotropic functions remains to be elucidated. Sox2, like other Sox family members, performs

its regulatory functions more efficiently when paired with an interacting partner. Although Oct4 has been well demonstrated as being such a partner in pluripotent stem cells, the identities of Sox2 partners in other tissues are largely unknown. Understanding the molecular mechanisms governing Sox2 functions will facilitate the use of pluripotent stem cells for clinical and biomedical applications, with particular relevance to the modelling and treatment of various neurological disorders.

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WJSC 6th Anniversary Special Issues (2): Embryonic stem cells

Dendritic cells derived from pluripotent stem cells: Potential of large scale production

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Abstract

Human pluripotent stem cells (hPSCs), including human embryonic stem cells and human induced pluripotent stem cells, are promising sources for hematopoietic cells due to their unlimited growth capacity and the pluripotency. Dendritic cells (DCs), the unique immune cells in the hematopoietic system, can be loaded with tumor specific antigen and used as vaccine for cancer immunotherapy. While autologous DCs from peripheral blood are limited in cell number, hPSC-derived DCs provide a novel alternative cell source which has the potential for large scale production. This review summarizes recent advances in differentiating hPSCs to DCs through the intermediate stage of hematopoietic stem cells. Step-wise growth factor induction has been used to derive DCs from hPSCs either in suspension culture

of embryoid bodies (EBs) or in co-culture with stromal cells. To fulfill the clinical potential of the DCs derived from hPSCs, the bioprocess needs to be scaled up to produce a large number of cells economically under tight quality control. This requires the development of novel bioreactor systems combining guided EB-based differentiation with engineered culture environment. Hence, recent progress in using bioreactors for hPSC lineage-specific differentiation is reviewed. In particular, the potential scale up strategies for the multistage DC differentiation and the effect of shear stress on hPSC differentiation in bioreactors are discussed in detail.

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Key words: Pluripotent stem cells; Dendritic cells; Bioreactor; Hematopoietic differentiation; Large scale production

Core tip: Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are promising sources for hematopoietic cells. This review summarizes recent advances in differentiating hESCs and hiPSCs to dendritic cells (DCs), which are unique immune cells in the hematopoietic system and can be loaded with tumor specific antigen and used as vaccine for cancer immunotherapy. While autologous DCs from peripheral blood are limited in number, human PSC (hPSC)-derived DCs provide a novel alternative cell source for clinical application. Different strategies and effects of shear stress on large-scale production of hPSC-derived DCs in bioreactors are also discussed.

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INTRODUCTION

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced PSCs (hiPSCs), have unlimited self-renewal ability and can give rise to all cell types from three germ layers^[1-5]. While the applications of hESCs are limited by their origin, hiPSCs can be derived from individual patients by reprogramming the somatic cells using pluripotent genes or even small molecules^[6,7]. The derived patient-specific hiPSCs can be indefinitely expanded in culture and differentiated into hematopoietic cells, providing an universal cell source for autologous cell replacement without immune rejection and the models to recapitulate genetic hematological diseases for pathological studies^[8]. Therefore, hiPSCs have emerged as a broad platform to develop medicines for cell therapy, to establish disease models, and to screen compounds for drug discovery, not only for research but also for commercialization^[9,10].

Various blood components have been generated from hPSCs, including red blood cells, platelets, leukocytes, natural killer cells, erythroblasts, T cells, and B cells^[11-13]. Dendritic cells (DCs) are potent antigen presenting cells which can be produced as vaccines for cancer immunotherapy^[14]. Based on the unique ability to mediate immunity, about 200 DC trials have been reported in treating various types of cancer such as lung cancer and breast cancer^[15]. However, autologous DCs are limited in cell number ($\sim 10^8$) and subject to large donor-to-donor variability^[16]. In addition, DC defects have been observed in circulating peripheral blood mononuclear cells (PBMCs) for some cancer patients^[17]. DCs derived from hPSCs can potentially overcome these drawbacks. An unlimited number of DCs with little variability can be derived from hPSCs, which are independent of circulating blood. Thus, hPSC-derived DCs are especially useful for cancer patients who have functional defects in PBMC-derived DCs. Functional DCs have been derived from both hESCs and hiPSCs recently, providing novel alternative sources to autologous DCs^[18-20]. Given the unique proliferative capacity, hPSC-based process has the potential for mass production of DCs at a scale of more than 10^{10} cells^[9].

For large-scale production, bioreactor provides a powerful tool to fulfill the unlimited proliferation capacity of hPSCs to derive hematopoietic cells. Bioreactors not only allow the scale up of the process, but may also regulate the differentiation pathway due to the unique hydrodynamic environment, especially the presence of shear stress^[21,22]. Several types of bioreactors have been used for hPSC expansion and differentiation, including spinner flasks, rotating wall vessels, and perfusion bioreactors^[23-25]. These bioreactors provide a closed and homogenous culture environment along with the capability for online monitoring and control of culture parameters that fulfill regulatory requirements^[26]. For example, spinner flask has been tested for hematopoietic differentiation from hESC through embryoid body (EB) formation^[27]. EBs in suspension bioreactors have more uniform size distribution,

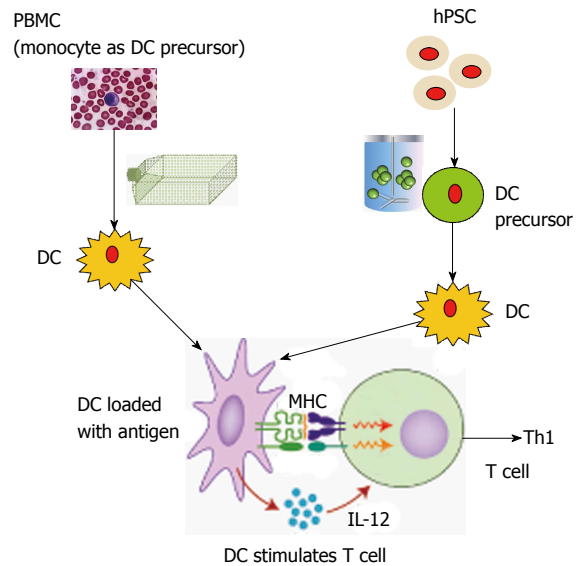


Figure 1 Schematic comparison between conventional dendritic cell vaccine production and human pluripotent stem cell-based dendritic cell vaccine production. Conventional dendritic cell (DC) vaccine production is usually from peripheral blood mononuclear cells (PBMCs) in patients. T-flasks, Cell Factories or bags are used for DC maturation. From human pluripotent stem cells (hPSCs), DC precursors (similar to monocytes isolated from PBMCs) can be generated in large scale in bioreactors and then mature into DCs. In theory, this approach can generate an unlimited number of DCs. To stimulate T cell response, the activated DCs can release cytokines such as interleukin (IL)-12 to trigger T helper type 1 (Th1) immune response. DCs are also able to capture and process antigens, converting proteins to peptides that are presented on major histocompatibility complex (MHC) molecules and recognized by T cells. The induced Th1 immune response can target on the cancer cells, which express tumor-specific antigens.

less agglomeration, and similar percentage of the differentiated hematopoietic cells compared to static culture. To provide cell adhesion surfaces, microcarriers have also been applied in hPSC expansion and differentiation due to the scale up potential in stirred bioreactors^[28].

In this review article, we discuss the potential of hPSCs as the cell source to generate DCs [through the intermediate stage of hematopoietic stem cells (HSCs)] (Figure 1) and the current progress in DC differentiation from hPSCs. The scale up potential of the differentiation procedure in bioreactors and the potential effect of hydrodynamic environment on hPSC differentiation are also discussed. Combining the guided biological differentiation following the developmental pathway with the engineering principle in scalable bioreactors, the potential of hPSCs in producing DCs to treat cancers can be better fulfilled.

WHY AN ALTERNATIVE SOURCE OF HEMATOPOIETIC CELLS IS IMPORTANT?

Limitation of autologous HSCs

HSCs are critical sources for various blood cells including DCs. Therefore, HSC transplantation of human leukocyte antigen-matched bone marrow, cord blood, or mobilized peripheral blood CD34⁺ cells has been the standard medical treatment for cancer patients to repopulate

hematopoietic system. However, current allogeneic HSC transplantation is accompanied with high frequency of graft-versus-host diseases due to immune response and high risk of infection^[8]. Autologous HSC transplantation has a lower rate of immune rejection, but the cells are not available for patients with genetic defects. Both allogeneic and autologous HSCs from somatic sources are limited in cell number, which significantly affects the therapeutic outcomes. To avoid transplantation-related mortality due to the delayed neutrophil engraftment, a large number of HSCs are required for infusion to patients. For example, a doubled cord blood unit (3.5×10^7 nucleated cells/kg) was applied to increase cell number, which however, did not show a significant impact on neutrophil recovery^[29]. *Ex vivo* expansion strategies have been extensively studied using soluble cytokines, but only 2 to 5-fold increase in long-term repopulating cells was achieved and modest effect on neutrophil recovery was observed^[30]. New approaches are being explored to provide niche factors that target on molecular pathways such as Notch or Wnt but still resulted in limited long-term engraftment^[29,31]. Besides repopulating bone marrow, HSCs have been used to produce mature blood cell types such as red blood cells and DCs for transfusion or immune therapy^[14,32]. However, the number of mature blood cells needed is enormous and current cell expansion technology is not efficient or economical. For example, for DC therapy, which requires a cell number of 10^8 per patient, the cost of generating sufficient PBMC-derived DCs for each patient is as high as \$93000^[20]. In contrast to the current HSC sources, hPSC is an ideal cell source that has the potential to generate a large number of immune-compatible hematopoietic cells in a scalable bioreactor system.

Limitation of autologous DC vaccine

Among the mature blood cells, DCs are the most potent immune cells for antigen presentation and the only cells with the ability to induce a primary immune response in resting naïve T lymphocytes^[33]. Numerous DC trials have been reported, and the trials in cancer immunotherapy showed encouraging results^[34,35]. One such trial in breast cancer was performed in 27 patients with human epidermal growth factor receptor 2 (HER2)/neu overexpressing ductal carcinoma. Sensitization of T-helper cells was observed in 22 of 25 patients. In addition, responses of anti-HER2/neu peptides were observed up to 52-month post-immunization^[34]. Some other DC trials also achieved positive outcomes, including the trials using Dendreon's lapuleucel-T^[36-38]. These promising results encourage further study with multiple doses of DC vaccines in various cancer patients.

DCs for clinical trials can be generated from CD34⁺ cells in bone marrow using granulocyte-macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor (TNF)- α , or CD14⁺ monocytes derived from PBMCs using GM-CSF and interleukin (IL)-4^[14]. Comparison of CD34⁺ cells-derived DCs and monocyte-derived DCs from the same patients demonstrated similar morphology and performance in mixed lymphocyte reaction (MLR),

while differences in some surface markers such as CD86 and human lymphocyte antigen-DR were observed^[39]. Difference in the capacity to activate CD8⁺ T cells was also reported in these two populations^[40]. Because DC functions are affected by patient health, cytokine selection, and isolation procedures, DCs from both sources have been used in clinical trials^[41]. However, CD34⁺ cells are usually found in small numbers and a longer time is needed to generate sufficient DCs (14 d vs 7 d), thus monocyte-derived DCs have been used more often in clinical trials^[42]. Production of autologous DCs involves the purification of monocytes from PBMCs, generating immature DCs from monocytes, and differentiating immature DCs into mature DCs^[42,43]. For clinical use, a scalable culture system that meets current Good Manufacturing Practices (cGMP) guidelines is required. Such large scale closed-systems have been developed based on ElutraTM cell separation device and cell culture in Teflon bags, which can produce an order of 10^8 mature DCs^[16]. This number generally provides one dose for one patient and multiple preparations are required for multiple doses. Importantly, circulating PBMC-derived DCs from some cancer patients have been shown to have poor ability in stimulating T cell proliferation and reduced capacity of capturing antigens^[17]. Both the number limitation and DC defects in some cancer patients have motivated the demand for an alternative DC source.

hPSCs: A NEW SOURCE FOR HSCs?

Hematopoietic differentiation from hPSCs has been investigated recently^[44,45]. Although both hiPSC and hESC can be differentiated to hematopoietic cells with comparable efficiency, generating hematopoietic cells from the patient-specific iPSCs has the advantage of immunologic compatibility^[46]. Methods for hematopoietic differentiation from hPSCs have been performed either by co-culturing on stromal feeder layers or in the form of EBs, an aggregate-like structure mimicking embryonic development^[44]. Currently, the most commonly used stromal feeder is murine bone marrow stromal line OP9, which augments the hematopoiesis by promoting the survival of hematopoietic precursors and progenitors^[47]. It has been suggested that mKlrre, Notch ligand, or other unidentified factors may contribute to the observed effects of OP9 in hPSC differentiation^[48]. However, OP9 cells cannot be used to produce hematopoietic cells for clinical application due to their animal origin. EB-based differentiation is more suitable for clinical use due to the absence of murine stroma and the adaptability in suspension culture. However, it is difficult to control the differentiation efficiency and the derived cell phenotype for the EB-based differentiation because of the heterogeneity of EB size and morphology. Therefore, novel methods that can make homogenous EBs such as forced aggregation have been developed recently^[49].

To date, CD34⁺ cells generated from hPSCs using the existing methods are less clonogenic and less proliferative than CD34⁺ cells isolated from somatic sources^[44].

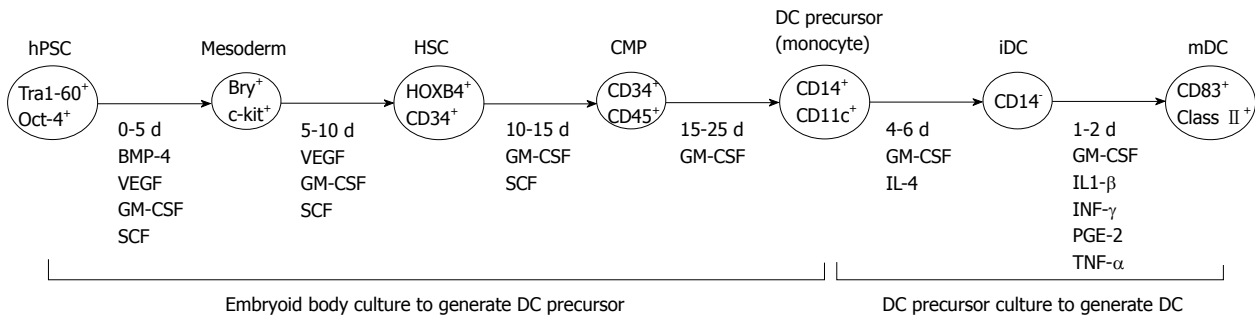


Figure 2 Schematic differentiation process from human pluripotent stem cells to dendritic cells. The differentiation into dendritic cells (DCs) was initiated from mesoderm specification. Then, the cells become hematopoietic stem cells (HSCs) after treatment with growth factors, followed by the stage of more committed common myeloid progenitors (CMP). CMP will then become monocyte-like cells as DC precursors. DC precursors will become immature DCs (iDCs), which further differentiate into mature DCs (mDCs). Definite markers can be used to identify each stage over the course of differentiation. Bry: Mesoderm marker brachyury; BMP-4: Bone morphogenetic protein-4; VEGF: Vascular endothelial growth factor; GM-CSF: Granulocyte-macrophage colony-stimulating factor; SCF: Stem cell factor; IL-4: Interleukin 4; IL-1 β : Interleukin-1 beta; INF- γ : Interferon gamma; PGE-2: Prostaglandin E2; TNF- α : Tumor necrosis factor alpha.

Furthermore, the engraftment capacity of hPSC-derived HSCs was poor compared to cells from somatic source^[50]. Despite the poor engraftment capacity, the erythroid, myeloid, natural killer cells, and DCs from hPSC-derived CD34⁺ cells were qualitatively similar to their somatic counterparts^[51-53]. It has been suggested that transfusion and immune therapy may be the immediate clinical applications for hPSC-derived HSCs, which require high derivation efficiency and a clinical-scale culture system suitable for mass production of these cells^[11].

hPSCs: A NEW SOURCE FOR DC

DC differentiation from hPSCs: promise and progress

Similar to deriving HSCs, there are two methods for DC differentiation from hPSCs: (1) through the formation of EBs in suspension culture; and (2) by co-culture with mouse OP9 stromal cell line^[54]. The differentiation into DCs starts from mesoderm specification induced by growth factors including vascular endothelial growth factor (VEGF) and bone morphogenetic protein (BMP)-4 (Figure 2)^[20,52]. Mesoderm cells further develop into HSCs (characterized by HOXB4⁺ and CD34⁺ cells) and then become common myeloid progenitors (CMPs) (characterized by CD34⁺ and CD45⁺ cells). CMPs are differentiated into monocyte-like DC precursors (CD14⁺, CD11c⁺) indicated by single cells (*i.e.*, DC precursors) released from the aggregates. After reaching the DC precursor stage, the differentiation procedure is almost identical to PBMC-derived DCs.

For hESC-derived DCs based on EB formation, only about 25% DCs with low CD83 expression, which indicates the relative immature DC stage, were obtained from spontaneous differentiation from EBs^[18]. A similar method was tested in the presence of BMP-4 to enhance mesoderm differentiation^[55]. The purity of DCs was improved to more than 80% CD11c⁺ cells, but CD83 was still less than 50%. A serum-free EB-based protocol was assessed using four growth factors including VEGF, stem cell factor (SCF), BMP-4, and GM-CSF^[52,56]. BMP-4 and VEGF are critical growth factors which synergistically

enhanced hematopoietic differentiation by activating the Nodal/Activin pathway^[57]. About 75% of cells expressed DC markers CD86 and CD83, and the derived DCs had similar stimulatory function compared to PBMC-DCs. This promising protocol serves as the baseline process for Geron's potential product GRNVAC2, *i.e.*, DCs derived from hESCs, which is the second generation of DCs following GRNVAC1, *i.e.*, DCs derived from PBMC. The four-growth factor (VEGF, SCF, BMP-4, and GM-CSF) protocol was also evaluated for DC differentiation from hiPSCs. High-purity DCs (> 70% CD83⁺ cells) were generated, but a subset of cell population co-expressed CD141 and XC chemokine receptor 1 (XCR1), which was phenotypically different from CD141⁺ XCR1⁺ hESC-derived DCs^[58]. The expression of XCR1 in DCs may be better suited to the induction of antitumor responses due to the augmentation of antigen-driven expansion of CD8⁺ cytotoxic T lymphocytes^[59]. For the OP9 co-culture protocol, hESCs were plated onto OP9 feeder layer to facilitate hematopoietic differentiation and about 50% of DCs was achieved^[60]. However, this protocol only achieved 20% purity of hiPSC-derived DCs^[19]. Apparently, the OP9 protocol needs to be improved for efficient DC differentiation from hiPSCs.

Functional assessments were performed for hPSC-derived DCs in comparison with PBMC-derived DCs, including specific cytokine secretion, allogeneic T-cell response by MLR assay, endocytosis ability, and antigen-specific T cell response^[19,52,55]. For example, hESC-derived DCs produced similar level of IL-6 and IL-12p70 compared to PBMC-derived DCs^[52]. Strong allogeneic T-cell response and phagocytosis ability were also demonstrated for both hESC-DCs and hiPSC-DCs^[19,55]. Human telomerase reverse transcriptase antigen-specific T-cell stimulation was observed with significant IFN- γ production which was greater than the response stimulated by PBMC-DCs^[52]. Further enhancement of stimulatory function was also demonstrated for hESC-DCs after transfection with mRNA encoding IL-12p70^[56]. Despite these successes, a fully understanding of the functions of hPSC-derived DCs, especially DCs derived from hiPSCs,

Table 1 Bioreactor systems for hematopoietic differentiation from pluripotent stem cells

Bioreactor type	PSC line	Performance	Ref.
Spinner flask	hESC lines: H9 and H1	15-fold expansion in cell number compared to 4-fold in static culture; 5%-6% CD34 ⁺ CD31 ⁺ cells	Cameron <i>et al</i> ^[27]
RWV	Mouse ESC line: R1	Hematopoietic differentiation in RWV generated more Sca-1 ⁺ cells; while spinner flasks generated more c-kit ⁺ progenitors	Fridley <i>et al</i> ^[25]
Microcarrier-based spinner flask	hESC lines: WA01 (H1), WA09 (H9), HuES3, MA09; hiPSC line: IMR90-1	DE-53 microcarriers were used. EBs were formed 10 times more efficiently compared to 2-D culture; CFUs were similar to static culture	Lu <i>et al</i> ^[67]
Perfusion bioreactor	Mouse ESC line: D3 Mouse ESC line: CCE	Perfusion promoted hematopoietic differentiation; Perfusion in 3-D fibrous scaffolds supported 2-3 fold higher cell density compared to static culture; comparable CFU percentage	Wolfe <i>et al</i> ^[77] , Li <i>et al</i> ^[24]

hESC: Human embryonic stem cell; ESC: Embryonic stem cell; EBs: Embryoid bodies; hiPSC: Human induced pluripotent stem cell; RWV: Rotary wall vessel; CFU: Colony-forming units.

is still required for potential clinical applications.

Scalability of DC differentiation from hPSCs

Compared to the OP9 co-culture system, the EB-based protocol (free of feeder cells) is more suitable for large-scale production in stirred bioreactors for potential clinical applications^[61]. Because the DC precursors are released from EBs as single cells, the DC precursors are also able to be expanded and differentiated in suspension. Hence, the process of DC differentiation from hPSCs through EB formation is scalable in bioreactors. Different from traditional static tissue culture vessels, hPSCs in bioreactors are exposed to dynamic physiochemical environments. Especially, the presence of shear stress affects not only cell growth and viability, but also the cell phenotype and lineage commitment^[23,62]. Understanding the impact of hydrodynamic environment on hPSC differentiation is a critical step to scale up the process for producing DCs from hPSCs.

POTENTIAL SCALABLE PRODUCTION OF hPSC-DERIVED DCs IN BIOREACTORS

Bioreactors for hPSC-derived cells

Dynamic bioreactors can be used to enhance mass transfer coefficient, alter the kinetics of receptor-ligand binding, and control the aggregate collision^[21,62,63]. Thus, stem cell aggregation, metabolism, and cell phenotype can be modulated in the hydrodynamic environment of bioreactors. Several scalable EB-based culture systems, including those for hematopoietic differentiation, have been developed recently (Table 1)^[61]. The initial bioreactor culture was performed in slow turning lateral vessel (STLV) for spontaneous EB formation^[64]. The STLV with a low shear stress supported better cell expansion than static culture although massive EB agglomeration was observed. Later, STLV was compared with the spinner bioreactor, which gave better cell proliferation^[65]. Bioreactor configuration has also been shown to affect hematopoietic differentiation from PSCs. For example, EB formation and the subsequent hematopoietic differentiation in spinner flasks generated more c-kit⁺ progenitors while

more Sca-1⁺ cells were observed in the STLV, possibly due to different hydrodynamic environments in these two types of bioreactors^[25]. Pre-formed EBs were also seeded in spinner bioreactors for hematopoietic differentiation and 5%-6% CD34⁺ cells were detected^[27]. The spinner bioreactor was further improved with oxygen control and size-controlled cell clumps as the inoculum^[66]. In general, compared to static cultures, dynamic bioreactors promoted cell expansion with a similar or higher differentiation efficiency. However, the differentiation efficiency is still low (< 30%), and needs significant improvements for clinical applications. This can be done by using a specific lineage differentiation protocol rather than spontaneous differentiation.

Recently, microcarriers have been used to support hematopoietic differentiation of hPSCs to improve EB-forming efficiency^[67]. Microcarrier culture provides a high surface-to-volume ratio, leading to a high cell density. The process is also suitable for scale up in stirred bioreactors. The hPSCs grown on DE-53 microcarriers were able to form EBs with 10-fold higher efficiency compared to hPSCs grown in 2-D cultures^[67]. After replating, these EBs developed into hemangioblasts which can differentiate into hematopoietic and endothelial cells. This system can be further developed into a cGMP-compatible scalable system to generate blood cells for clinical applications. However, there are several challenges for microcarrier cultures, including microcarrier clumping, cell damage caused by shear stress, and difficult operation for cell-carrier separation^[68].

Microencapsulation of hPSCs in hydrogel is another approach of suspension culture, which can avoid EB aggregation or microcarrier clumping while protecting cells from shear stress^[69,70]. Using specific biomaterials such as alginate, agarose, and hyaluronic acid, microencapsulation can preserve 3D cell-cell and cell-matrix contacts, which simulate *in vivo* stem cell niches for efficient hPSC differentiation^[70,71]. However, there are some limitations in microencapsulation cultures. For example, gas and mass diffusion inside the hydrogel could be limited; monitoring and observing the culture are difficult; and the additional cell releasing process is required^[68]. For all these suspension cultures (*i.e.*, EBs, microcarriers, and microencapsu-

Table 2 Effects of shear stress in bioreactors on pluripotent stem cells expansion and differentiation

Shear stress	Effect on pluripotency	Effect on differentiation	Ref.
Agitation in stirred bioreactors	Bioreactor-differentiated ESCs retained the ability to express pluripotent markers	EBs in bioreactors differentiated into cardiomyocytes	Shafa <i>et al</i> ^[73]
Agitation in stirred bioreactors (3 dyne/cm ² vs 6 dyne/cm ²)	Shear stress maintained certain pluripotent markers (e.g., Nanog, Rex-1)	Reduced spontaneous differentiation	Gareau <i>et al</i> ^[72]
Agitation in stirred bioreactors	A subpopulation of bioreactor-differentiated ESCs expressed the pluripotent markers	Differentiation into osteogenic and chondrogenic cell types	Taiani <i>et al</i> ^[74]
Agitation in stirred bioreactors (glass ball impeller, < 1.52 dyne/cm ²)	Homogeneous aggregate size distribution	Cells maintained the differentiation potential into hematopoietic cells	Wang <i>et al</i> ^[79]
Rotary orbital shaking (< 2.5 dyne/cm ²)	Shear stress up-regulated genes specific for endoderm and mesoderm differentiation	Spontaneous three-germ layer differentiation	Sargent <i>et al</i> ^[63]
Perfusion flow (1.5-15 dyne/cm ²)	Shear stress promoted early differentiation of ESCs	Shear stress promoted hematopoietic and endothelial differentiation	Wolfe <i>et al</i> ^[77]
Agitation in microcarrier-based bioreactors	HES-2 line and hiPSC line IMR-90 were shear sensitive, showing the down-regulation of pluripotent markers	Shear stress induced spontaneous differentiation	Leung <i>et al</i> ^[76]

ESC: Embryonic stem cell; EBs: Embryoid bodies; hiPSC: Human induced pluripotent stem cell.

lation), the effect of shear stress on hPSC differentiation needs to be further studied for efficient differentiation in bioreactors.

Effects of shear stress in bioreactors

Shear stress is an important parameter that can be used to regulate hPSC expansion and differentiation (Table 2)^[68,72]. During the differentiation of hPSCs into cardiomyocytes or osteoblasts, the PSC aggregates cultured in stirred bioreactors preserved the Oct-4 expressing pluripotent cells, which were absent in the cell population differentiated in static culture^[73,74]. It was postulated that shear stress modulated gene expression through mechano-transduction and that the non-canonical Wnt pathway might play an important role in bioreactor-induced pluripotency. Mechanical strain also suppressed spontaneous differentiation and induced autocrine or paracrine signaling through transforming growth factor- β superfamily ligand to activate Smad 2/3^[75]. However, in both the microcarrier-based stirred bioreactors and the EB-based rotary orbital bioreactors, shear stress in the hydrodynamic environment was found to promote spontaneous differentiation of PSCs^[63,76]. Perfusion-induced shear stress (1.5-15 dyne/cm²) was also shown to promote hematopoietic differentiation from PSCs by up-regulating fetal liver kinase-1 (VEGF receptor) expression^[77]. The effect of shear stress on fate decision of hPSCs has not been fully understood and requires future investigation.

The shear stress exerted on the cells during initial seeding may affect EB-forming efficiency in the dynamic culture. For EB formation, a low shear stress could lead to inadequate nutrient diffusion and massive EB agglomeration, while a high shear stress could lead to the dissociation of receptor-ligand binding and may also cause significant cell death^[78]. A few studies tried to avoid direct EB formation in spinner bioreactors by seeding pre-formed EBs^[27,65]. However, this approach is not practical for large-scale production. The rotary speed (20-60 rpm) in a rotary orbital suspension culture has been found to affect EB size distribution, where a mild shear stress (<

2.5 dyne/cm²) led to homogenous EBs^[63]. A mild shear stress (< 1.5 dyne/cm²) in the spinner flask equipped with a pendulum-shape impeller at 40-75 rpm also produced homogeneous hiPSC aggregates^[79]. To minimize the initial cell death and EB aggregation, novel agitation scheme based on intermittent mixing may also need to be applied.

Scale up challenges for producing DCs from hPSCs

DC differentiation from hPSCs, which lasts about 32 d, has multiple stages of differentiation with a different cocktail of growth factors at each stage (Figure 2). Specifically, the differentiation procedure comprises of EB stage and single-cell stage, each requiring a different agitation rate as the EBs and the single cells might have different sensitivities to shear stress. Although hPSC-derived EBs have been expanded in bioreactors, the lineage specification of DC differentiation from hPSCs in bioreactors has not been demonstrated. For conventional production of DC vaccines, isolated monocytes from PBMCs are cultured as DC precursors to generate DCs. To integrate with monocyte isolation from PBMCs, autologous DC production for clinical use is usually performed in large T-flasks, roller bottles, and bags^[80-82]. The bags are widely used because they are easy to be connected with cell separation system, enabling DC production in a complete closed-system^[16,81]. However, to integrate hPSC-DC precursor culture (single cell stage) with dynamic hPSC-EB culture (aggregate stage), stirred spinner bioreactors may be required. The concept of process integration, which includes reprogramming, expansion, and differentiation in one fully integrated process, has been demonstrated for iPSC-derived cardiac cells^[83]. For DCs, it would be difficult and inconvenient to transfer the EB-derived DC precursors from spinner flasks to roller bottles or bags. Continuous differentiation of hPSC-derived DC precursors in the same culture vessel offers obvious advantages for large scale production. Due to different sensitivities to shear stress at various differentiation stages, different agitation programs may be required for day 0-2 (EB for-

mation stage), day 2-25 (EB culture stage), and day 25-32 (single cell stage). Therefore, an integrated bioreactor system can significantly enhance the process efficiency and scalability for hPSC-derived DCs.

Online monitoring the differentiation status of hPSCs is also a crucial element in bioreactor-based DC production. Given that hPSC differentiation usually generates a heterogeneous cell population, including the residue undifferentiated cells that can form tumor *in vivo*, novel reliable technologies for downstream cell separation in clinical scale also need to be developed^[84]. Establishing the correlation of cell-secreted molecules with the differentiation outcome can facilitate the in-process monitoring. For DC differentiation, DC precursors are released from EBs and the frequency of undifferentiated cells is low. More importantly, DCs loaded with tumor-specific antigen can be irradiated for vaccine injection, thus there is minimal risk of tumor formation. Instead, cell irradiation will be a part of the production process and the procedure needs to be optimized to better preserve DC function.

CONCLUSION

Pluripotent stem cells have emerged as new cell sources for HSC-derived mature blood cells, especially DCs. PSC-derived DCs can overcome the limitations of autologous DCs from cancer patients, including the limited cell number and possible functional defects. Although the engraftment capacity of hPSC-derived HSCs has been poor compared to cells from somatic sources, mature blood cells, including DCs from hPSC-derived CD34⁺ cells, are qualitatively similar to their somatic counterparts. Efficient DC differentiation from hPSCs has been achieved through EB formation with high purity. To fulfill the potential of hPSC-derived DCs, large-scale production in bioreactors is a critical step toward clinical applications. The hydrodynamic environment in bioreactors, especially shear stress, is a potent regulator for hPSC expansion and differentiation, while the effect of shear stress on fate decision of hPSCs has not been fully understood. For DC differentiation in bioreactors, an integrated process from EB formation to DC maturation will offer significant advantages in process efficiency and scalability. However, it is challenging to integrate the multiple stages of DC differentiation from hPSCs, which needs a better understanding of the stage-specific responses to the hydrodynamic environment.

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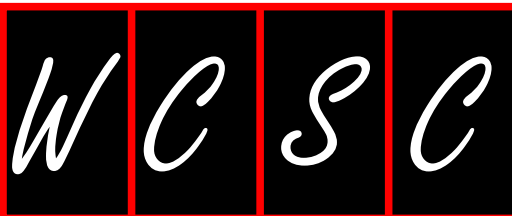
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WJSC 6th Anniversary Special Issues (3): Embryonic stem cells

Neural differentiation from pluripotent stem cells: The role of natural and synthetic extracellular matrix

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Abstract

Neural cells differentiated from pluripotent stem cells (PSCs), including both embryonic stem cells and induced pluripotent stem cells, provide a powerful tool for drug screening, disease modeling and regenerative medicine. High-purity oligodendrocyte progenitor cells (OPCs) and neural progenitor cells (NPCs) have been derived from PSCs recently due to the advancements in understanding the developmental signaling pathways. Extracellular matrices (ECM) have been shown to play important roles in regulating the survival, proliferation, and differentiation of neural cells. To improve the function and maturation of the derived neural cells from PSCs, understanding the effects of ECM over the course of neural differentiation of PSCs is critical. During neural differentiation of PSCs, the cells are sensitive to the properties of natural or synthetic ECMs, including biochemical composition, biomechanical properties, and structural/topographical features. This review summarizes recent advances in neural differen-

tiation of human PSCs into OPCs and NPCs, focusing on the role of ECM in modulating the composition and function of the differentiated cells. Especially, the importance of using three-dimensional ECM scaffolds to simulate the *in vivo* microenvironment for neural differentiation of PSCs is highlighted. Future perspectives including the immediate applications of PSC-derived neural cells in drug screening and disease modeling are also discussed.

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Key words: Pluripotent stem cells; Neural differentiation; Extracellular matrix; Three-dimensional; Drug screening

Core tip: Neural cells derived from human pluripotent stem cells (hPSCs), including oligodendrocyte progenitor cells and neural progenitor cells, emerge as an unlimited and physiologically relevant cell source for drug screening, disease modeling, and regenerative medicine. Natural and synthetic extracellular matrices play an important role in regulating neural differentiation, cell migration, and the derived neural cell maturation. Recent advances in neural differentiation of hPSCs on extracellular matrices in 2-D and 3-D systems are reviewed in this paper. The immediate applications of the derived neural cells in drug screening and disease modeling are also discussed.

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INTRODUCTION

Human pluripotent stem cells (hPSCs), including human

embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), have extensive proliferation potential and the unique ability to produce any type of somatic cells^[1,2]. Due to their self-renewal ability, hPSCs potentially can provide unlimited numbers of neural cells for cell therapy and drug discovery^[3,4]. For example, oligodendrocyte progenitor cells (OPCs) derived from hESCs have been tested in Geron's Phase I clinical trial approved by Food and Drug Administration in 2010 to treat spinal cord injury (SCI)^[5,6]. OPCs derived from hiPSCs have also been shown to myelinate and rescue a mouse model of congenital hypomyelination^[7]. Compared to other animal cells and tissues, hPSCs can provide physiologically relevant cells to deliver more efficacious medicines and to provide accurate models for drug screening^[8,9]. For example, *in vitro* model of amyotrophic lateral sclerosis (ALS) can be established from the motor neurons differentiated from hPSCs, which are sensitive to the toxic effect of glial cells carrying an ALS-causing mutation^[10]. Compared to hESCs, hiPSCs can be derived from individual patients, providing "personalized" medicine and the *in vitro* models to study pathological neural development and disease progression^[11]. For neurological diseases where somatic neural cells are limited in number, hPSCs emerge as a powerful tool for drug screening, disease modeling, and regenerative medicine.

The ability to obtain high-purity and functionally mature neural cells is the pre-requisite to fulfill the potential of hPSCs in neurological disease treatments. Differentiating hPSCs into OPCs or neural progenitor cells (NPCs) with a high purity has been demonstrated, but their function and maturation are still under investigation^[12-14]. Extracellular matrix (ECM) plays an important role in neural differentiation of hPSCs and the maturation of the derived neural cells^[15]. ECM proteins, through the interaction with integrins expressed on the neural cells, modulate cell survival, migration, proliferation, and the differentiated cell function^[16]. Besides ECM composition, the mechanical property of ECMs is also found to regulate neural lineage commitment of hPSCs recently. To better understand the *in vivo* development and the "niches", *i.e.*, microenvironment, of neural tissue development^[15], three-dimensional (3-D) ECMs, both natural and synthetic, have been investigated for efficient neural differentiation of hPSCs. 3-D ECM scaffolds provide not only physical support for cell adhesion, but also the structural and biomechanical cues that can be transduced into biochemical signals, affecting cellular composition during neural differentiation^[17,18]. By regulating biochemical composition, biomechanical properties, and physical structure of 3-D ECMs, neural differentiation of hPSCs can be effectively controlled.

This review summarizes recent advances and the development of protocols for *in vitro* differentiation of hPSCs to OPCs and NPCs with high purity and desired function. To provide the 3-D microenvironment that more resembles *in vivo* tissues than traditional 2-D cultures, 3-D neural differentiation systems based on various natural and synthetic ECMs have been extensively

studied and are discussed in this review, with an emphasis on the effects of ECMs on neural lineage commitment of hPSCs. Current progress in the application of hPSC-derived neural cells for drug screening is also discussed and highlighted.

PLURIPOTENT STEM CELL-DERIVED OLIGODENDROCYTE PROGENITOR CELLS

Oligodendrocytes derived from OPCs can remyelinate axons upon maturation. However, transplanting OPCs instead of mature oligodendrocytes is a better strategy to restore neural function^[19]. While OPCs from somatic tissues are limited in cell number, OPCs derived from hPSCs provide novel alternative autologous or allogeneic cell sources. There are two types of OPC differentiation protocols from hPSCs in general: epithelial growth factor (EGF)-dependent protocol and platelet-derived growth factor (PDGF)-dependent protocol (Table 1)^[20]. OPCs were initially derived from hESCs through embryoid body (EB) formation in the presence of fibroblast growth factor (FGF)-2, retinoic acid (RA), and EGF for 4 wk followed by attaching the neural spheres onto Matrigel-coated surface for another 2 wk (6-wk protocol)^[21]. A high-purity population of OPCs was achieved with the expression of more than 70% NG2, oligodendrocyte transcription factor 1 (OLIG1), OLIG2, and (sex determining region Y)-box 10 (SOX10) using this EGF-dependent protocol. When culturing the cells on human laminin in the absence of mitogen EGF, the derived OPCs displayed minimal neuronal and astrocyte markers, and could mature into oligodendrocytes, which expressed Gal C, O4, and myelin basic protein. Transplanting hESC-OPCs was shown to remyelinate axons and restore the locomotor function in a rat contusion model^[19]. The transplanted OPCs phenotypically replaced lost oligodendrocytes, remyelinated axons, and also secreted neurotrophic factors to establish a repair environment in the lesion^[22,23]. This EGF-dependent OPC differentiation protocol has been successfully used in a manufacturing process in a cGMP (*i.e.*, current Good Manufacturing Practices) facility to produce OPCs for treating SCI patients in Phase I clinical trials^[6]. Although the preliminary safety data were obtained, additional trials are required to demonstrate the efficacy of the hESC-derived OPCs. Different protocols have been developed later using different induction factors, including sonic hedgehog (Shh) protein, PDGF, insulin-like growth factor (IGF)-1, bone morphogenetic protein antagonists such as noggin, neurotrophic factors such as neurotrophin (NT)-3 and ciliary neurotrophic factor, with or without EGF^[24-28]. High-purity OPCs were obtained, and they also showed remyelination capacity in animal study^[29]. The main drawback of these PDGF-dependent protocols is their lengthy (10-14 wk) and complicated procedures with multiple growth factors and multiple steps of suspension and adherent cultures, which are difficult and expensive to scale up for

Table 1 Protocols and *in vivo* studies for oligodendrocyte progenitor cells differentiation from human pluripotent stem cells

Growth factors	Cell source	Cell characteristics	Ref.
EGF-dependent protocol			
RA/EGF (short FGF-2 exposure)	hESC lines: H1 and H7	Olig1 (80%-90%), Sox10 (76%-84%), NG2 (95%); remyelinated in a rat thoracic contusion model	Nistor <i>et al</i> ^[21] , 2005; Keirstead <i>et al</i> ^[19] , 2006; Li <i>et al</i> ^[13] , 2013
RA/EGF (short FGF-2 exposure)	hiPSC lines: Royan hiPSC1, hiPSC8	> 90% Olig2, Sox 10, > 80% NG2 and PDGFR α ; tested in a rat model of optic chiasm demyelination	Pouya <i>et al</i> ^[33] , 2011
RA/EGF (short FGF-2 exposure)	hESC H7 line	OPCs remyelinated in a rat cervical contusion model	Sharp <i>et al</i> ^[94] , 2010
RA/EGF (short FGF-2 exposure)	hiPSC lines: 201B7, 253G1	O4 ⁺ cells were observed in EGF-dependent protocol	Ogawa <i>et al</i> ^[20] , 2011
PDGF-dependent protocol			
PDGF/FGF-2 (short EGF exposure)	SNUhES1 line	PDGFR α (81%), A2B5 (90.4%), NG2 (91.3%) and O1 (81%); myelinate axons in co-cultures with fetus hippocampal neurons.	Kang <i>et al</i> ^[24] , 2007
RA/Shh/FGF-2/PDGF	hESC lines: H1, H9 H14	> 80% PDGFR α ⁺ , also co-express Olig2, Nkx2.2, Sox10 and NG2	Hu <i>et al</i> ^[26] , 2009
RA/Shh/FGF-2/PDGF	hiPSC lines: K04, C14, and C27	70%-90% Olig2 ⁺ /Nkx2.2 ⁺ , OPCs myelinated the brains of myelin-deficient shiverer mice	Wang <i>et al</i> ^[7] , 2013
FGF-2/EGF/PDGF/CNTF plus laminin;	hESC lines: HS360 and HS362;	> 90% NG2 ⁺ , > 80% PDGFR α ⁺ ; multilayered myelin sheet formation around axons was detected in co-culture with neuronal cells	Sundberg <i>et al</i> ^[28] , 2010
Shh, PDGF, IGF-1, EGF, FGF-2 and CNTF plus RA and laminin	Regea 06/040 and Regea 08/023		Sundberg <i>et al</i> ^[27] , 2011
Noggin/FGF-2/FGF-4/PDGF/EGF	hESC H1 line	> 95% cells expressing Sox10, A2B5, PDGFR α , NG2, O4, O1; increased neurological response in a rat contusion model	Kerr <i>et al</i> ^[29] , 2010

RA: Retinoic acid; EGF: Epidermal growth factor; FGF-2: Fibroblast growth factor 2; hESC: Human embryonic stem cell; hiPSC: Human induced pluripotent stem cell; PDGFR: Platelet-derived growth factor receptor; PDGF: Platelet-derived growth factor; Shh: Sonic hedgehog; CNTF: Ciliary neurotrophic factor; IGF-1: Insulin-like growth factor-1.

generating cells needed for clinical studies.

The differentiation of iPSCs to oligodendrocytes was initially performed with mouse iPSCs for the possible application in SCI^[30]. A lower percentage of O4⁺ cells was obtained compared to the differentiation from mouse ESCs^[31]. However, the variability of iPSC lines due to different tissues of origin and reprogramming methods may account for the difference^[32]. The differentiation of hiPSCs to oligodendrocytes was performed using two types of hESC-OPC protocols based on PDGF- or EGF-induced differentiation^[20]. The O4⁺ oligodendrocytes were only observed in the EGF-dependent protocol with a low induction efficiency (< 0.01%). Later, the protocol developed by Nistor *et al*^[21] was tested for hiPSC differentiation, and more than 90% of the differentiated cells expressed OPC markers (OLIG2, NG2, and O4), similar to that obtained with hESC-OPCs. The derived OPCs were transplanted into a demyelinated rat model and showed maturation into oligodendrocytes and the ability of remyelination^[33]. An *OLIG* gene targeting protocol was also developed for hiPSCs, providing the possibility of genetic correction of patient-specific hiPSCs for cell therapy^[34]. High-purity (70%-90%) Olig2⁺/Nkx2.2⁺ OPCs were obtained from hiPSCs treated with RA, Shh, FGF-2 and PDGF, and these OPCs were shown to myelinate the brains of myelin-deficient shiverer mice^[7]. Given the progress made for OPC differentiation from hiPSCs, there is an urgent need for a clinical relevant system to generate a large amount of hiPSC-OPCs for drug screening and autologous transplantation. ECM is an important component during OPC differentiation, affecting both the differentiation efficiency and the derived cell function (Table 2). Thus, understanding the cell-ECM interactions

and development of defined ECM substrates are critical steps for future clinical applications^[13].

EFFECTS OF ECM ON OPC DIFFERENTIATION FROM PSCS

For various types of OPC differentiation protocols, replating the neural progenitors on ECM-coated surface is always part of the procedure^[21,26]. The most common ECMs that have been used for OPC differentiation include laminin, fibronectin, alone or with poly-D-lysine, and Matrigel, which comprises mostly of laminin (Table 2). Oligodendrocytes were reported to express the laminin receptor $\alpha 6 \beta 1$ integrin^[25]. Laminin is thus a potent promoter of oligodendrocyte survival and myelination. Direct comparison of various ECM proteins including fibronectin, laminin, and Matrigel was performed on OPCs isolated from embryonic day 15 rat spinal cords. All three ECMs were found to promote OPC survival, proliferation, migration, and maturation as compared to poly-D-lysine^[35]. Recently, another ECM protein, vitronectin, was shown to promote oligodendrocyte differentiation from hESCs by synergistically interacting with Shh protein^[36]. Besides $\alpha 6 \beta 1$, vitronectin receptors $\alpha v \beta 1$, $\alpha v \beta 3$, and $\alpha v \beta 5$ are also differentially expressed at different OPC developmental stages and play an important role in modulating OPC migration, proliferation, and differentiation^[37]. Especially, vitronectin-derived synthetic peptide acrylate surface (VN-PAS), which contains the active binding site of vitronectin, has been shown to support high-purity OPC derivation from hPSCs (Figure 1)^[13]. Compared to Matrigel-coated surface, VN-PAS supported higher NG2 expression with similar expressions of nestin and

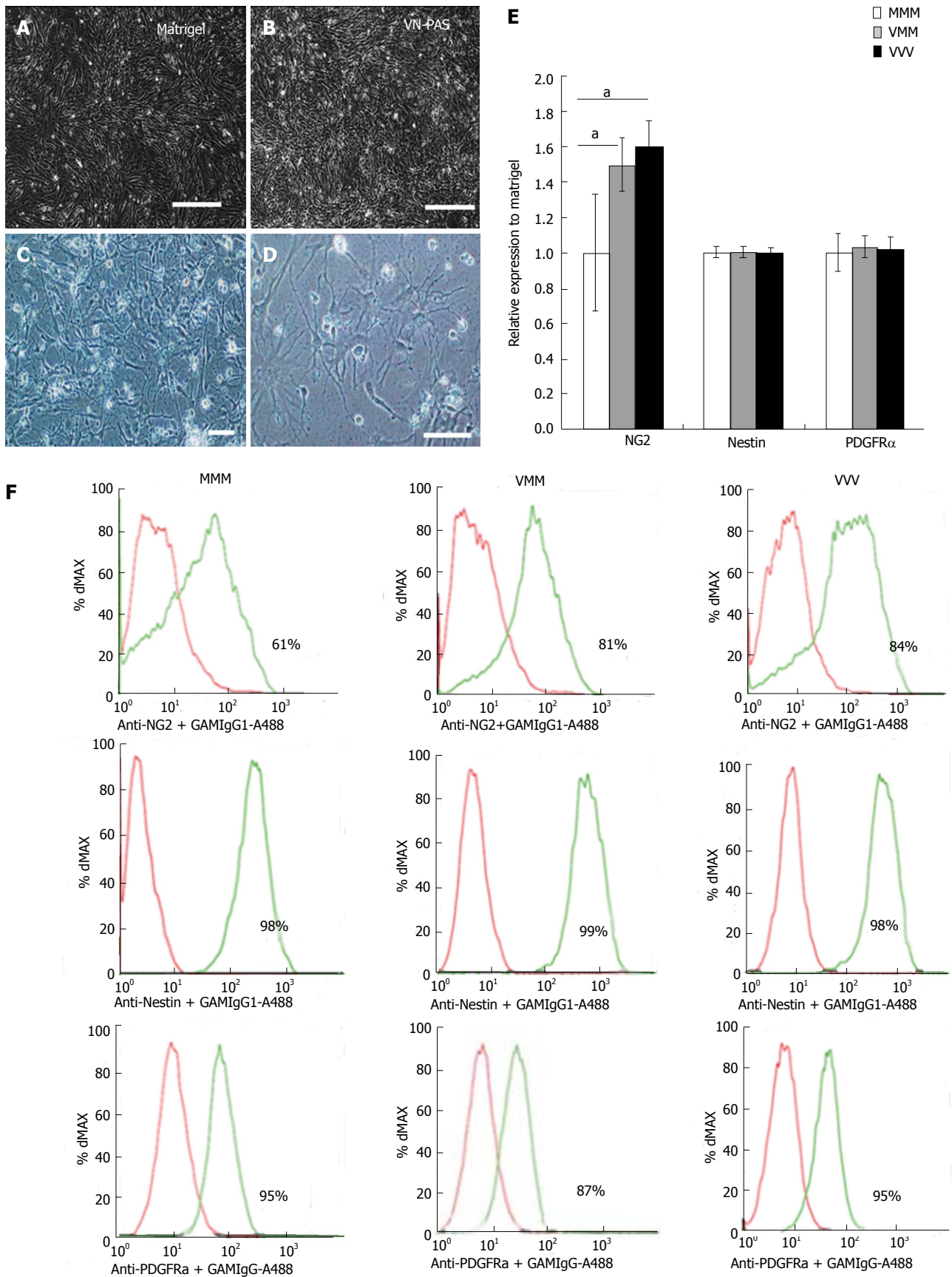


Figure 1 Oligodendrocyte progenitor cells derived from human pluripotent stem cells. A: Morphology of day 41 Oligodendrocyte progenitor cells (OPCs) derived from cells grown on Matrigel; B: Morphology of day 41 OPCs derived from cells grown on vitronectin-derived synthetic peptide acrylate surface (VN-PAS); scale bar: 200 μ m; C and D: Oligodendroglial morphology after OPC maturation; C: Low magnification; D: High magnification, scale bar: 100 μ m; E: OPC marker expression; MMM: all the steps of human pluripotent stem cell (hPSC) expansion and differentiation were performed on Matrigel; MVV: hPSC expansion on Matrigel and differentiation on VN-PAS; VVV: All the steps of hPSC expansion and differentiation were performed on VN-PAS. ^a $P < 0.05$ vs MMM. F: Flow cytometry histograms of OPC markers; PDGFR α : Platelet-derived growth factor receptor alpha. This figure is adapted from Li *et al.*^[13]

Table 2 Effects of extracellular matrices proteins on neural differentiation of pluripotent stem cells

ECM protein	Integrins	Cell source	Neural differentiation	Ref.
Laminin	$\alpha 6\beta 1$	HESC lines: TE03 and TE06	Neuronal generation and neurite outgrowth were significantly greater on laminin and laminin-rich Matrigel than fibronectin, poly-D-lysine, and collagen I	Ma <i>et al</i> ^[16] , 2008
Matrigel (rich in laminin)	$\alpha 3\beta 1$			
Fibronectin	$\alpha 5\beta 1$	Oligodendrocyte progenitor cells from rats	Promoted OPC survival, proliferation, migration, process extension, and OPC purity	Hu <i>et al</i> ^[35] , 2009
Vitronectin	$\alpha v\beta 1, \alpha v\beta 3, \alpha v\beta 5$	hESC lines: Miz-hES4, Miz-hES6	Promoted oligodendrocyte differentiation in the presence of RA/Shh/Noggin	Gil <i>et al</i> ^[36] , 2009
Collagen IV	$\alpha 1\beta 1, \alpha 10\beta 1$	Rabbit neural progenitor cells	Conducive for both neuronal and glial cell differentiation	Raghavan <i>et al</i> ^[61] , 2013
Collagen I	$\alpha 2\beta 1$	Rabbit neural progenitor cells	Conducive for both neuronal and glial cell differentiation	Raghavan <i>et al</i> ^[61] , 2013
Vitronectin-derived synthetic peptide acrylate surface	$\alpha v\beta 5$	hESC line: H1	Promoted oligodendrocyte progenitor differentiation; higher NG2 expression compared to Matrigel	Li <i>et al</i> ^[13] , 2013
Heparan sulfate	Binding heparin	Rabbit neural progenitor cells	Addition of heparan sulfate to collagen mixtures improved neuronal differentiation	Raghavan <i>et al</i> ^[61] , 2013

ECM: Extracellular matrix; hESC: Human embryonic stem cells; RA: Retinoic acid; Shh: Sonic hedgehog; OPC: Oligodendrocyte progenitor cells.

platelet-derived growth factor receptor alpha, demonstrating the active role of ECM-integrin interactions in OPC differentiation. In addition to the single ECM protein, decellularized ECM derived from bone marrow mesenchymal stem cells (MSCs) has also been evaluated for its ability to support neural cell growth^[38]. Compared to poly-D-lysine, MSC-ECM enhanced the differentiation into astrocytes and oligodendrocytes beside neurons, prolonged survival, and better protected the cells from nutrient and growth factor deprivation.

Besides natural ECM proteins, synthetic ECMs have also been developed to better control biochemical and biomechanical microenvironments. Synthetic ECMs such as chitosan and poly (lactic) acid have been used to promote myelination by providing suitable environment to activate Schwann cell function post SCI^[39]. OPCs have been shown to be mechanosensitive; the survival, proliferation, and migration of OPCs in polyacrylamide gels were optimal on intermediate stiffness (0.7-1 kPa) while differentiation efficiency increased with the substrate stiffness^[40]. Chitosan was tested as the substrate for oligodendrocyte differentiation from neural stem cells (NSCs), where stiff surface (> 7 kPa) promoted NSC differentiation into oligodendrocyte while soft surface (< 1 kPa) promoted oligodendrocyte maturation and myelination^[41]. Hybrid-scaffolds combining synthetic ECMs with cell-derived ECMs would be a better strategy as they could provide both biomechanical stability and the large amount of neurotropic factors in treating spinal cord^[42]. ECMs not only modulate the late-stage OPC differentiation, but also provide a cell delivery strategy to enhance the *in vivo* remyelination and tissue regeneration^[42]. However, the effects of ECMs during differentiation of hiPSC into OPCs and using ECM in cell delivery of hiPSC-derived OPCs have not been well studied.

PLURIPOTENT STEM CELL-DERIVED NEURAL PROGENITOR CELLS

NPCs and NSCs are able to differentiate into neurons, astrocytes, and oligodendrocytes, with neuronal lineage

as the dominant population in most cases. Robust neural differentiation has been observed from various hESC and hiPSC lines, although variations among cell lines exist (Table 3)^[12,43,44]. The differentiation of hPSCs into NPCs has been performed either by monolayer induction or by the formation of EBs in suspension, with inducing factors including RA, FGF-2, EGF and Shh, *etc*^[45-49]. Recently, the synergistic induction using two inhibitors of SMAD signaling, noggin and SB431642, resulted in efficient neural differentiation for various hPSC lines^[12,50]. SMADs are intracellular proteins that transduce extracellular signals from TGF- β ligands to the nucleus where they activate downstream gene transcription. The derived neural progenitors demonstrated the ability to further differentiate into dopaminergic neurons, when treated with Shh and FGF8, and motor neurons, when treated with brain-derived neurotrophic factor, ascorbic acid, Shh and RA^[12]. Both monolayer induction and EB formation methods produced high-purity (> 80%) NSCs or NPCs. However, the populations obtained in different studies had different potential to differentiate into mature neuronal types. For example, FGF-2/EGF expanded hiPSC-derived NSCs showed a high tendency to differentiate into γ -aminobutyric acid neurons while RA/FGF-2 induced hESC-derived NPCs differentiated easily into motor neurons^[46,47].

Specific neuronal cell types are required for treating particular neurological diseases. For example, protocols of motor neuron differentiation have been developed by several groups due to their potentials to treat SCI, ALS, and muscular atrophy, *etc*^[11,51]. For the application in treating Alzheimer's disease, the hiPSC-derived neuronal cells were shown to express amyloid precursor protein and capable of secreting A β protein^[52]. To treat stroke-damaged brain, early-stage neural progenitors expressing nestin, Pax6, and Musashi have been used in several studies^[45,53,54]. Human ESC-derived NPCs were transplanted into the cortex rats after permanent distal middle cerebral artery occlusion. Some improvements in sensorimotor functions were observed but more complicated functions were not restored^[45]. HiPSC-derived NPCs have

Table 3 Protocols and *in vivo* studies for neural progenitor cells differentiation from human pluripotent stem cells

Growth factors	Cell source	Cell characteristics	Ref.
EB-based protocol			
FGF-2/RA/ascorbic acid, db-cAMP, HA _g	16 hiPSC lines	13 of the cell lines produced functional motor neurons. Treat with BDNF, GDNF, CNTF to produce motor neurons (4%-15% ISL ⁺ neurons)	Boulting <i>et al</i> ^[43] , 2011
RA/FGF-2	hESC lines: H7, hCSC14, hCSC14-CL1	Produce neuronal progenitors (> 95% nestin and Musashi-1), can become cholinergic neurons, GABA neurons, <i>etc</i>	Nistor <i>et al</i> ^[47] , 2011
Isolated Rosette expanded with EGF/FGF-2	hiPSC lines	Generate long-term expandable neuro-epithelial like stem cells (LT-NES); in stoke model, the cells improved recovery of fine forelimb movements	Oki <i>et al</i> ^[53] , 2012
RA/Shh	hESC lines H1 and H9	To generate motor neurons (50% HB9 ⁺ motor neurons)	Hu <i>et al</i> ^[51] , 2009
Monolayer-based protocol			
Noggin and SB431542	hESC line: H9	> 80% PAX6 ⁺ cells; Shh/FGF8 for midbrain dopamine neurons; BDNF, Shh, RA, ascorbic acid for motor neurons	Chambers <i>et al</i> ^[12] , 2009
	hiPSC lines: iPS-14, iPS-27		
Noggin and SB431542	hESC line: H9	Treat BDNF, GDNF, NT-3 for forebrain neurons which secrete A β for drug screening.	Yahata <i>et al</i> ^[52] , 2011
	hiPSC line: 253G4		
Noggin only; FGF-2 may be added at later stage	hESC line: H1, H7, H9	> 90% nestin, musashi, and PSA-NCAM; For TH neurons, add Shh/FGF8/ascorbic acid; followed by BDNF, GDNF, ascorbic acid and laminin	Gerrard <i>et al</i> ^[48] , 2005
FGF-2	hESC line: SA002 and AS034	> 90% nestin, NCAM; For neuronal lineage, add Shh/FGF8; after differentiation TH ⁺ cells, MAPab ⁺ cells and astrocytes existed	Axell <i>et al</i> ^[49] , 2009

EB: Embryoid body; FGF-2: Fibroblast growth factor-2; RA: Retinoic acid; db-cAMP: Dibutyl- α -cAMP; HA_g: A small molecule agonist of the sonic hedgehog pathway; hESC: Human embryonic stem cell; hiPSC: Human induced pluripotent stem cell; BDNF: Brain-derived neurotrophic factor; GDNF: Glial cell-derived neurotrophic factor; CNTF: Ciliary neurotrophic factor; GABA: Gamma-Aminobutyric acid; Shh: Sonic hedgehog; NT-3: Neurotrophin-3; PSA-NCAM: Polysialylated-neural cell adhesion molecule; TH: Tyrosine hydroxylase; NCAM: Neural cell adhesion molecule.

also been shown to engraft with little neuroblasts or morphologically mature neurons in a rat model^[55,56]. Recently, transplantation of hiPSC-derived NSCs exhibited functional recovery and electrophysiological properties of mature neurons, and was proved to be a safe approach for neuron replacement in stroke-damaged brain^[53]. However, the cell engraftment and *in vivo* maturation are yet to be improved. Transplantations of NPCs derived from hiPSCs for treating other neurological diseases such as ALS and muscular atrophy have also been demonstrated in proof-of-principle studies^[47,57]. The neural progenitors survived and engrafted *in vivo*, and the nestin-positive cells differentiated into neuronal phenotype and motoneuron-like structure in both wild-type rats and the ALS rats harboring a mutated human *SOD1* (*G93A*) gene^[57]. To eliminate the risk of tumorigenicity of the residual undifferentiated hPSCs, intermediate NPC and NSC lines were established from hPSCs, which can be maintained for more than 100 passages^[46]. There are growing interests in functional NPC differentiation from hPSCs to generate neural cells with clinically relevant quality and quantity for preclinical and potential clinical studies^[58]. Current challenges include the functional maturation of NSCs and NPCs both *in vitro* and *in vivo*^[58]. Large-scale generation of a specific neural subtype also remains a major challenge for neuronal differentiation of hPSCs. Recreating the stem cell niches enriched with ECMs is being pursued to address these challenges^[15].

EFFECTS OF ECM ON NPC DIFFERENTIATION FROM PSCS

ECM proteins have been shown to regulate the survival,

proliferation, and neurite outgrowth of hESC-derived NPCs in a dose-dependent manner through integrin-ECM signaling (Table 2)^[16]. Similar to OPCs, NPCs also express integrin $\alpha 6 \beta 1$ and its ligand laminin is a major ECM protein that regulates NPC differentiation. Neuronal generation and neurite outgrowth were significantly greater on laminin and laminin-rich Matrigel substrates than other substrates including fibronectin, poly-D-lysine, and collagen I^[16]. Delivering NPCs in laminin- or fibronectin-based constructs into injured brain showed the improved survival, migration, and behavioral recovery at 8 wk post-transplant^[59]. Endogenous ECMs derived from the RA-treated EBs also accelerated neural differentiation, demonstrating the signaling capacity of ECM environment associated with the lineage commitment^[60]. The native ECMs derived from PSC aggregates had a high content of fibronectin, laminin, collagen IV and vitronectin (Figure 2), which after decellularization can be used as 3-D scaffolds to promote stem cell adhesion, proliferation and differentiation. Such ECM scaffolds contain the balanced composition with the sequestered biological factors which provide the unique signaling to mediate the coordinated cellular events of stem cells. The composition of ECM proteins consisting of laminin, collagen IV, and heparan sulfate was found to regulate the balance of neuronal and glial cell differentiation; the ECM containing a higher portion of laminin and heparan sulfate induced more neuronal differentiation^[61]. Neural differentiation of PSCs is associated with the switch from E-cadherin expression to N-cadherin expression. Hence, recombinant ECM components based on E-cadherin and N-cadherin hybrid substratum were also shown to support neural differentiation of ESCs and iPSCs^[62].

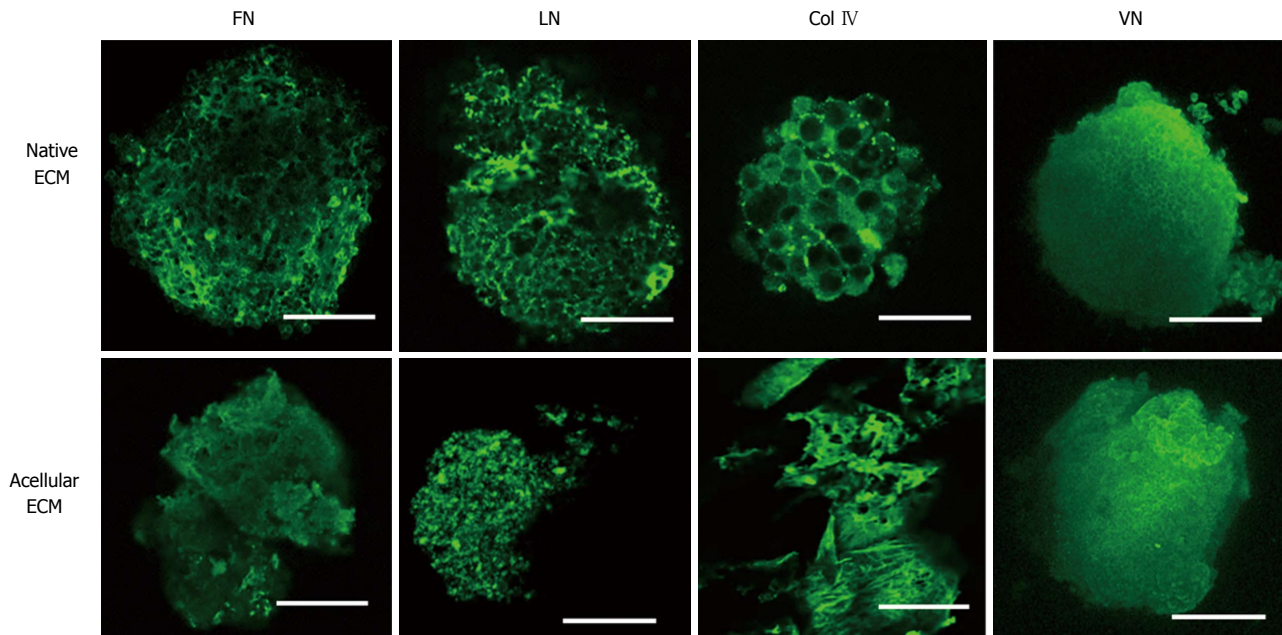


Figure 2 Three-dimensional extracellular matrix scaffolds derived from pluripotent stem cell aggregates. Confocal images of fibronectin (FN), laminin (LN), Collagen IV (Col IV), and vitronectin (VN) expression pre- and post-decellularization [acellular extracellular matrix (ECM) and native ECM, respectively]. Scale bar: 100 μ m. For native Col IV, scale bar: 50 μ m. The ECM scaffolds can be used for neural differentiation. Images are adapted from Sart *et al.*^[60].

Besides the ECM composition, the mechanical property of ECMs such as stiffness also affects neural differentiation. HPSCs are sensitive to biomechanical cues of the microenvironment^[63-65] and respond quickly to stiffness change^[65,66]. For hPSCs, a stiff surface was found to promote cell attachment and proliferation with dense F-actin expression while a soft surface led to cell detachment^[67]. For neural lineage, soft hydrogels (100-500 Pa) promoted neuronal lineage while hard hydrogels (1-10 kPa) promoted glial differentiation^[68-70]. Similarly, soft ECMs with a stiffness similar to that of the neural tissue (100-700 Pa) promoted the generation of early neural ectoderm from hPSCs, while this effect was less pronounced for hard ECMs (7.5 kPa)^[71]. In studies simulating the biomechanical environment in each germ-layer, the scaffolds with high (1.5-6 MPa), intermediate (0.1-1 MPa), and low elastic moduli (< 0.1 MPa) were found to promote mesodermal, endodermal, and ectodermal differentiation of hPSCs, respectively^[66,72]. ECMs may function as force sensors and transduce the biomechanical signals through the ECM-integrin-cytoskeleton pathway^[73]. Therefore, the biomechanical elasticity of ECMs is a potent regulator for neural lineage commitment of hPSCs.

THREE-DIMENSIONAL NEURAL DIFFERENTIATION OF PSCS

Because cells *in vivo* are exposed to a 3-D ECM environment, 3-D neural differentiation in natural or synthetic ECM scaffolds has been studied to mimic the architecture and biological role of the ECM in modulating stem cell fate decision^[17,74]. Different 3-D synthetic ECM scaffolds

including hydrogels, microfibrillar, and nanofibrillar matrices have been used for neural differentiation from PSCs or PSC-derived neural precursors (Table 4)^[75-78]. For example, using chitin-alginate 3-D microfibrillar scaffolds together with RA and noggin, nestin-expressing neural progenitors were derived from three independent hiPSC and hESC lines^[75]. Neuron growth factor-grafted poly(ϵ -caprolactone)-poly(γ -hydroxybutyrate) scaffolds were demonstrated to improve iPSC differentiation into neurons while inhibiting differentiation into other lineages^[79]. In another example of a 3-D synthetic hydrogel-based system, PuraMatrixTM, hESC-derived neuronal cells developed more branched neurite structures and formed more electrically active networks as compared to 2-D differentiation, better resembling the *in vivo* tissues^[76]. Electrospun polyurethane fibrous scaffolds have been shown to preferably differentiate hESCs into the neuronal lineage over the glial lineage^[80]. A 3-D system involving an air-liquid interface was shown to generate a self-organized three-dimensional neural tissue guided by endogenous developmental cues on hydrophilic polytetrafluoroethylene membrane^[81]. Tissue-engineered fibrin scaffolds were developed to enhance PSC-derived NPC survival and direct differentiation into neurons^[82]. All these studies demonstrated that 3-D scaffolds physically influenced neural lineage commitment from PSCs.

The contact guidance and topography effects of 3-D scaffolds on neural differentiation were revealed in several studies recently^[77,83]. The 3-D microfibrillar poly(ethylene terephthalate) (PET) scaffolds have been shown to support neural differentiation of PSCs induced in an astrocyte-conditioned medium^[84,85]. Compared to 2-D differentiation, 3-D differentiation in microfibrillar matri-

Table 4 Three-dimensional natural and synthetic extracellular matrices scaffolds for neural differentiation of pluripotent stem cells

Scaffolds	Cell source	Neural differentiation	Ref.
Poly(lactic-co-glycolic acid) and poly(L-lactic acid) scaffolds	hESC	Enhanced numbers of neural structures and staining of nestin and β -tubulin III were observed	Levenberg <i>et al</i> ^[74] , 2005
Synthetic hydrogel matrix PuraMatrix	hESC-derived neuronal cells	HESC-derived neurons, astrocytes, and oligodendrocytes grew, matured and migrated in hydrogel; neuronal cells had electrically active connections.	Ylä-Outinen <i>et al</i> ^[76] , 2012
Poly(epsilon-caprolactone)-poly(beta-hydroxybutyrate) scaffolds	Mouse iPSCs	Improved iPSCs to differentiate into neurons and inhibited other differentiations.	Kuo <i>et al</i> ^[79] , 2012
Polycaprolactone nanofiber matrices	HESC-derived neural precursors	Aligned fibrous matrices showed higher rate of neuronal differentiation compared to random micro- and nano-fibers (62%-86% <i>vs</i> 27%-32%).	Mahairaki <i>et al</i> ^[77] , 2011
Polyurethane nanofibrous scaffolds	hESC line SA002	Neuronal differentiation was preferred over astrocyte differentiation.	Carlberg <i>et al</i> ^[80] , 2009
Tissue-engineered fibrin scaffolds	Mouse ESC-derived NPCs	Enhanced NPC survival and directed differentiation into neurons.	Johnson <i>et al</i> ^[82] , 2010
PET microfibrous scaffolds	Mouse ESC D3 line	Enhanced neuronal differentiation indicated by nestin, Nurr1, and tyrosine hydroxylase compared to 2-D culture.	Liu <i>et al</i> ^[84,85] , 2013, 2013
Multiwalled carbon nanotube modified PET microfibrous scaffolds	Mouse ESC D3 line	Enhanced neuronal differentiation compared to unmodified scaffolds	Zang <i>et al</i> ^[83] , 2013
Chitin-alginate 3-D microfibrous scaffolds	hESC line: HUES 7 hiPSC lines: PD-iPS5 and hFib2-iPS4	Efficient neuronal differentiation: > 95% nestin ⁺ ; able to mature into neurons (> 90% β -tubulin III ⁺)	Lu <i>et al</i> ^[75] , 2012
3-D ECM scaffolds derived from ESC aggregates	Mouse ESC D3 line	ECM scaffolds derived from RA-treated EBs enhanced nestin and β -tubulin III expressions	Sart <i>et al</i> ^[60] , 2013
Inverted colloidal crystal (ICC) scaffolds containing alginate, poly(gamma-glutamic acid), and surface peptide; or chitin-chitosan-gelatin ICC scaffolds	Mouse iPSCs	Accelerated neuronal differentiation (β -tubulin III expression) of iPSCs.	Kuo <i>et al</i> ^[86,87] , 2013, 2013

hESCs: Human embryonic stem cells; iPSCs: Induced pluripotent stem cells; NPC: Neural progenitor cells; PET: Poly(ethylene terephthalate); ECM: Extracellular matrix; RA: Retinoic acid; EB: Embryoid body.

ces resulted in a higher percentage of nestin-positive cells (68% *vs* 54%) and upregulated the expressions of nestin, Nurr1, and tyrosine hydroxylase. Multiwalled carbon nanotubes (MWCNTs) were used to coat and provide nano-features on the surface of 3-D PET fibers, which significantly enhanced neuronal differentiation of ESCs compared to the surface without MWCNTs (Figure 3)^[83]. Without MWCNTs, cells were flatly spread out on the PET membrane with few neurites formed. In contrast, with MWCNT, more neurons were observed across the surfaces of carbon nanotubes, forming a neural network with extensive neurite bridges between adjacent cells both on 2-D PET membrane and 3-D PET matrices. The 3-D differentiation in PET scaffolds was also demonstrated in stirred bioreactors for potential scale up^[85]. The effects of fiber diameter and fiber orientation of polycaprolactone fiber matrices were evaluated for hESC-derived neural precursors^[77]. The NPCs adhered on the aligned fibers showed a higher rate of neuronal differentiation as compared to cells cultured on random micro- and nano-fibers (62%-86% *vs* 27%-32%). The alginate, poly(-glutamic acid), and surface peptide based inverted colloidal crystal (ICC) scaffolds were shown to provide hexagonal crystals of polystyrene microspheres with interconnected pores, in which topography together with the surface peptide improved the differentiation of iPSCs into neuron cells^[86]. Chitin-chitosan-gelatin scaffolds with ICC geometry were also found to accelerate neuronal differentia-

tion of iPSCs compared to free-form constructs^[87]. The topography with different surface gratings can increase the rate of neural differentiation of hPSCs, although the mechanisms that transduce the topographical signals into cell phenotype remain unknown^[88]. By ingenious design of novel 3-D scaffolds, the neural differentiation from PSCs or the derived NPCs can be promoted.

DRUG SCREENING BASED ON HPSC-DERIVED NEURAL CELLS

Current drug screening methods using immortalized human lines or rodent models cannot accurately represent how various drugs would initiate the response in humans due to the physiological differences between animal and human as well as the lack of native metabolic and biological functions^[89]. Although the sensitivity of human primary cells (*e.g.*, human cardiomyocytes) may give better response, these somatic cells are often limited by the available cell numbers. Estimates indicate that every 1% increase in predictability of toxicity in human would save up to \$100 million in the pharmaceutical industry^[90]. A human cell-based drug screening platform is thus desirable for drug discovery and mechanistic studies of various neurological diseases. Human PSCs, especially iPSCs, provide a great platform to generate allogeneic or patient-specific neural cells that are physiologically relevant for drug screening and disease modeling^[8]. For example, A β -

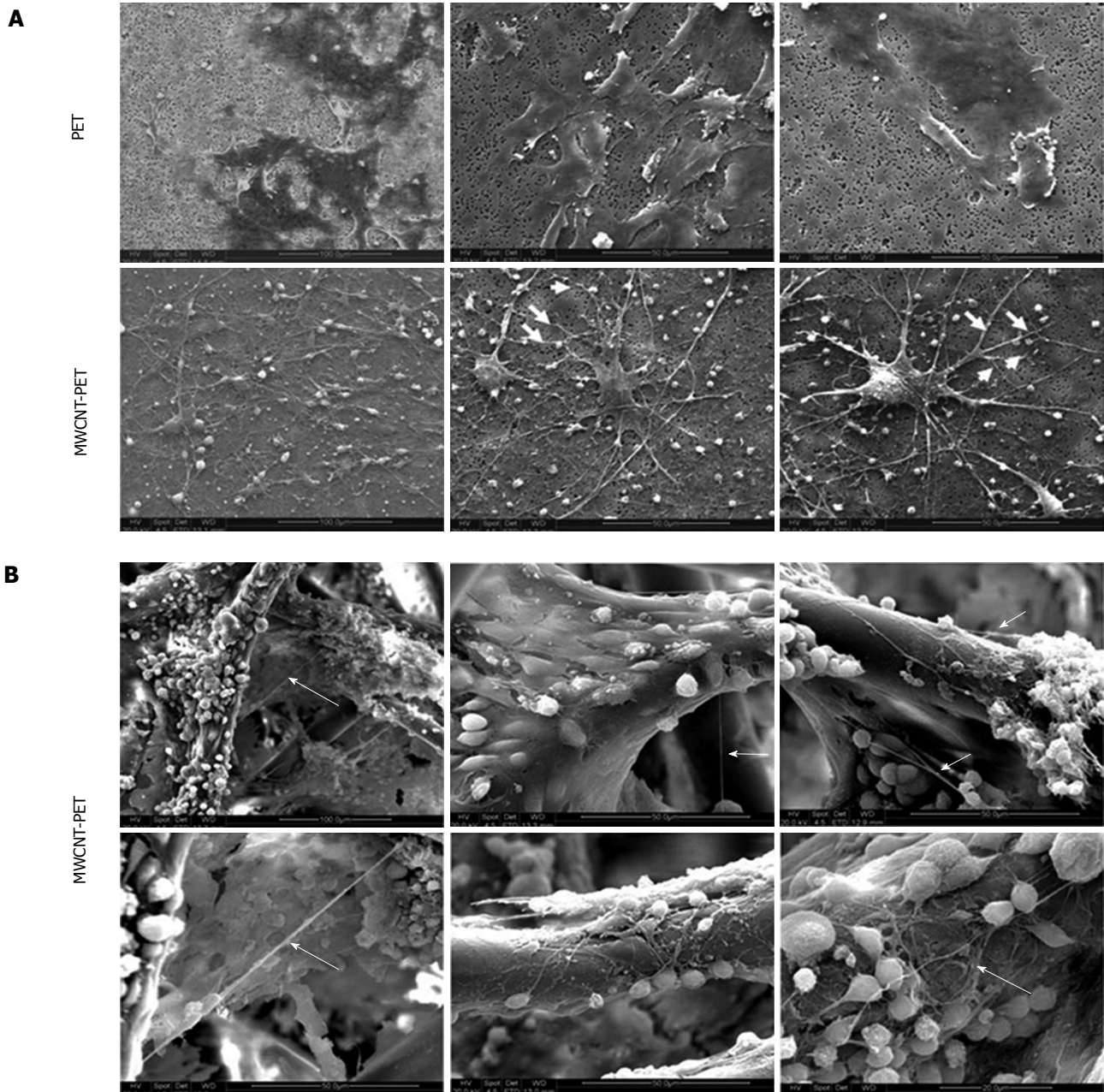


Figure 3 Neural differentiation of pluripotent stem cells. A: Neural cells derived from murine embryonic stem cells (mESCs) cultured on 2-D PET surface with or without multiwalled carbon nanotube (MWCNT) coating; B: Neural cells derived from mESCs cultured in 3-D PET scaffolds with MWCNT coating. Arrows point to neurite fibers. Images are adapted from Zang *et al.*^[83]

secreting neurons were derived from hiPSCs and used for screening anti- $A\beta$ drugs for the treatment of Alzheimer's disease^[52]. β -secretase inhibitor and γ -secretase inhibitor were shown to inhibit $A\beta$ 40 and $A\beta$ 42 secretion from hiPSC-derived neuronal cells. Overexpressing synuclein in hESC-derived dopamine neurons led to the selective cell death; thus drugs interacting with this process or reducing the accumulation of synuclein in cells can be used to treat Parkinson's disease^[91]. Quantitative analysis of neural cells derived from hiPSCs harboring mutations associated with neurodegenerative disorders (*e.g.*, Parkinson's, ALS and schizophrenia) indicated the defects in cell growth, migration, and function compared to healthy

donors^[90]. These disease-relevant cells are more suitable for assessing the outcome of drug treatment. For examples, anti-psychotic drug loxapine has been shown to improve neuronal connectivity in Schizophrenia models established from hiPSCs^[92]. The selective loss of motor neurons derived from iPSCs of spinal muscular atrophy patients was also decreased by treating with drugs such as valproic acid and tobramycin^[91].

High-throughput analysis and high-content imaging platforms need to be developed for efficient screening. Various automated platforms, including IN Cell Analyzer (GE Healthcare), Cellomics Arrayscan (ThermoFisher), and ImageXpress (Molecular Devices), have been devel-

oped to collect information about cell physiology and function, including cell viability and apoptosis, cell number and proliferation, cell migration *etc*^[90]. 3-D culture conditions are necessary to recreate the phenotype better representing *in vivo* neural tissues. The main challenge of hPSC-based drug screening is that the cells generated from hPSCs are developmentally immature^[91]. Thus, functional maturation of hPSC-derived cells is being actively pursued in the field. Compared to 2-D platform of drug screening, 3-D ECM scaffold-based screening has been shown to be more predictive in terms of cell sensitivity to the drugs^[93]. Hence, efficient 3-D neural differentiation systems that can enhance neural cell functions are in a great demand. High-throughput electrophysiology is also a critical component in drug screening because it can provide functional readouts during the screening. Therefore, the pharmaceutical industry is developing the platform such as PatchXpress to assess the effect of ion channel modulators. Given the challenges in cell therapy and transplantation, disease modeling and drug screening have been considered as two immediate applications of hPSCs.

CONCLUSION

Neural cells (including oligodendrocyte progenitors and neural progenitors) derived from hPSCs have great potential in drug screening, disease modeling, and regenerative medicine. High-purity neural cells can be derived from hPSCs induced by various biological and biochemical cues. Natural and synthetic ECMs, including their composition, mechanical properties, and physical structures play important roles in regulating cell survival, proliferation, migration, and differentiation. Therefore, there is an urgent need to optimize ECMs for efficient neural differentiation and functional maturation, especially 3-D ECM scaffolds, which can interact with other niche factors (*e.g.*, cytokines, accessory cells and nutrients) and provide the physiologically relevant microenvironment to guide neural tissue development. Understanding the biochemical and biomechanical interactions of hPSCs and the ECMs should accelerate the applications of hPSCs, especially in the immediate applications in drug screening.

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Familial Alzheimer's disease modelling using induced pluripotent stem cell technology

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disease in which patients exhibit gradual loss of memory that impairs their ability to learn or carry out daily tasks. Diagnosis of AD is difficult, particularly in early stages of the disease, and largely consists of cognitive assessments, with only one in four patients being correctly diagnosed. Development of novel therapeutics for the treatment of AD has proved to be a lengthy, costly and relatively unproductive process with attrition rates of > 90%. As a result, there are no cures for AD and few treatment options available for patients. Therefore, there is a pressing need for drug discovery platforms that can accurately and reproducibly mimic the AD phenotype and be amenable to high content screening applications. Here, we discuss the use of induced pluripotent stem cells (iPSCs), which can be derived from adult cells, as a method of recapitulation of AD phenotype *in vitro*. We assess their potential use in high content screening assays and the barriers that exist to realising their full potential in predictive efficacy,

toxicology and disease modelling. At present, a number of limitations need to be addressed before the use of iPSC technology can be fully realised in AD therapeutic applications. However, whilst the use of AD-derived iPSCs in drug discovery remains a fledgling field, it is one with immense potential that is likely to reach fruition within the next few years.

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Key words: Human induced pluripotent stem cells; Alzheimer's disease; Neurodegenerative diseases; High-throughput screening assays; Cholinergic neurons; Drug discovery; Stratified medicine

Core tip: Alzheimer's disease (AD) affects 36 million people worldwide and is set to double by 2030. Progress in understanding AD has been hindered by a lack of suitable *in vitro* and *in vivo* models reflected in > 90% drug attrition rates. Induced pluripotent stem cells are an alternative source of neural cells that can be derived from patients' somatic cells and exhibit AD pathophysiological phenotypes. These cells are amenable to HTS formats required for drug discovery applications. Harnessing this combined potential would provide an unprecedented opportunity to significantly reduce timeframes and costs associated with developing novel therapeutics, ultimately improving patient outcomes.

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INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease in which patients exhibit gradual loss of

memory that impairs their ability to learn or carry out daily tasks. The classic, post-mortem neuropathology exhibited in AD largely consists of amyloid plaques and neurofibrillary tangles^[1], however, there is significant controversy within the field as to the causative mechanism(s). Worldwide nearly 36 million people have AD or related dementia, with a reported 7.7 million new dementia sufferers worldwide per year. The global cost of neurodegenerative diseases was over United States \$600 billion in 2010 and affects people in all countries, with 58% living in low- and middle-income countries^[2]. In the United Kingdom alone, specific neurodegenerative diseases (including AD and Parkinson's disease), have a combined patient population in excess of 800000 and the cost for provision of care was an estimated £23bn in 2012^[3].

Diagnosis of AD is difficult, particularly in early stages of the disease, and largely consists of cognitive assessments, with only one in four patients being correctly diagnosed^[2]. Lack of knowledge of disease pathology is a major disadvantage in diagnosis and prescribing treatments since drug regimens are not the same for all dementias or patients. Moreover, development of a successful drug for the treatment of AD has, as yet, eluded pharmaceutical companies as current medicines treat only symptoms and not the cause(s) of AD. For example, in just over a decade there have been over 100 failed medicines for treatment of AD, including recent late stage failures of solanezumab and bapineuzumab with just five approved medications available to treat the symptoms of various stages of AD (three in United Kingdom). Therefore, a failure in pre-clinical to clinical development exists and can be attributed to several key factors; existing animal models or cellular models are inadequate, insufficient knowledge of drug action on human physiology and a lack of pharmacologically relevant biomarkers. Consequently, there is a pressing need for technologies that can provide definitive assays that can confirm disease pathology as well as predict novel or optimal drug regimens.

Since the creation of induced pluripotent stem cells (iPSCs) from human adult somatic cells in 2007^[4], the potential applications of stem cells in regenerative medicine are considerable. Human pluripotent stem cells (that include iPSCs and embryonic stem cells) are self-renewing, which permits them to be grown indefinitely, and retain the potential to give rise to all cell types of the body. iPSCs are an ideal alternative cell source as they can be derived (reprogrammed) from somatic cells from any individual and are genetically identical to the donor, making them invaluable for use in cell-based models for human disease (Figure 1). Reprogramming of somatic cells is a highly inefficient and lengthy methodology and, as such, certain parameters should be considered when making disease specific iPSCs. These include; source of somatic cells (*e.g.*, dermal fibroblast, blood cells), method of cellular reprogramming (*e.g.*, retroviral, episomal) and

the robustness of differentiation protocols for mature cell types. Here, we focus on AD-specific iPSCs and their derivatives to illustrate how they might be used in various applications in regenerative medicine. For a detailed overview of reprogramming, we refer the reader to another review^[5].

Crucially, previous research demonstrates that iPSC-derived neural cells harvested from individuals suffering from a range of neurodegenerative disorders exhibit similar abnormal disease characteristics *in vitro*^[6-9]. This observation presents an invaluable opportunity for the use of diseased cell lines in *in vitro* studies to further our understanding of disease modelling, early toxicity screening and in the development of novel therapeutics. Performance of a literature search using the NCBI database, PubMed, under specific search terms [disease modeling AND ips cells NOT "review" (Publication Type)] in original research publications reveals that the field of disease modelling using iPSCs has increased at a substantial rate since the creation of iPSCs in 2007 (Figure 2). A year-on-year increase in the number of publications from 2009 ($n = 20$) to 2011 ($n = 114$) is observed, however, in 2012 this trend appeared to slow. In 2013, a reduction in papers is recorded ($n = 52$) which could indicate that the field is maturing, whereby the initial raft of papers reflected high impact method-based publications (*i.e.*, the production of diseased iPSCs), whereas current work is focussed on disease modelling and drug discovery, which are lengthy studies. The number of original research articles containing iPSCs for disease modelling of AD patients was very small and there are only 8 research papers that have utilised AD-derived iPSCs between 2011-2013. This demonstrates that the use of iPSCs to model AD is still in its infancy and may reflect the difficulty of isolation of these cells and identification of appropriate donor patients. This review will discuss the pathology and cellular targets of AD, how we can utilise iPSCs as a model to investigate AD, applications and limitations of these cells in high throughput analyses and future opportunities in personalised medicine.

DISEASE PATHOLOGY

AD can be divided into familial or sporadic genetic events with early- or late-onset. Whilst the majority of AD cases manifest as late-onset sporadic form, familial cases present a unique opportunity to investigate the inheritance of genes contributing a higher risk of AD. The familial form of AD is associated with mutations in amyloid precursor protein (APP), presenilin-1 and presenilin-2. Risk of AD is also observed to be increased where mutations in apolipoprotein E4 (APOE4) or triggering receptor expressed on myeloid cells 2 (TREM2) are present. Genes associated with the pathology of AD include APP, which results in β -amyloid plaques (A β), and microtubule associated protein Tau (MAPT),

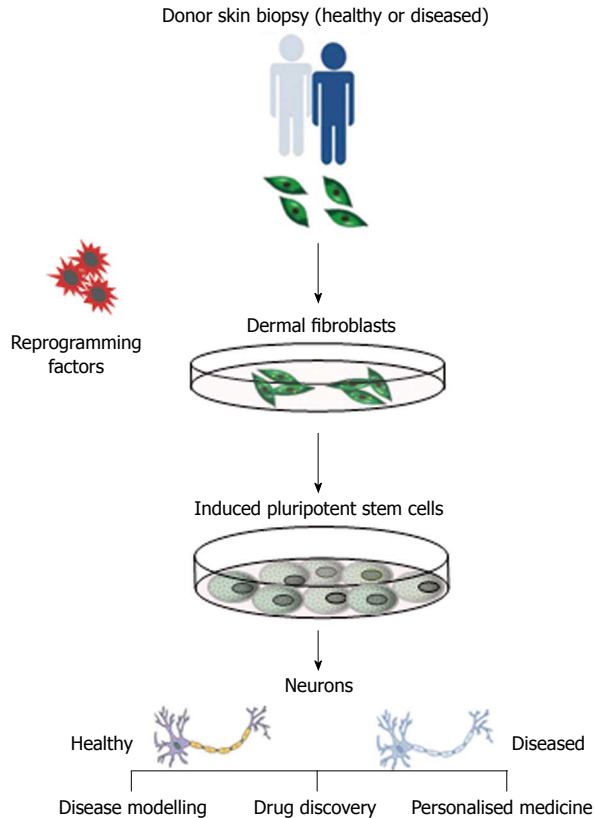


Figure 1 Isolation of disease specific induced pluripotent stem cells. Reprogramming of dermal fibroblasts from patients with Alzheimer's disease into induced pluripotent stem cells provides an infinite source of cells to apply directed differentiation protocols to generate disease-specific neurons that exhibit phenotypic disease traits. This presents a unique opportunity to utilise these cells in the exploitation of drug discovery, disease modelling and personalised medicine.

which results in hyperphosphorylated tau aggregates (tau tangles) within neurons of AD patients^[10]. Despite tau tangles being identified as a pathological feature of AD, mutations in this gene are unusual in such patients. AD is characterized by extracellular amyloid deposition, intracellular neurofibrillary tangle formation, and neuronal loss. Below, we discuss the contribution of these genes to the pathology of AD. Other confounding factors in AD include oxidative stress, mitochondrial function, inflammation and microglia function.

Amyloid precursor protein

A significant pathological feature of AD is the presence of extracellular plaques in the brain comprised of β -amyloid ($A\beta$) peptides derived from the amyloid precursor protein^[11,12]. APP is located on chromosome 21 in humans and is associated with dementia in Down syndrome patients, who exhibit a triplication of this chromosome (trisomy 21). Whilst APP in AD has been studied in significant detail, the events leading to $A\beta$ deposition are less well defined and likely to involve stimulation of APP expression *via* the neuroinflammation-promoting cytokines IL-1 and S100B^[12]. Drugs developed to target $A\beta$ deposits for the treatment of AD

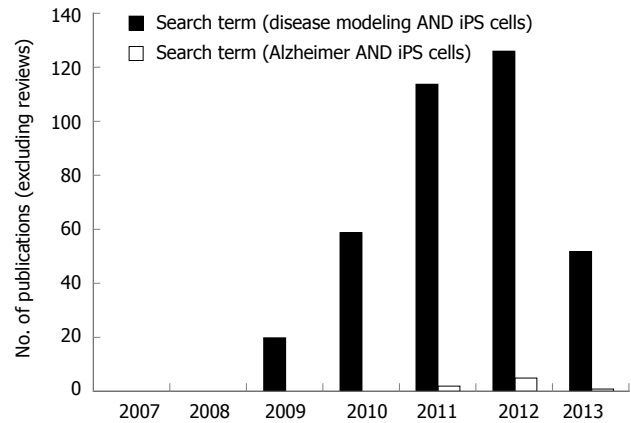


Figure 2 Publication statistics on original research papers using disease specific induced pluripotent stem cells between 2007 and 2013. Analysis of the search terms [disease modeling AND ips cells NOT "review" (Publication Type)] (blue bars) and (Alzheimer AND ips cells) (red bars) for research papers published on NCBI database (PubMed) between 2007 and 2013.

have proved relatively unsuccessful. This may be due to the fact that overexpression of APP is associated with other events, such as glial activation, suggesting that the deposition of $A\beta$ is associated with, rather than being a causal factor of, AD. As such, APP is now generally disparaged as a drug target for AD treatment with hyperphosphorylated tau aggregates now being a major focus.

Microtubule Associated Protein Tau

The Microtubule Associated Protein Tau (MAPT; Tau) functions to assemble and stabilize microtubules within neurons, playing an important part in regulation of neuronal polarity, axonal transport and neurite outgrowth^[10]. Phosphorylation of Tau allows regulation of binding and stability within neurons and aberrant phosphorylation or dephosphorylation in specific residues of the Tau protein lead to pathology, collectively known as tauopathies. The main component of the protein aggregates found in tauopathies is hyperphosphorylated tau protein within neurons. Although the exact mechanisms are unclear, the neurofibrillary tangles (NFT) associated with tauopathies may also involve conformational changes in Tau protein. Whilst tau in NFT forms the basis for pathology of tauopathies it has been suggested that tau oligomers act as a toxic species by providing a template for the misfolding of native tau and spreading from cell to cell leading to propagation of the disease^[13]. Research is now focused on the targeting of Tau oligomers for drug therapies for the treatment of AD.

Apolipoprotein E4

Apolipoprotein E consists of 3 isoforms of which apoE4 is a genetic risk factor for late-onset familial and sporadic forms of AD and is also associated with dementia in DS, Huntington's disease, vascular dementia and cerebrovascular disease^[14]. APOE4 exhibits multifunctionality in lipid and lipoprotein transport systems, mainly in the metabolism of dietary lipids^[15]. Carriers

of polymorphic variants of APOE4 are between 4- and 10-times more likely to exhibit late onset AD. In the CNS, APOE4 is produced by glial cells and interacts with receptors of the low-density lipoprotein family. APOE4 binds to A β peptide and onset of AD is likely to reflect the inability of APOE4 to aggregate and clear A β in the brain, although other factors such as the effect of APOE4 on synaptic plasticity, lipid transport, neuroinflammation may also account for this^[16]. Since the APOE4 isoform can be assessed prior to onset of neurodegeneration it is considered a promising target for drug therapy^[17].

Presenilin-1 and -2

Presenilin-1 (PSEN1) and PSEN2 are major components of the atypical aspartyl protease complex that is required for γ -secretase complex activity and cleavage of APP. Mutations in PSEN1 are the major cause of early onset AD and also account for the most severe forms of the disease^[18]. Early onset AD in PSEN1 mutation carriers can occur as early as 30 years of age, although the mean age of onset is over 58 years. More than 180 mutations have been described in PSEN1, of which the majority are missense mutations^[18]. PSEN2 mutations are less common and 14 specific mutations have been associated with AD^[19]. Mutations within the PSEN proteins affect APP synthesis and proteolysis leading to an increase in the ratio of A β 42 peptide compared to A β 40, the former a more toxic form of A β peptide that is more prone to oligomerisation and fibril formation^[19,20]. Drug treatments have focussed on γ -secretase modulators capable of decreasing the ratio of A β 42 to A β 40 peptides^[21].

Triggering Receptor Expressed on Myeloid Cells 2

Variants in Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) have been identified that triple the risk of developing late onset AD^[22]. TREM2 is a cell surface receptor, which triggers activation of the immune response in association with DAP12^[23]. In the CNS, TREM2 is expressed by microglial cells and functions to activate phagocytosis in these cells and to suppress neuroinflammation and cytokine production^[22]. Several functions of TREM2 include aiding clearance of A β and synapse remodelling. Whilst the exact mechanism of TREM2 in late onset AD is unclear it is likely that mutations in this gene contribute to disease pathogenesis *via* insufficient clearance of A β and increased localised inflammation.

AD MODELLING USING HUMAN IPSCS

The single most important factor in the utility of iPSCs in AD modelling, is that mature cell type(s) affected by the disease, *e.g.*, neurons, exhibit phenotypic characteristics of the disease. Numerous studies have demonstrated that iPSCs can be used to model genetic diseases by showing that cells affected by the disease recapitulate

these traits *in vitro*. iPSC AD modeling is still in its infancy and only a few studies have demonstrated successful generation and characterization of AD patient-derived neurons (Figure 2). Five out of eight publications reported isolation of iPSC-derived neurons from patients with familial AD, however a key development in the field showed that reprogramming could similarly be used to recapitulate patient specific phenotypes *in vitro* of sporadic forms of the disease^[6,24,25]. iPSC-derived neurons generated from familial AD patients with mutation of the APP gene and sporadic AD showed, relative to non-demented controls, elevated levels of A β , phosphorylated tau and glycogen synthase kinase 3B^[6].

A known pathology of AD progression is significant neurodegeneration in the cortical regions, with all regions of the brain registering degenerative changes as the disease progresses. Initial reports using iPSC-derived neurons from patients with familial AD utilised heterogeneous neuron populations^[6,8]. Although results demonstrated an increase in A β 42 secretion from mutant PSEN1, PSEN2 and APP iPSC-derived neurons compared to control cells both studies observed inconsistencies in Tau expression. For example, no Tau expression or tangles were observed in the Yagi *et al*^[8] study, whereas increased levels of phosphorylated Tau were observed in both familial AD-derived neurons and one of the two sporadic AD-derived neurons compared to non-demented control neurons in the Israel *et al*^[6] study. In addition, a recent paper reported increased levels of intracellular neuron specific amyloid aggregates in cells derived from familial (APP-E693 Δ) and one of two sporadic AD derived neurons^[24]. These disparities may reflect the disparate differentiation periods used in the studies and differences in the proportion of cholinergic neurons within the populations. However, it is also possible that these differences reflect inherent variability of iPSCs, which is discussed further below.

In a seminal study, iPSCs derived from patients with Down's Syndrome (a model for early onset AD) were used to generate, highly enriched populations of cholinergic neurons in significant numbers. Following differentiation times of 28-100 d following neural induction of iPSCs, analysis of these cells showed production of neuron specific A β secretion, amyloid aggregate formation and altered Tau protein localisation and phosphorylation^[26,27]. Another key finding from this report (and others) demonstrates that early AD pathologies, such as the formation of A β 42 aggregates, occur in relatively short culture periods *in vitro* opposed to years *in vivo*. Furthermore, iPSC-derived neurons are able to respond functionally to various modulators highlighting their potential use in validation and identification in drug discovery^[8,25].

LIMITATIONS OF IPSCS AS MODELS OF DISEASE

At present, a number of limitations need to be ad-

dressed before the full potential of iPSC technology in predictive efficacy, toxicology and disease modelling can be realised. Human iPSCs are effectively man-made cells that are similar to embryonic stem cells, which themselves only exist *in vivo* for a matter of days. These nuances may be reflected in the challenges faced in the differentiation of pluripotent stem cells into mature cell derivatives, despite a good understanding of the molecular mechanisms that occur during development. In order to fully exploit opportunities in disease modelling, but in particular in HTS formats, robust, efficient and cost-effective methods are fundamental. Differentiation protocols that require cocktails of growth factors are costly and are susceptible to significant batch-batch variation, however, alternative methods to acquire differentiated phenotypes are being explored, such as the use of more cost effective small molecules^[28].

A significant research focus in the pluripotent stem cell field has been the development of robust differentiation protocols to enrich for specific mature cell types and populations. However, homogenous cell populations are difficult to obtain in practice and are unlikely to reflect the true pathophysiology of the disease. In addition, modelling complex, idiopathic diseases such as AD, likely requires exposing the cells to biological, chemical or environmental factors to reveal pathophysiological phenotypes. For example, Israel *et al.*^[6] demonstrated a favorably enriched neuron population (90%), however since neurons and synapses are largely dependent upon endocytic activity they found it necessary to co-culture with astrocytes.

In addition, it has been shown by hierarchical cluster analysis that AD-derived neurons are akin to fetal neurons and, therefore, not fully mature^[6]. Although, this is considered one of the major hurdles to overcome in modelling degenerative diseases, the recapitulation of a fetal phenotype presents an opportunity to isolate specific progenitors, which can be used to study developmental aberrations in congenital/developmental disorders. Conversely, for the study of late-stage onset diseases, such as sporadic AD, adult disease phenotypes might not be exhibited under standard differentiation conditions. As such, further work is necessary to identify appropriate differentiation methods for the derivation of adult neurons *in vitro*.

An advantage with the use of patient specific iPSCs means that each iPSC-derived cell reflects this genetic variation. Despite this being a clear advantage in the toxicological evaluation of patient populations to novel therapeutics, conclusions from studies using iPSCs from donors with different genetic backgrounds may be problematic. For example, are any phenotypic differences observed due to the mutation of interest or the genetic background of the patients? At present, parameters such as gender-, age- and ethnicity-matching are used in the selection of control donors, however, genome-wide studies show that each person has single nucleotide polymorphisms that may have disease relevance.

Therefore, a fundamental feature in the use of iPSCs in regenerative applications is careful consideration of appropriate control patients. A further aspect to consider is the reprogramming event required to derive iPSCs from donors. It is well known that epigenetic variations can, and often do, occur during the reprogramming stage of iPSC derivation. Therefore, iPSC clones must be fully characterised prior to use in therapeutic analysis.

HIGH THROUGHPUT SCREENING OF NOVEL THERAPEUTICS FOR AD: *IN VITRO* CLINICAL TRIALS

Development of novel therapeutics for treatment of disease is a lengthy and costly process with extremely high attrition rates of > 90%, in particular, CNS therapeutics exhibit one of the lowest success rates^[29]. Current practices involve evaluation of the safety and efficacy of new drugs in animal and *in vitro* models of relevant tissues and biological processes. Existing *in vitro* cell models attempt to recapitulate core pathologies or targets of AD. For example, Georgievska *et al.*^[30] recently described inhibition of Tau phosphorylation in response to AZD1080, an inhibitor of Glycogen synthase kinase-3 β , using a mouse 3T3 fibroblast cell line transfected with human Tau. Stable over expression of Tau has also been achieved in the human SH-SY5Y neuroblastoma cell line^[31], similarly, over expression of APP695wt in the SH-SY5Y cell line was used to determine A β 40 secretion in response to AZD3839 in pre-clinical studies^[32]. The use of animal cells, however, lacks human context and the cancer-derived SH-SY5Y cell line may not accurately reflect the cellular processes associated with AD. A recent paper highlighted the importance of the endoplasmic reticulum (ER) in protein catalysis and correlated the presence of amyloid- β plaques with age-related diminished ER function. The author went on to call for better drug discovery cell models which enable enhancement of ER function to be detected through embedding fluorescent reporter proteins within an exon of a target gene^[16]. In short, these methods of target validation focus on the recapitulation of only one key feature of AD in an often-irrelevant cell line, failing to account for other components of the signalling pathway. Primary neurons offer more relevant pre-clinical cell models and are capable of synapse formation, but are costly, difficult to transfect and are typically animal derived^[33]. Transgenic animal models and cell lines have undoubtedly aided our knowledge of AD mechanisms and predictive pharmacology, however, these are hindered by inter-species differences and lack of clinical relevance and genetic heterogeneity, which has resulted in poor clinical translation.

The derivation of iPSCs from patients with AD would, however, enable the applicable recapitulation of AD phenotype in a dish, since iPSCs retain the patient's genotype. Circumventing cross species differences and

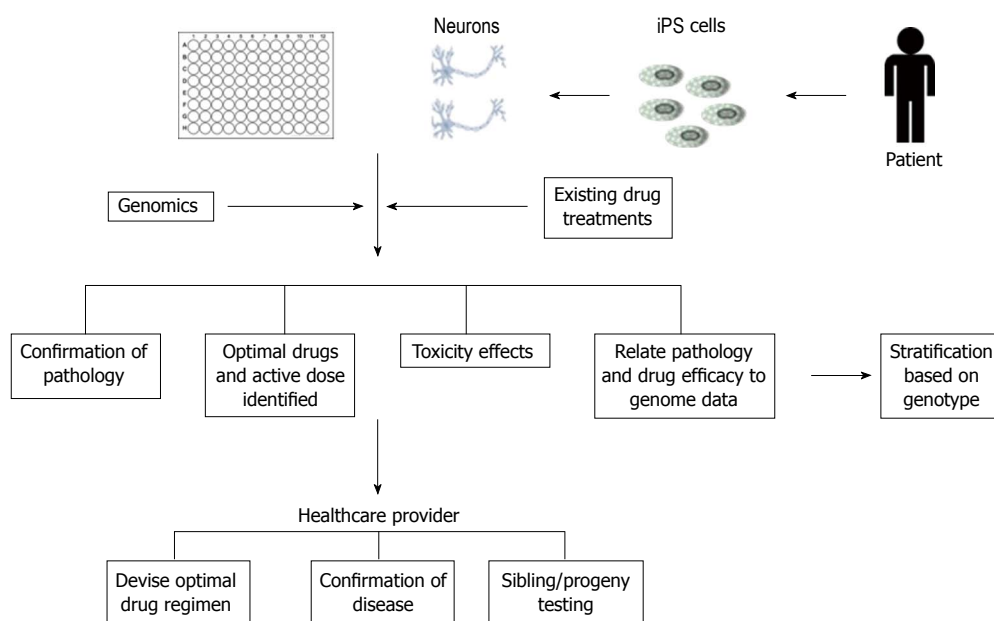


Figure 3 Example of some of the inputs and outputs in Alzheimer's disease high content screening applications. iPS: Induced pluripotent stem.

negating any ethical constraints associated with the use of human embryonic stem cells would create increased translational value. Indeed, neurons derived from disease specific iPSCs have been used to validate the potency of candidate drugs in the treatment of neurological pathologies^[34]. Of further importance, studies have shown treatment of AD iPSC-derived neurons with β -secretase inhibitors, but not γ -secretase inhibitors, causes significant reductions in phosphorylated Tau expression and GSK-3 β levels^[6,8,25]. The accessibility of iPSCs allows many compounds to be tested simultaneously, reflecting a real-life scenario of patients taking a variety of prescription and non-prescription drugs.

Harnessing this potential could provide an unprecedented opportunity to improve preclinical predictions by allowing therapeutics to be tested in multiple cell lines derived from a cohort of patients^[35]. This may also allow the repositioning, reprofiling or repurposing of old, failed and existing drugs. The use of patient-derived iPSCs could be highly amenable to high throughput screening (HTS) practices using multi-well formats to enable rapid analysis of thousands of compounds. Early identification of toxic or inefficacious compounds would, thus, prevent expensive animal studies and subsequent clinical failures. Traditional HTS techniques have focussed on biochemical assays measuring enzyme activity and protein interactions using absorbance, luminescence or fluorescence based readings. For example, Haugabook *et al*^[36] describe the use of a sandwich ELISA (in 96-well formats) to detect aggregation of amyloid plaques, a key contributor to the formation of senile plaques in AD. HTS assays have also been developed to enable detection of A β 42 aggregation using a GFP fusion construct expressed in *E. Coli*, in which compound inhibition of A β 42 aggregation resulted in the emission

of a fluorescent signal^[37,38]. As a result of these methods often lacking cellular context, high content screening (HCS) in whole cells has been recognised as a powerful tool for drug discovery and has been adopted largely by the pharmaceutical industry due to the large volume of multiparametric data that can be obtained^[39]. HCS encompasses the automated acquisition of fluorescent images and image analysis using mathematical algorithms to extract and quantify phenotypic information, including signal shape, intensity and cellular localisation, which can be statistically analysed^[40]. To increase throughput and reduce human error, additional processes such as compound storage, dosing and immunofluorescent staining can also be automated. The principle of HCS in neuronal cultures has already been demonstrated^[41-43]. Neurite loss is one of the core pathologies of AD and application of HCS to quantify neuronal outgrowth has already been achieved and proven to be faster than traditional manual tracing methods^[41,43]. Assessment of chemical toxicity has also been demonstrated by HCS in three neuronal cell lines, whereby proliferation was detected by BrdU incorporation (an indicator of actively proliferating cells) and cell counts were obtained with Hoechst 33342 nuclear dye in a 96-well plate format^[44]. HCS has applications in additional areas of neuroscience including neurogenesis, cell signalling and inclusion formation as reviewed by Dragunow^[45]. An example of HCS applications in AD therapeutics is shown in Figure 3.

Overall, powerful high-throughput and -content screening assays are in place that can be applied to multiple areas of drug discovery, but clinical success is hindered by a lack of relevant cell models in the pre-clinical stages. High throughput toxicity screening using human iPSC-derived cardiomyocytes has been reported

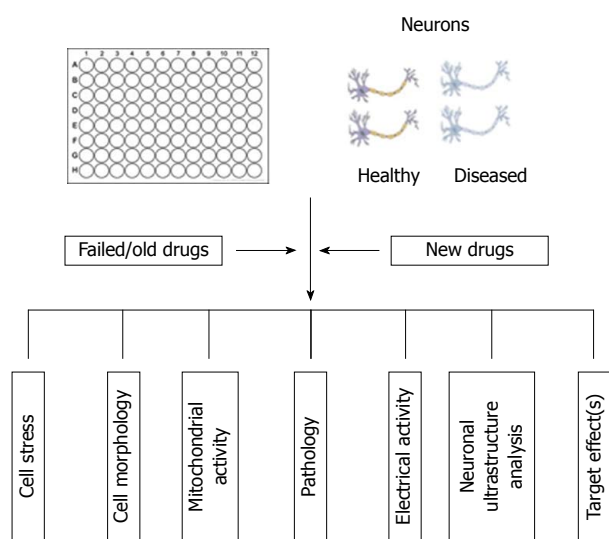


Figure 4 Example of how high content screening of patient-derived induced pluripotent stem cells could aid stratification of existing drug treatments and help identify genetic profiles associated with specific disease phenotypes.

using electrode sensors to acquire oscillating impedance measurements to detect the contraction and relaxation or beating of iPSC-derived cardiomyocytes in a 96-well plate format^[46]. Arrhythmia data obtained from iPSC-derived cardiomyocytes treated with cardiac modulators was qualitatively comparable to results obtained from more traditional, low throughput microelectrode arrays in parallel experiments. Therefore, the potential use of iPSC technology in high throughput drug discovery has been demonstrated but to date has not been described in the literature for iPSC-derived neurons. The UK Government and pharmaceutical industry have recognised the potential for iPSC AD models in HCS and by late 2013 several calls for funding such technology have been announced. As a result, we expect to see significant activity in this field and the development of HCS platforms for AD.

FUTURE PERSPECTIVES: PERSONALISED MEDICINE

The potential to use patient-specific cells to generate pluripotent cells, which can be maintained indefinitely and subsequently differentiated into desired cell types, presents a real opportunity for stratified (personalised) medicine applications (Figure 4). For example, this will allow scientists and clinicians to model, *in vitro*, the progression of AD (or other degenerative diseases) for each individual patient, perform “customised” pharmacologic screening to determine the optimal therapeutic regimes and implement genomic testing of large cohorts of patients, representing different ethnic/genetic backgrounds in order to inform pharma of susceptible populations. There is a clear unmet drug need for the treatment of AD and the utility of iPSC technology will provide a

more efficacious model to reassess (or rescue) former drug candidates that either have been withdrawn from use or aborted at a late stage of development for safety reasons. In short, the use of disease specific iPSC derived neural cells, in conjunction with high throughput/content screening methods, offer improved clinically relevant cell models that will significantly reduce time-frames and costs associated with the development of novel therapeutics, ultimately improving the number of new medicines to the market to treat patients with neurodegenerative diseases.

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Human induced pluripotent stem cells: A new source for brown and white adipocytes

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ate white and brown adipocytes and we discussed their therapeutic capacities for obesity and lipodystrophy diseases. Then, we described the main approaches to derive human induced pluripotent stem cells-mesenchymal stem cells (hiPSC-MSCs). Finally, we underlined the low adipogenic capacity of hiPSC-MSCs compared to adult-MSCs and proposed several hypothesis to explain this feature.

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Abstract

Mesenchymal stem cells (MSCs) derived from human induced pluripotent stem cells (hiPSCs) provide a novel source for generating adipocytes, thus opening new avenues for fundamental research and clinical medicine. We present the adipogenic potential of hiPSCs and the various methods to derive hiPSC-MSCs. We discuss the main characteristic of hiPSC-MSCs, which is their low adipogenic capacity as compared to adult-MSCs. Finally, we propose several hypotheses to explanation this feature, underlying a potential critical role of the micro-environment. We favour the hypothesis that the range of factors or culture conditions required to induce adipocyte differentiation of MSCs derived from adult tissues and from embryonic-like cells could differ.

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Key words: Human induced pluripotent stem cells; Mesenchymal stem cells; Brown adipocytes; White adipocytes; Obesity; Lipodystrophy

Core tip: In this mini-review, we summarized the potential of human induced pluripotent stem cells to gener-

INTRODUCTION

Mesenchymal stem cells (MSCs) derived from human induced pluripotent stem cells (hiPSCs) provide a novel source for generating adipocytes. hiPSC-MSCs satisfy the minimal criteria for defining MSCs proposed by the International Society for Cellular Therapy, *i.e.*, express certain cell surface markers, adhere to tissue culture plastics and exhibit differentiation towards osteogenic, chondrogenic and adipogenic lineages^[1]. However, hiPSC-MSCs have also been referred to as MSC-like, mesenchymal progenitors or fibroblast-like cells by different authors, including us, because hiPSC-MSCs are not identical to MSCs isolated from adult tissues, as we will discuss. For simplicity, we use the general term MSCs in this review. We present hiPSCs as a unique source of adipocytes, thus opening new avenues for fundamental research and clinical medicine. We also underline the low adipogenic capacity of hiPSC-MSCs compared to adult-MSCs, based on our expertise regarding the study of human MSCs isolated from iPSCs and adipose tissue.

In mammals, two adipose tissue functional types

coexist, *i.e.*, brown and white adipose tissues, which are both involved in the energy balance while having opposite functions. White adipose tissue (WAT) is mainly involved in energy storage and mobilization in the form of triglycerides. In contrast, brown adipose tissue (BAT) burns fat and is specialized in energy expenditure. BAT is a key thermogenic organ since brown adipocytes convert nutrients into heat by uncoupling respiration from ATP synthesis. BAT implants were recently shown to improve the metabolic conditions in obese mice and to restore normoglycemia and glucose tolerance in streptozotocin-induced diabetic mice^[2]. These recent findings offer promising prospects for basic research and clinical medicine-increasing energy expenditure *via* recruitment of brown adipocyte progenitors could be a valuable therapeutic approach to counteract obesity and its associated metabolic complications. However, BAT represents a minor fraction of the adipose tissue in humans, is found throughout the body and disappears from most areas with age, persisting only around deeper organs^[3]. Human BAT is hard to isolate in this regard, so an alternative cellular source is required to generate brown adipocytes.

Congenital or acquired lipodystrophies result from the loss, degeneration or misdistribution of white adipose tissue, leading to diabetes, severe defects in lipid homeostasis and ectopic fat accumulation. Transplantation of white adipose tissue in a lipoatrophy mouse model has been shown to improve the metabolic parameters^[4]. Therefore, isolation of hiPSCs-MSCs differentiating into brown and white adipocytes could provide an unlimited source of adipocytes for autologous cell-based therapy to treat obesity and lipodystrophy diseases.

Differentiation of iPSCs into MSCs, and then into adipocytes, also offers a unique opportunity to determine adipocyte properties according to their embryonic origins, but this issue has yet to be investigated in humans. Indeed, individual white adipose tissue depots are not equivalent, as functional differences have been reported in mice and humans^[5]. These distinct properties of individual adipose tissue depots could play a role in obesity-related complications and might explain why the spread of only certain depots is associated with severe metabolic disorders. It has also been observed that the different fat depots are not altered in a similar manner in genetic- and drug-induced lipodystrophies^[6]. These regional differences were conserved during *in vitro* propagation and adipocyte differentiation of MSCs, strongly suggesting that MSCs from different fat depots are indeed inherently different^[7]. Recent published data, including ours, suggested that MSC embryonic origins could play a role in these intrinsic differences. Surprisingly little is known about the developmental origin of adipocytes in rodents, and nothing is known in humans. Gesta *et al.*^[8] compared gene expression profiles of intra-abdominal and sub-cutaneous adipose tissues in mice and found major differences in the expression of several genes involved in embryonic development and pattern specification. We recently demonstrated that subsets of adipocytes have mesodermic

and neuroectodermic origins depending on the location of adipose tissue depots in mouse and quail^[9,10]. These data are in full agreement with the demonstration of Takashima *et al.*^[11] that MSCs originate both from mesoderm and neuroectoderm. Interestingly, the potential of neuro- and meso-derived MSCs to differentiate and participate in tissue repair differs depending on the embryotic origin^[12].

DIFFERENTIATION OF HUMAN-INDUCED PLURIPOTENT STEM CELLS INTO ADIPOCYTES

Differentiation of hiPSCs offers a unique opportunity to purify human MSCs for the purpose of generating brown and white adipocytes, as well as adipocytes of different embryonic origins from the same patients' somatic cells. Following the pioneer work of Yamanaka's group on the generation of iPSCs by reprogramming human fibroblasts^[13], the capacity of hiPSCs to generate functional adipocytes was first reported by Nakao's group. The authors showed that hiPSCs have an adipogenic potential comparable to human embryonic stem cells. Interestingly, hiPSC-adipocytes can maintain their functional properties for several weeks after transplantation into nude mice^[14,15]. These data revealed that hiPSC-adipocytes could potentially be used to correct metabolic parameters in patients. In these experiments, differentiated hiPSCs, but not MSCs, were transplanted into mice. Indeed, hiPSC differentiated cultures are enriched with adipocytes after adipogenic induction, but also contain several other cell types that are undesirable for transplantation, including immature neural cells and undifferentiated iPSCs that can form teratomas several weeks after transplantation. As indicated by Noguchi and colleagues, transplantation of mature adipocytes alone results in graft loss that could be improved by transplanting adipocyte progenitors^[15]. Therefore, purification of hiPSC-MSCs with a high adipogenic capacity is required prior to an hiPSC-based therapeutic approach. Nishio *et al.*^[16] developed a procedure to generate functional brown adipocytes at a high frequency from hiPSC using a hematopoietic cocktail to induce hiPSC differentiation. Remarkably, hiPSC-brown adipocytes were able to improve glucose tolerance after transplantation in mice. This report established a link between brown adipocytes and hematopoietic cells, and indicated that hiPSCs could potentially be used to generate brown adipocytes with therapeutic properties. As recently reported, we designed a procedure to derive MSCs with a capacity to differentiate into both brown and white adipocytes^[17]. In this latter study, the use of small molecules during hiPSC differentiation revealed that TGF β and retinoic acid pathways regulate the generation of MSC subtypes having a brown or white adipogenic potential, respectively. Differentiation of hiPSCs offers the opportunity to characterize the earliest steps of adipogenesis and identify signalling pathways regulating brown and white fat cell lineages. However, as discussed below,

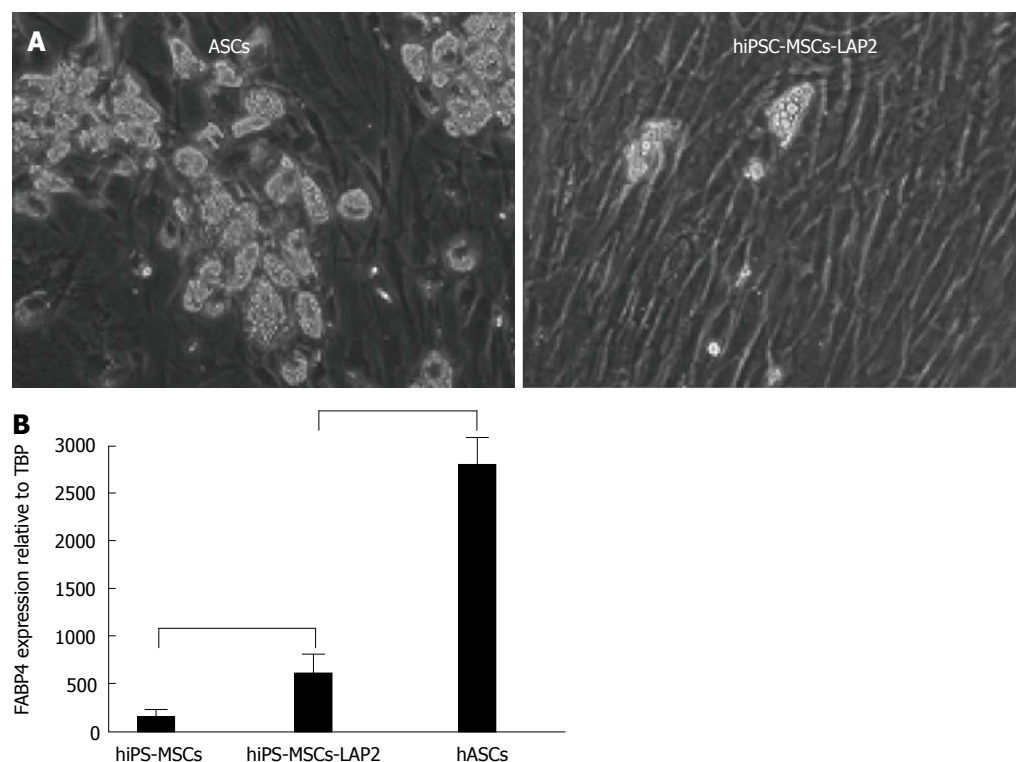


Figure 1 Adipogenic capacities of mesenchymal stem cells derived from human induced pluripotent stem cells and from human adipose tissue (adipose-derive stem cells). A: Microphotographic images showing adipocytes generated from hiPSC-MSCs transduced with C/EBPβ LAP (left) and from adult ASCs (right); B: Adipocyte differentiation potentials quantified via FABP4 adipogenic gene expression. MSCs: Mesenchymal stem cells; hiPSCs: Human induced pluripotent stem cells; ASCs: Adipose-derive stem cells.

hiPSC-derived MSCs have a low potential to differentiate into adipocytes compared to hMSCs derived from adult adipose tissue.

ADIPOGENIC CAPACITY OF hiPSC-DERIVED MSCS

Chen *et al.*^[18] first underlined the limited capacity of hiPSC-MSCs to undergo adipocyte differentiation, a feature that has often been observed by authors but not always highlighted. Ahfeldt *et al.*^[19] were able to generate pure brown or white adipocytes from hiPSCs, but only following genetic modification of MSCs. These artificially programmed hMSCs are of great interest from a therapeutic standpoint, but cannot be applied to investigate ontogenesis of human adipocytes. The fact that MSCs must be transduced with adipogenesis master genes clearly illustrates the low adipogenic potential of hiPSC-derived MSCs. This low adipogenic potential is illustrated in Figure 1. As shown, transduction of hiPSC-MSCs with a vector expressing an adipogenic gene, such as C/EBP-LAP, dramatically enhanced the MSC adipogenic potential, as monitored with the FABP4 adipogenic marker, but still at a lower level compared to MSCs derived from adipose tissue, also named adipose-derive stem cells (ASCs). Interestingly, some authors claim that the low differentiation capacity is limited to adipogenesis since hiPSC- and hESC-MSCs are able to differentiate

towards chondrogenic and osteogenic lineages at high levels^[20-22]. MSCs are abundant in adipose tissue, can be easily harvested using liposuction and have a considerable expansion potential *ex vivo*, particularly when isolated from young donors^[23]. Adipose tissue derived MSCs are currently being investigated in autologous transplantations to improve revascularization and tissue perfusion in ischemic limbs^[24-26]. Results so far suggest that the efficiency of adipose tissue-MSCs in regenerative medicine could rely more on their cytokine secretory functions and potential use as immunomodulators than on their differentiation potential^[27]. The therapeutic potential of MSCs derived from hiPSCs and from human adipose tissue should therefore now be carefully compared.

In recent years, various groups have successfully derived MSCs from hESC and hiPSC using a range of methods. There are two main approaches to differentiate pluripotent stem cells into MSCs (Figure 2).

One strategy involves embryoid body (EB) formation. In this approach, suspension cultures allow pluripotent stem cells to form 3-dimensional structures called EB (Figure 2A). This step models the *in vitro* embryonic development with the commitment of cells into the three primary germ layers. The duration of this stage may range from 7 to 14 d. EBs are then seeded onto culture dishes and, after a proliferation step, outgrowth cells are maintained in a mesenchymal culture medium. Subsequently, adherent cells display a fibroblast-like morphology and acquire specific MSC markers after se-

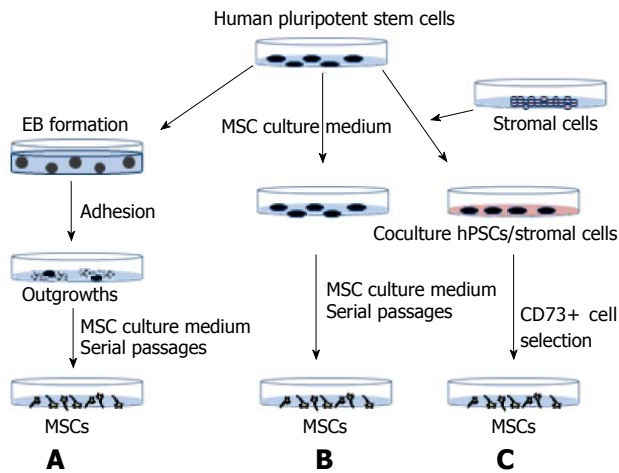


Figure 2 Mesenchymal stem cell derivation strategies. MSCs were derived from hiPSCs following (A) or not (B) embryoid body formation. Alternatively, hiPSCs were maintained in co-culture with murine stromal cells, and then MSCs were derived following selection of CD73-positive cells (C). MSCs: Mesenchymal stem cells; hiPSCs: Human induced pluripotent stem cells.

rial passages^[28,29]. An alternative strategy involves direct differentiation of pluripotent stem cells without the EB step (Figure 2B). Pluripotent stem cells are dissociated into single-cell suspensions and then maintained in mesenchymal medium for several weeks. The resulting cell cultures are enriched for MSCs through serial cell passaging^[30]. Another version of this protocol relies on the spontaneous differentiation of pluripotent stem cells into MSCs^[31]. Different research groups have developed additional steps to improve this method. For instance, before single-cell suspension, hiPSCs are committed towards MSC differentiation *via* treatment with small molecules, such as inhibitors of the TGF β pathway^[18]. Using small molecules with the EB method has been shown to promote the generation of two MSC subtypes having a selective potential towards brown and white adipocyte lineages^[17]. Growing hiPSCs on a fibrillar-collagen matrix has also been shown to improve their differentiation into MSCs^[32]. Coculture of pluripotent stem cells with murine stromal cells, followed by the selection of CD73-positive cells^[33], is another way to derive MSCs (Figure 2C). Interestingly, MSCs from mesoderm or from neuroectoderm origins can be derived depending on the stromal cells used^[34,35]. Finally, MSCs derived from different hiPSC lines generated from different donors, or from the same hiPSC clone using different derivation approaches, have the same adipogenic features^[30,31]. MSC adipogenic characteristics are therefore not dependent on the derivation method used.

Several hypotheses could be put forward to explain the low hiPSC-MSC adipogenic capacity compared to adult MSCs. Interestingly, the low differentiation potential is not restricted to hiPSC-MSCs since MSCs derived from human embryonic stem cells (hESCs) display the same feature, thus ruling out the possibility that the low hiPSC-MSC adipogenic capacity could be due to the reprogramming process or to an epigenetic mechanism. The fact

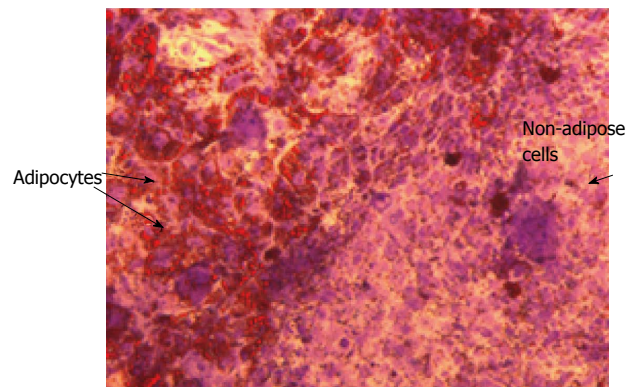


Figure 3 A niche for adipocytes when mesenchymal stem cells are maintained in the human induced pluripotent stem cells environment. Differentiated hiPSCs stained with Oil Red O, a specific stain for lipid droplets, to reveal adipocytes, and with violet crystal, a non specific stain, to reveal other cells. hiPSCs: Human induced pluripotent stem cells.

that hiPSCs generated from adipose-derived stem cells or from neural stem cells displayed a similar adipogenic capacity is in agreement with this hypothesis^[17]. One possibility is that the criteria to identify hiPSC-MSCs and adult MSCs could differ. For instance, individual cell surface markers used to characterize MSCs are also expressed by non-MSCs such as by fibroblasts^[36]. Therefore, it could be important to select hiPSC-MSCs based on the co-expression of several mesenchymal markers and not on CD73 expression only. We favour the hypothesis that the range of factors or culture conditions required to induce adipocyte differentiation of MSCs derived from adult tissues and from embryonic-like cells could differ. The low hiPSC-MSC adipogenic capacity is a reminiscence of an earlier observation reported by Han *et al.*^[37]. The authors observed that epididymal adipose tissue, which undergoes postnatal development in mouse, is composed of multipotent progenitor cells that meet the MSC criteria but lack an adipogenic capacity *in vitro*. In contrast to cells derived from other fat pads, epididymal fat cells require a three-dimensional culture conditions and a different micro-environment to undergo differentiation. These results underline that the micro-environment has a critical role in differentiation but could differ for adult and embryonic-like cells. We have observed that hiPSCs can generate nice adipocyte colonies when MSCs are not derived but are maintained in an iPSC environment. As shown in Figure 3, adipocytes are close to non-adipose cells in hiPSC-differentiated cultures. As isolated MSCs have been found to have a low adipogenic capacity, we propose that these non-adipogenic cells are required for full differentiation of MSCs into adipocytes. The identification of these non-adipogenic cells and factors that they secrete could be of a great interest.

CONCLUSION

The potential of hiPSCs to generate MSCs having an adipogenic capacity represents a powerful cellular model for studying brown and white adipocyte ontogenesis and

comparing the properties of adipocytes derived from mesoderm or neuroectoderm. This also provides a basis for investigating factors involved in the recruitment of MSCs having different potentials in normal and pathological contexts.

From a clinical standpoint, many issues have to be resolved before using hiPSC-MSCs in cell-based therapeutic for obesity and lipodystrophy diseases. However, the differentiation of iPS cells towards the adipogenic lineage offers a unique opportunity to purify white and brown adipocytes from patients, which could lead to the development of autologous transplantation procedures to treat obese and lipodystrophic patients. It would be essential to determine the factors underlying the low adipogenic capacity of hiPSC-MSCs. They are functionally distinct from adult hMSCs and the challenge will be to determine the cellular and molecular events necessary to prime hiPSCs towards the adipogenic lineage at a high level.

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Changes in human pluripotent stem cell gene expression after genotoxic stress exposures

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Abstract

Human pluripotent stem cells (hPSCs) represent heterogeneous populations, including induced pluripotent stem cells (iPSCs), endogenous plastic somatic cells, and embryonic stem cells (ESCs). Human ESCs are derived from the inner cell mass of the blastocyst, and they are characterized by the abilities to self-renew indefinitely, and to give rise to all cell types of embryonic lineage (pluripotency) under the guidance of the appropriate chemical, mechanical and environmental cues. The combination of these critical features is unique to hESCs, and set them apart from other human cells. The expectations are high to utilize hESCs for treating injuries and degenerative diseases; for modeling of complex illnesses and development; for screening and testing of pharmacological products; and for examining toxicity, mutagenicity, teratogenicity, and potential carcinogenic effects of a variety of environmental factors, including ionizing radiation (IR). Exposures to genotoxic stresses, such as background IR, are unavoidable; moreover, IR is widely used in diagnostic and therapeutic procedures

in medicine on a routine basis. One of the key outcomes of cell exposures to IR is the change in gene expression, which may underlie the ultimate hESCs fate after such a stress. However, gaps in our knowledge about basic biology of hESCs impose a serious limitation to fully realize the potential of hESCs in practice. The purpose of this review is to examine the available evidence of alterations in gene expression in human pluripotent stem cells after genotoxic stress, and to discuss strategies for future research in this important area.

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Key words: Human pluripotent stem cells; Gene expression alterations; Genotoxic stress; Ionizing radiation

Core tip: Genome-wide alterations in gene expression in human pluripotent stem cells (hPSCs) following genotoxic stress exposures may underlie the ultimate fate and outcome of practical utility of hPSCs which makes systematic studies of these effects a high priority in stem cell research.

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INTRODUCTION

Human pluripotent stem cells have been isolated from the inner cell mass of the preimplantation embryos (embryonic stem cells, hESCs)^[1], from fetal germ cells (embryonic germ cells, hEGCs)^[2], and, more recently, from breast tissues of healthy human subjects (endogenous plastic somatic cells, ePSCs)^[3]. Since the discovery of so-

called Yamanaka factors in 2006^[4], a variety of different types of adult human somatic cells were experimentally converted into so-called induced pluripotent stem cells (iPSCs) in many respects resembling hESCs. Recent advances in application of somatic cell nuclear transfer technology (SCNT) to human cells led to breakthroughs in producing human pluripotent stem cells almost indistinguishable from hESCs^[5,6]. Arguably, the most studied among different types of human pluripotent stem cells are hESCs. These cells readily demonstrate a stable developmental potential to form derivatives of all three embryonic germ layers, and can be kept in the undifferentiated state in culture for prolonged periods, if not indefinitely. Human pluripotent stem cells are promising candidates for development of novel models to study human developmental biology, to promote drug discovery, and to foster efforts for cell-based regenerative medicine. To realize the potential of hESCs in practice would require growing and expansion of these cells in culture, during which hESCs may face many challenges. For example, hESCs experience culture stress, and stress associated with genotoxic agents, ubiquitous in nature.

In real life situations, exposures to electromagnetic ionizing radiation (IR) stemming from cosmic rays, natural background radioactive isotopes, and many other sources are inevitable. Many studies indicate IR as being one of the most potent cytotoxic and genotoxic agents^[7,8]. One of the key manifestations of the biological effects of IR is the change in global gene expression, which may dictate the ultimate hESCs fate after genotoxic stress. Detailed analyses of the available evidence of alterations in gene expression in human pluripotent stem cells after IR exposures will help pave the way for future research and strategical planning in this important area of studies.

GENE EXPRESSION-SPECIFIC SIGNATURE OF HESCS

The global gene expression signature of hESCs has been examined by many modern assays, including serial analysis of gene expression (SAGE), DNA microarray analysis, and new-generation, massively parallel signature sequencing (NGS). As a result of these studies, some key genes that regulate pluripotency and self-renewal, were identified and verified as being expressed in all lines of undifferentiated hESCs, such as *POU5F1*, *SOX2*, *NANOG*, and several others^[9-11]. A remarkable heterogeneity and variability in gene expression was found in many functional classes of genes across multiple lines of hESCs, including but not limited to housekeeping genes, and some “stemness” genes, such as *STAT3* and *RUNX1*^[12]. A high degree of both quantitative and qualitative differences in gene expression among hESC lines exist for many genes; and some of these differences may contribute to measurable biological consequences. For example, different developmental outcomes may result from a relatively moderate, *i.e.*, less than 2-fold variation,

in the level of expression of *POU5F1* in hESCs. *REM2* is upregulated in hESCs and is necessary to maintain survival and pluripotency of hESCs by down-regulating p53 and cyclin D1^[13]. Human ES cells are distinct from somatic cells in the expression of members of the E2F family and RB family so-called pocket proteins, such as p105 (RB1), p107 (RBL1), and p130 (RB2) that are known to control expression of genes implicated in both DNA and nucleotide metabolism^[14]. Some other distinct subsets of genes are expressed at consistently higher levels in hESCs compared to normal differentiated human cells. Among these are both components of telomerase *TERT* and *TR*^[15], antioxidant genes, such as *SOD2* and *GPX2*^[15], and many DNA repair genes, such as *BRCA1*, *MSH3*, *MSH6*, *LIG3*, *DMC1*, *FEN1*, *RPA3*, *BLM*, *WRN*, *etc.*^[15,16], partly explaining higher fidelity of DNA repair in hESC after genotoxic stress exposures^[17,18]. Importantly, some genes encoding key proteins implicated in cell cycle control and DNA damage signaling were also observed to be more abundantly expressed in hESCs compared to IMR-90 fibroblasts. Among them are *ATR*, *CHEK1*, *PCNA*, *PRKDC* (DNA-PKcs), and others^[19]. Recently, it was demonstrated that levels of BCL-2 are lower, whereas those of pro-apoptotic PUMA are higher, in hESCs compared to human somatic cells^[20], which is in concert with the tendency of hESC to undergo programmed cell death under permissive conditions. Noteworthy, the hybrid sequencing technique identified that a substantial subset of 273 novel RNAs from gene loci is expressed in human pluripotent stem cells, but not in diverse fetal and adult tissues, further adding to the differences in gene expression signatures between human pluripotent stem cells and other types of cells^[21]. The unique epigenetic landscape of the former might contribute, at least in part, to those distinct transcription profiles observed in many studies^[22,23].

CHANGES IN PROTEIN-CODING GENE EXPRESSION IN IRRADIATED HESCS

The transcriptional responses of many types of fully differentiated somatic human cells exposed to IR have been studied by numerous labs in the past. Much less is known about how human pluripotent stem cells, such as hESCs, respond to genotoxic stresses at the level of whole genome gene expression. Studies into such gene expression alterations were conducted only recently; but, we still have only partial knowledge about hESCs transcriptional programs elicited by DNA damage/genotoxic stressors. Importantly, changes affecting the global gene expression networks have been strongly associated with ultimate cell fates/outcomes in human cells undergoing genotoxic stress exposures. Such perturbations are considered to be an integral part of human cell response to DNA damage-induced stress^[24,25].

Comprehensive studies specifically aimed at understanding how global gene expression alterations manifest

in human pluripotent stem cells are scarce, and cover very limited number of hESC lines, *i.e.*, the most widely used H9 and H1 hESC lines^[23,26,27]. DNA microarray technique was used to analyze the transcriptional changes in H9 cell line of hESCs 24 h after 0.4 Gy, 2 Gy, and 4 Gy of gamma-radiation^[26]. Quite unexpectedly, it has been found that the expression levels of a set of core transcription factors governing pluripotency, in particular, and stemness, in general, in hESCs are not changed significantly by IR exposures up to 4 Gy of gamma-radiation^[26]. The most common themes involved in manifestation of response of IR-exposed hESCs include p53 stress signaling, cell death/apoptosis, cell cycle regulation, developmental processes, and many others.

The key genes that were initially discovered as being IR-responsive in fully differentiated adult human cells, such as *CDKN1A*, *GADD45A*, *BTG2*, and some others, appear to be upregulated by genotoxic stress exposures in human pluripotent stem cells as well^[23,26]. The effect of induced expression of stress response genes is clearly dose-dependent, since low doses of genotoxic stressors may not elicit robust changes in transcriptional responses in hESCs^[28]. A modest dose (0.4 Gy) of gamma-radiation was found to have an impact on cell death, cancer, and p53 signaling pathways; IR exposure with this dose apparently failed to significantly reduce hESCs proliferation at 24 h post-IR^[26].

Importantly, much higher dose of 2 Gy of gamma-radiation led to changes in canonical TFG- β and Wnt/ β -catenin signaling, including *WNT10A* (up 2.1-fold), *WNT9A*, and *TGFBR2*^[26]. The perturbations in Wnt signaling axes following IR exposures could potentially affect the ultimate fate of irradiated hESCs, since Wnt genes are involved in key developmental pathways in human pluripotent stem cells^[29,30]. This dose induced *CDKN1A* overexpression by 2.3-fold in H9 hESCs^[26]. Noteworthy, the expression levels for many genes implicated in general metabolism functions (molecular transport *SLC6A13*, *SLC25A13*, cell morphology, amino acid metabolism, *etc.*) were significantly altered in hESCs by 2 Gy of IR exposures^[26].

Despite a high degree of similarity in gene expression profiles observed both after 2 Gy and 4 Gy of IR exposures, p53 and aryl hydrocarbon signaling, cancer-related processes, cell death, cell cycle and proliferation were found to undergo major modulations in hESCs after the higher dose (4 Gy). Among the highly induced IR-responsive genes were key genes implicated in p53 stress signaling, such as *CDKN1A*, *TP53INP1*, *HDM2* and TNF receptor genes^[26]. The minor gene expression alterations observed in the differentiation processes failed to lead to a loss of pluripotency even after 4 Gy of IR exposures. Unexpectedly, the expression changes of the core transcriptional factors operating in hESC were quite minor; hence, successful formation of teratomas was proven to be feasible to achieve even after 4 Gy. One of the key conclusions of this study is that the gene expression changes in H9 line of hESCs are dose-dependent

at a late timepoint after IR (24 h)^[26]. However, it remains to be addressed if this finding is still valid for other time points after IR exposures; and, if it can be generalized to other lines/types of human pluripotent stem cells.

Our more recent work examined the dynamic changes in global gene expression of H9 hESC line after 1 Gy of IR both at 2 and 16 h post-exposures^[23]. There were major differences in transcriptome alterations in hESCs and somatic human cell lines, such as fibroblasts, following IR^[23,31,32]. Overall, the scale of gene expression changes was rather modest, with a total of only 30 overexpressed genes observed in H9 hESC at an “early” timepoint after 1 Gy exposures. At the earliest, changes in expression cover almost entirely a limited subset of p53 stress signaling pathway genes^[23]. For example, the great preponderance of pro-apoptotic/cell cycle arrest gene up-regulation in H9 hESC line represent genes, such as *BTG2*, *CDKN1A*, *GADD45A*, *SESN1*, and *IER5*, that were shown previously to be IR-responsive in human somatic cells^[32-34]. Both cell cycle arrest (*GADD45A*, *PLK2*, *PLK3*, *IER5* implicated in execution of G(2)/M checkpoint) and pro-apoptotic genes (*BBC3*, *FAS*, *GDF15*, *HTATIP2*, *CARD8*, *TP53INP1*) were found to be induced by IR exposures at 2 h post 1 Gy of treatment^[23]. It is not clear if all these genes are overexpressed in all the cells within irradiated hESC populations, or there are distinct subpopulations of pluripotent stem cells that are destined to follow divergent paths (either recovery after IR-inflicted damage, or cell death). Single-cell methodological approaches may address this important issue in the near future. Detailed studies of gene expression changes at the later (16 h) post 1 Gy of IR identified 354 differentially expressed genes in H9 hESC line^[23]. Importantly, the overexpression of many pro-survival genes were observed, for example many members of the metallothionein superfamily, such as *MT1M*, *MT1L*, and *MT1H*^[23,32-34], and many genes belonging to general metabolism signaling. Some of the genes that tend to be overexpressed at 16 h post 1 Gy of IR encode known and putative transcription factors, such as *SP5*, *ZNF302*, *ZNF33A*, and *ZFYVE16*. The magnitude of expression of genes that were shown to be upregulated is within 1.5-fold to 25-fold over mock-irradiated hESC cultures^[23].

It is noteworthy that the gene expression profiles portraying dynamic transcriptomic changes as part of a broader radioresponse of hESCs cultures to 1 Gy of IR are distinct depending on time after genotoxic stress exposures^[23]. Only six genes (*CDKN1A*, *GDF15*, *SESN1*, *BTG2*, *ANKRA2* and *PLK3*) are differentially expressed at both early (2 h) and late (16 h) timepoints examined. This finding could potentially be explained by distinct molecular mechanisms operating in IR-exposed hESC populations at different timepoints after IR. Integration of the gene-rich metadata from other independent “omics” approaches (DNA/histone chemical modifications, non-coding RNAs, *etc.*) would definitely enable researchers to come up with a refined genotoxic stress-induced molecular signature that could be used as a bio-

marker of IR exposure of hESCs.

Recently, the studies in H1 line of hESCs exposed to 1 Gy of IR identified cell growth and proliferation, cell death, DNA-related processes, such as replication, recombination, and DNA repair as being the most genotoxic stress-affected biological pathways/themes^[27]. Therefore, it seems that there exists at least partial overlap in major sets of broadly defined processes/functions across distinct hESC lines^[23,26,27].

Surprisingly little is known on how low and very low levels of genotoxic stress exposures affect gene expression in hESCs. To the best of our knowledge, our group was the first recently to study the alterations in expression of stress-responsive genes following low and very low doses of IR, such as 0.01 Gy, 0.05 Gy, and 0.1 Gy^[28]. The results clearly indicate the heterogeneity of hESCs populations and warrant further genome-wide studies to support the development of “low-dose” specific signature of responses of hESCs.

Pluripotent human stem cells are known to present a high degree of heterogeneity in gene expression, but only recently the possible cause of such diversity was identified by detailed single-cell gene expression studies in hESC subsets defined by surface antigen expression^[35]. It was shown that hESC cultures exist as a continuum of intermediate pluripotent cell states^[35]. The bulk of the hESC population may express all key pluripotency transcription factors, such as *POU5F1*, *NANOG*, *SOX2*, *etc.* enabling successful differentiation into derivatives of all three germ layers upon permissive conditions^[35,36]. However, a small fraction of hESCs within population shows no lineage priming; these cells possess expression of a particular subset of intercellular signaling molecules with common regulation^[35]. Therefore, cultured hESCs can be considered as an inherently quasi-stable population with a multitude of pluripotent states that become committed for lineage specification at some point. The increased expression of developmental regulators in G1 cell cycle might be one of the factors influencing the heterogeneity of hESC populations^[37].

The notorious heterogeneity of any stem cell population was recently addressed by single cell quantitative RT-PCR method. It was found that each hESC has high expression in *POU5F1*, but *NANOG* expression levels varies significantly^[38]. In addition, geometrical position of individual hESCs within each colony can dictate the preponderance to differentiation along specific developmental pathway, such as ectoderm derivatives from the central part of the colony, trophoblast from the outer colony ring, *etc.*^[39]. This propensity is reflected by notorious differences in basal gene expression among single hESCs within colony^[39]. Whether genotoxic stress exposures increase or decrease such heterogeneity in gene expression among distinct hESCs is still unknown. However, the stochasticity of intranuclear molecular reactions and biochemical processes may control the ultimate decision of cell fate associated with DNA damage^[40].

CHANGES IN MICRORNA GENE EXPRESSION IN HESCS EXPOSED TO RADIATION

Gene expression alterations might be heavily influenced by epigenetic changes, such as DNA methylations, histone modifications, and perturbations in miRNA gene expression^[31]. It was found that dozens of miRNA genes were overexpressed after UV-exposures in hESCs, including genes belonging to miR-302 and miR-371-372 clusters thought to be human pluripotent stem cell-specific^[41]. Importantly, *miR-302a*, *miR-302b*, *miR-302c*, *miR-302d*, and *miR-372* genes were implicated in regulating the expression of p21 in hESCs, governing crucial self-renewal and cell cycle processes^[41,42].

The comprehensive data on epigenetic alterations in stressed hESCs are lacking; however, our recent study addressed hESC responses to IR exposures at a level of global microRNAome changes^[43]. By employing DNA microarray approach, we showed for the first time, that the microRNAome undergoes global alterations in hESCs after IR. We profiled expression of 1090 miRNA species in irradiated H1 and H9 lines of hESCs, and our analysis revealed statistically significant changes in expression of 54 genes following 1 Gy of IR exposures^[43]. Noteworthy, global microRNAome alterations in hESCs were both time-dependent and cell-line-dependent. “Late” transcriptional response at 16 h post-IR exposures of hESCs was shown to be quite robust at a level of global microRNAome. Just a few miRNA genes, such as miR-15b, miR-1973, *etc.*, were IR-responsive at 2 h post IR in both hESC lines we examined. The level of miRNA gene expression alterations at this “early” timepoint was modest at best (usually less than 2-fold)^[43]. Our global analysis of microRNAome changes reinforced the idea that miRNA gene expression after genotoxic stress exposures maintains the pluripotent state of surviving hESCs; and, for the most part, implicates the cell cycle-, and alternative splicing-related biological processes. Importantly, the identification of novel molecular targets of genotoxic stress exposure in hESCs will aid in understanding the underlying mechanisms governing the fundamental principles of human pluripotent stem cell behavior and plasticity for application in health science and as a remedy to cure diseases.

CHANGES IN GENE EXPRESSION IN HESCS EXPOSED TO GENOTOXIC DRUGS

In general, data on sensitivity and gene expression changes in human pluripotent stem cells in response to different genotoxic agents/drugs are still very limited. Studies were performed on comparison of the sensitivities of hESCs, their fibroblast-like derivatives, and matched human iPSCs and their parental and filial fibroblast-like cells

to one of the genotoxic drugs most widely used in clinical practice, such as etoposide which is a known poison of DNA topoisomerase II^[44]. It was found that human pluripotent stem cells are exquisitely sensitive to this genotoxic agent compared to differentiated cells, with DNA damage occurring as a result of stem cell exposure to only 0.5 µg/mL concentration of etoposide^[44]. Incubation of hESCs with 0.2 µmol/L etoposide for 16 h resulted in 80% hESC death^[45]. The minor surviving fraction of hESC that recovered after etoposide treatment displayed undifferentiated morphology, even though the ability of these cells to differentiate into derivatives of all three germ layers was not directly examined^[45]. The altered expression of key apoptosis regulators such as *TP53* and *BBC3* can at least partly explain a rapid and extensive induction of apoptosis in etoposide-treated hESC cultures^[46].

A high degree of sensitivity of hESC cultures to camptothecin, an inhibitor of DNA topoisomerase I, was recently observed^[47]. Camptothecin exerts its cytotoxic effects by inducing DNA double-strand breaks (DSBs) in S-phase cells^[48]. Even though *CDKN1A* mRNA was induced almost 5-fold compared to sham-treated H9 hESC cultures (1 µmol/L camptothecin, 3 h post exposure), the level of p21 protein remained undetectable^[47]. This report also supports the prevailing view that P53 signaling pathway is crucial in execution of apoptosis and in preventing the propagation of DNA damage in genotoxic stress-exposed hESC cultures.

Very recently, the adriamycin-induced DNA damage response in hESCs was characterized with ChIP-seq and microarray analysis^[49]. About 1,326 genes were responsive to adriamycin in H9 line of hESCs, with TP53-target genes being implicated mostly in cell death, cell cycle ($P < 10^{-6}$), and cell motility and migration ($P < 10^{-4}$). TP53 was found to target highly distinct subsets of genes during genotoxic stress exposures compared to induced differentiation in hESCs, resulting in specific outcomes that partly overlap, but largely differ^[49]. Importantly, genotoxic stress - induced targets of TP53 in hESCs, human colon cancer cells, and human normal cells, such as fibroblasts and keratinocytes, are surprisingly different^[50-52]. Therefore, changes in DNA damage-elicited gene expression are governed not only by stimulus-specific upstream signaling, but cellular milieu as well.

Induction of apoptosis was observed as a default response to moderate and high levels of genotoxic stress in hESCs in many studies^[20,53-55]. One of the radiomimetic drugs, neocarzinostatin, was shown to elicit a robust programmed cell death at concentrations as low as 0.1 µg/mL in H1 line of hESCs^[20]. There were dramatic differences in how pro-apoptotic gene expression alterations manifest; for example, the levels of *BAX* remained unchanged, whereas *BBC3*, *FAS*, *APAF1*, and *NOXA* changed more than 2-fold^[20]. High mitochondrial priming of hESCs which is mostly dependent upon the specific characteristics of gene expression in human pluripotent cells may explain, at least in part, hESCs sensitivity to

DNA damage - induced apoptosis.

GENE EXPRESSION ALTERATIONS IN HUMAN INDUCED PLURIPOTENT STEM CELLS EXPOSED TO IONIZING RADIATION

The systematic studies of how human iPSCs (hiPSCs) change their global gene expression in response to genotoxic stresses including IR exposures are yet to be performed. However, previous experiments suggested that the stress gene expression in hiPSCs closely resemble that in hESCs after IR in many respects^[19]. Firstly, the expression level of core transcription factors governing pluripotency, such as *OCT4* and *NANOG* was not changed significantly in hiPSCs following 1 Gy of IR^[19]. Secondly, more than two-fold overexpression of *CDKN1A*, *GADD45A*, *PPM1D*, *SESN1*, *SESN2*, and *HDM2* genes were observed, suggesting that TP53 signaling is activated after IR exposures in hiPSCs^[19]. Thirdly, no changes in the level of total ATM, CHEK2 and NBS1 were detected after genotoxic stress in these cells which was in contrast with the increase in total TP53^[19]. In general, observed changes in gene expression, if any, are in concert with alterations in hESC, but the absolute levels of specific alterations may differ^[19]. Undoubtedly, future studies using different approaches and protocols to create hiPSCs from different donors and various tissues will strengthen our understanding of transcriptional changes in human pluripotent stem cells after stresses of a variety of genotoxic agents, not only IR exposures.

CONCLUSION

In summary, human pluripotent stem cells display unique molecular and gene expression features defining both their self-renewal and pluripotent capabilities, and high propensity to undergo cell death upon moderate to severe genotoxic stress exposures. The apoptotic mode of cell death appears to be the main driving force clearing damaged human pluripotent stem cells from stressed cell populations. Whereas, the high efficacy of DNA repair, and robust induction of antioxidant and/or pro-survival pathways at the level of altered global gene expression in cells that are destined to recover after genotoxic stress may play a primary role in protecting a subpopulation of human pluripotent stem cells from death and transfer of damaged genetic material to progeny. Future directions in studying human pluripotent stem cells should ask if these surviving cells carry any “molecular memory”, or molecular changes associated with prior genotoxic stress exposure. In the planning, evaluation, and subsequent implementation of human pluripotent stem cell-based research activities, detailed gene expression analyses integrated with other global “omics” approaches will undoubtedly inform future basic science, cell regenerative-based and disease modeling studies.

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Cell signalling pathways underlying induced pluripotent stem cell reprogramming

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Abstract

Induced pluripotent stem (iPS) cells, somatic cells reprogrammed to the pluripotent state by forced expression of defined factors, represent a uniquely valuable resource for research and regenerative medicine. However, this methodology remains inefficient due to incomplete mechanistic understanding of the reprogramming process. In recent years, various groups have endeavoured to interrogate the cell signalling that governs the reprogramming process, including LIF/STAT3, BMP, PI3K, FGF2, Wnt, TGF β and MAPK pathways, with the aim of increasing our understanding and identifying new mechanisms of improving safety, reproducibility and efficiency. This has led to a unified model of reprogramming that consists of 3 stages: initiation, maturation and stabilisation. Initiation of reprogramming occurs in almost all cells that receive the reprogramming transgenes; most commonly *Oct4*, *Sox2*, *Klf4* and *cMyc*, and involves a phenotypic mesenchymal-to-epithelial transition. The initiation stage is also characterised by increased proliferation and a metabolic switch from oxidative phosphorylation to glycolysis. The maturation stage is considered the major bottleneck within the process, resulting in very few "stabilisation competent" cells progressing to the final stabilisation phase. To reach this stage in both mouse and human cells, pre-iPS cells must activate endogenous expression of the core circuitry of

pluripotency, comprising *Oct4*, *Sox2*, and *Nanog*, and thus reach a state of transgene independence. By the stabilisation stage, iPS cells generally use the same signalling networks that govern pluripotency in embryonic stem cells. These pathways differ between mouse and human cells although recent work has demonstrated that this is context dependent. As iPS cell generation technologies move forward, tools are being developed to interrogate the process in more detail, thus allowing a greater understanding of this intriguing biological phenomenon.

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Key words: Pluripotency; Reprogramming; Induced pluripotent stem; Cell signalling; Embryonic stem

Core tip: Induced pluripotent stem (iPS) cells present great promise, both to research and to medicine. However, we know very little regarding the mechanisms that occur throughout the iPS cell reprogramming process and thus the process remains inefficient. In this review, we discuss the 3 stages of reprogramming, initiation, maturation and stabilisation, and clarify the signalling pathways underlying each phase. We draw together the current knowledge to propose a model for the interactions between the key pathways in iPS cell reprogramming with the aim of illuminating this complex yet fascinating process.

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INTRODUCTION

Pluripotency, the ability of a single cell to give rise to all

Table 1 Factors that have been shown to achieve induced pluripotent stem cell reprogramming

Reprogramming factor	Human/mouse	Ref.
Oct4	Both	Takahashi <i>et al</i> ^[18,19]
Sox2	Both	Takahashi <i>et al</i> ^[18,19]
cMyc	Both	Takahashi <i>et al</i> ^[18,19]
Klf4	Both	Takahashi <i>et al</i> ^[18,19]
Nanog	Human	Yu <i>et al</i> ^[20]
Esrrb	Mouse	Feng <i>et al</i> ^[73]
Glis1	Both	Maekawa <i>et al</i> ^[49]
E-cadherin	Mouse	Redmer <i>et al</i> ^[43]
shp53	Both	Hanna <i>et al</i> ^[39]
Lin28	Both	Hanna <i>et al</i> ^[39]
UTX	Both	Mansour <i>et al</i> ^[82]

cells within an entire living organism, is of great biological interest both in terms of understanding developmental mechanisms as well as the medical potential that pluripotent stem cells possess. However, our understanding of the cell signalling networks underlying this complex process still remains incomplete. The first pluripotent stem cells were isolated from mouse blastocysts simultaneously by 2 groups in 1981^[1,2]. This was replicated 17 years later using human blastocysts^[3]. Embryonic stem (ES) cells have since been isolated from other species including rhesus monkeys^[4] and rats^[5,6]. Both human and mouse ES cells have provided an invaluable resource to understand the basic biology of the pluripotent state.

A “core circuitry” of homeodomain transcription factors, *Oct4*^[7], *Sox2*^[8] and *Nanog*^[9], governs pluripotency in both mouse and human ES cells^[10]. These transcription factors are expressed both *in vivo* in the inner cell mass (ICM) of the blastocyst and *in vitro*, in pluripotent cells. These 3 factors closely interact within the cell; for example *Oct4* and *Sox2* have been shown to form a heterodimeric transcription complex^[11-13] and all 3 factors share target genes^[14,15]. This interaction facilitates the precise regulation of the core circuitry necessary to maintain the pluripotent state; for instance *Oct4* overexpression leads to endoderm and mesoderm differentiation whereas blockade of *Oct4* induces trophoblast differentiation^[7]. This may be explained by its biphasic role in *Nanog* regulation whereby low levels of *Oct4* result in upregulation of *Nanog* whereas higher levels of *Oct4* result in downregulation of *Nanog*^[15]. Similarly, small increases in *Sox2* expression or ablation of *Sox2* expression both induce multilineage differentiation^[16]. Blockade of *Nanog* does not induce differentiation, thus indicating that *Nanog*’s role in the core circuitry of pluripotency is to stabilise the pluripotent state rather than acting as a housekeeper. However, *Nanog* knockdown does lead to an increased capacity for differentiation into primitive ectoderm^[9].

The core pluripotency circuitry is also autoregulatory since all 3 factors have been shown to regulate the expression of each other as well as themselves^[14,15,17]. Interestingly, SOX2 is dispensable for the activation of *Oct4*/*Sox2* target genes since forced expression of *Oct4* is able to rescue pluripotency in *Sox2*^{-/-} cells, however, *Sox2*

expression is necessary to maintain *Oct4* expression^[8]. Although it is clear that OCT4, SOX2 and NANOG occupy the top level of the pluripotency hierarchy, these core factors also regulate a wide range of genes associated with pluripotency signalling networks including *Stat3*, *Zic3*, *Tdgf1*, *Lefty/Ebaf*, *Dkk1* and *Frat2*^[14].

With the emergence of this complex molecular interplay of dosage dependency between hierarchical transcription factors in the maintenance of the somewhat unstable pluripotent ground state, it seems surprising that simply over-expressing these factors in somatic cells can induce the pluripotent state. However, the collective seminal studies of Yamanaka and Thomson show this to be feasible in their descriptions of reprogramming somatic cells to induced Pluripotent Stem (iPS) cells^[18-20].

The original iPS cell reprogramming strategy published by Takahashi *et al*^[19] 7 years ago remains robust and largely unaltered to the present day. The “Yamanaka factors”, *Oct4*, *Sox2*, *Klf4* and *cMyc* were constitutively expressed using genome integrating retroviruses in both mouse^[18] and subsequently human^[19] fibroblasts, and under ES cell culture conditions were able to induce pluripotency. To date, this methodology is still widely used, however, various adaptations to the method of vector delivery and reprogramming factors (Table 1) have been made. Advances in vector delivery have generally been made to either improve efficiency or safety, by preventing integration of the transgenes into the genome. For example, iPS cells have now been successfully generated using episomal plasmids^[21], Sendai viruses^[22] and piggyBac transposons^[23] to deliver the reprogramming factors and even proteins^[24] or small molecules^[25] alone. Many divergent cell-types have been successfully reprogrammed to pluripotency including neural stem cells^[26], neural progenitor cells^[27], keratinocytes^[28], B lymphocytes^[29], meningeal membrane cells^[30], peripheral blood mononuclear cells^[31] and pancreatic β cells^[32]. Often the minimal factors necessary to reprogram a cell depend on the endogenous “stemness” of the starting cell, for example, neural stem cells can be reprogrammed using *Oct4* alone since they express high levels of the other Yamanaka factors^[26].

The common aspiration is that iPS cells will provide an autologous source of cells for a multitude of regenerative medicine therapies in the future and clinical trials using iPS cells have begun^[33]. However, the most immediate utility of iPS cell technologies is the ability to study patient-derived cells in the lab. iPS cells present the opportunity to study a range of diseases in novel ways by isolating and reprogramming patient-specific cells and then differentiating them into the cell type of interest. For example, iPS cells have been generated from patients suffering from a wide range of disorders including Duchenne muscular dystrophy, Parkinson’s disease, Huntington’s disease, type I diabetes and Down’s syndrome (reviewed in^[34]). In addition, cells such as disease-specific cardiomyocytes, which would be difficult to obtain from patients, can also be generated and used to test specific drugs^[35]. In summary, the generation of iPS cells has

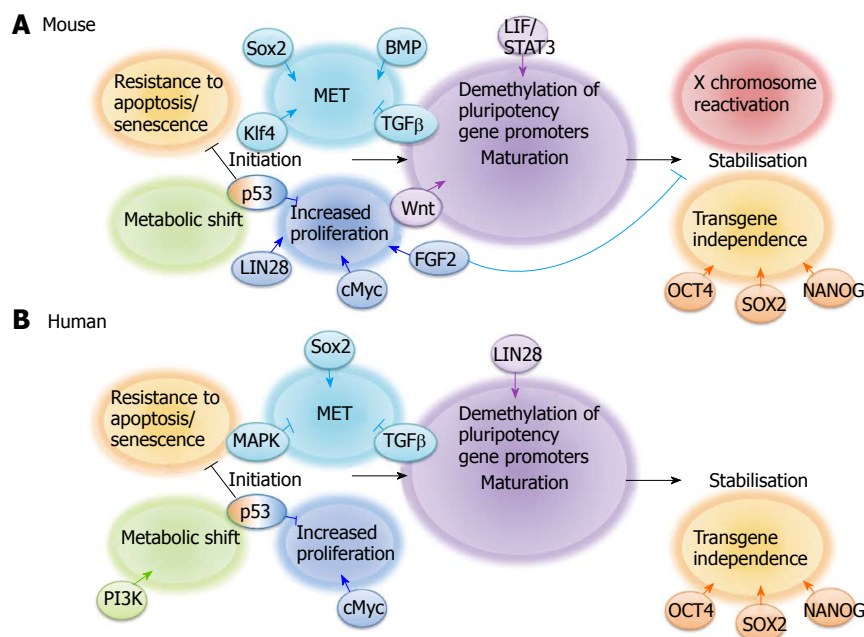


Figure 1 The key stages in (A) mouse and (B) human induced pluripotent stem cell reprogramming and the signalling pathways that regulate them.

stimulated the growth of a hugely active new area of research with promise to revolutionise medicine. However, the reprogramming process remains extremely inefficient and the basic molecular understanding of a process that does not appear to readily occur in nature is only just being unravelled. A greater understanding of the basic biology will lead to more efficient methodologies for iPS cell reprogramming *in vitro* and also potentially lead to strategies to therapeutically manipulate differentiated cells *in vivo* to become stem cells and repair or regenerate diseased tissues.

IPS REPROGRAMMING IS A STEPWISE PROCESS

Much progress has been made in recent years to define the molecular mechanisms involved in iPS cell reprogramming. This has led to the general acceptance of the model proposed by Samavarchi-Tehrani *et al.*^[36] that reprogramming consists of 3 phases: initiation, maturation and stabilisation (Summarised in Figure 1). Throughout reprogramming various changes occur not only to the cell phenotype but also to gene and non-coding RNA expression, epigenetic status and metabolism. In this review we will focus on cell signalling during the 3 stages of iPS cell reprogramming whilst other aspects are reviewed elsewhere by Papp *et al.*^[37] and Jia *et al.*^[38].

INITIATION

The initiation phase of reprogramming occurs in virtually all successfully transfected cells^[39] and is characterised by somatic genes being switched off by methylation, an increase in cell proliferation, a metabolic switch from

oxidative phosphorylation to glycolysis, reactivation of telomerase activity and a mesenchymal-to-epithelial transition (MET)^[40]. MET is a feature of both mouse^[41] and human^[42] somatic cell reprogramming and involves the loss of mesenchymal characteristics such as motility and the acquisition of epithelial characteristics such as cell polarity and expression of the cell adhesion molecule E-CADHERIN, perhaps explaining why *E-cadherin* can replace *Oct4* in the reprogramming process^[43]. MET and the opposite transition, epithelial-to-mesenchymal transition (EMT), are key features of embryogenesis^[44], tumour metastasis^[45] and both mouse^[46] and human^[47] ES cell differentiation. Interestingly, the MET that marks the initiation of cellular reprogramming is reversible since removal of the reprogramming factors from mouse “pre-iPS” cells after induction of reprogramming has been shown to lead to reversion of the cells to a mesenchymal phenotype^[36], thus demonstrating that continued transgene expression is necessary to allow cells to progress to the maturation stage.

Mechanistically, *Sox2* suppresses expression of *Snail*, an EMT inducer^[48], and *Klf4* induces *E-cadherin* expression, thus promoting MET^[41]. In addition, Mackawa *et al.*^[49] have shown that the Glis family zinc finger 1 protein *Glis1* can substitute *cMyc* in the reprogramming cocktail by inducing MET, thus initiating iPS cell reprogramming. MET can also be induced by chemicals, for example, various groups have demonstrated the ability of transforming growth factor (TGF)β inhibition to enhance the initiation stage of both mouse^[50,51] and human^[42] somatic cell reprogramming. This observation is supported by the finding that addition of recombinant TGFβ abrogates iPS cell formation^[42] and is likely due to the EMT-inducing action of TGFβ signalling, which then prevents the MET that is critical to successful iPS cell reprogram-

Table 2 Small molecules that enhance induced pluripotent stem cell reprogramming

Small molecule	Function	Ref.
BIX-01294	Histone methyltransferase inhibitor	Shi <i>et al</i> ^[51]
Bayk8644	Calcium channel agonist	Shi <i>et al</i> ^[51]
RG108	DNA methyltransferase inhibitor	Shi <i>et al</i> ^[51]
5-Aza-2'-Deoxycytidine	DNA methyltransferase inhibitor	Huangfu <i>et al</i> ^[89]
Dexamethasone	Steroid glucocorticoid	Huangfu <i>et al</i> ^[89]
Valproic acid	HDAC inhibitor	Huangfu <i>et al</i> ^[89]
Trichostatin A	HDAC inhibitor	Huangfu <i>et al</i> ^[89]
SAHA	HDAC inhibitor	Huangfu <i>et al</i> ^[89]
PD0325901 + CHIR99021	MAPK inhibition and GSK3 inhibition	Shi <i>et al</i> ^[51] , Silva <i>et al</i> ^[77]
SB 431542+ PD0325901	TGFβ inhibitor And MAPK inhibitor	Lin <i>et al</i> ^[42]
A-83-01	TGFβ inhibitor	Li <i>et al</i> ^[41] , Zhu <i>et al</i> ^[53]
E616452	TGFβ inhibitor	Ichida <i>et al</i> ^[90]
AMI-5	Protein arginine methyltransferase inhibitor	Yuan <i>et al</i> ^[13]
Kenpaullone	Unknown "novel function"	Lyssiotis <i>et al</i> ^[91]

Adapted from Feng *et al*^[73]. SAHA: Suberoylanilide hydroxamic acid; AMI: Arginine N-Methyltransferase Inhibitor.

ming. TGFβ signalling promotes EMT *via* a wide variety of mechanisms, including mediating the disassembly of junctional complexes, reorganising the cell cytoskeleton, and EMT gene activation^[52]. Various TGFβ inhibitors have been used to promote reprogramming, including A-83-01^[41,53], E616452^[25,50] (also known as RepSox) and SB431542^[42] (Table 2). In addition to promoting MET, TGFβ inhibitors promote *Nanog* expression^[50], thus providing 2 potential mechanisms for their ability to enhance reprogramming. Mitogen-activated protein kinase (MAPK) signalling, activated by TGFβ, further induces the expression of mesodermal genes^[52]. Inhibitors of MAPK signalling such as PD0325901 have therefore been used in combination with TGFβ inhibitors to promote MET^[42].

Bone morphogenetic protein (BMP) signalling also plays an important role in the initiation stage of mouse iPS cell reprogramming by promoting MET *via* upregulation of epithelial genes such as *E-cadherin*, *Ocludin* and *Epithelial cell adhesion molecule*^[36]. Chen *et al*^[54] have shown that BMPs can replace *Klf4* in the reprogramming cocktail, allowing mouse embryonic fibroblasts (MEFs) to be reprogrammed using *Oct4* alone. However, constitutive BMP activation prevents human somatic cell reprogramming. This was discovered through the observation that a naturally occurring *Alk2* mutation, which causes fibrodysplasia ossificans progressiva in humans, prevents iPS cell reprogramming and that this blockade can be rescued by inhibition of the ALK2 receptor^[55].

Increased proliferation has been observed in cells undergoing reprogramming as early as 3 d after induction of reprogramming^[56] and is likely to be initiated by *cMyc* transgene expression^[57]. *Lin28* expression and *p53* knockdown also increase the efficiency of iPS cell reprogramming by stimulating cell proliferation^[39]. Specifically, LIN28 has been shown to regulate cell cycle genes such as *Cyclin A*, *Cyclin B* and *Cdk4*^[58] whilst *p53* induces cell

cycle arrest *via* p21 and thus *p53* knockdown promotes proliferation^[59].

Fibroblast growth factor (FGF) signalling has also been implicated at the initiation stage^[60]. Araki *et al*^[61] show that *Fgf4* is upregulated on day 3 after induction of reprogramming in MEFs and Jiao *et al*^[60] show that FGF2 can improve the reprogramming efficiency in the early phases of mouse somatic cell reprogramming, whereas it has adverse effects in the later stages. Mechanistically, this group have shown that FGF2 promotes the early stages of reprogramming through accelerating cell proliferation, facilitating MET and eliminating extracellular collagens. In addition to an increased proliferation rate, the minority of cells that undergo successful reprogramming also exhibit resistance to apoptosis and senescence, by transgene expression^[56]. Recent studies have shown that miR-302 expression allows cells to overcome reprogramming-induced senescence^[62] and that silencing of the INK4/ARF locus is also likely to be involved, since INK4/ARF blockade improves reprogramming efficiency^[63,64]. The INK4/ARF locus encodes tumour suppressor genes that activate the retinoblastoma and *p53* pathways. Its inactivation therefore blocks apoptosis and senescence and facilitates reprogramming.

The initiation phase is also characterised by a metabolic switch from oxidative phosphorylation to glycolysis^[65] that occurs around 7 d after induction of reprogramming^[66] and involves phosphatidylinositol-3-kinase (PI3K)/AKT signalling^[53,67]. For example, Chen *et al*^[67] have demonstrated that the PI3K/AKT pathway was activated during reprogramming in parallel with the upregulation of glycolytic gene expression, showing specifically that AKT activated 2 key glycolytic regulators, AS1060 and PFKFB2. Zhu *et al*^[53] have also shown that PS48, an activator of the PI3K/AKT pathway, is able to enhance reprogramming by upregulating glycolytic genes. By switching their metabolism from oxidative phos-

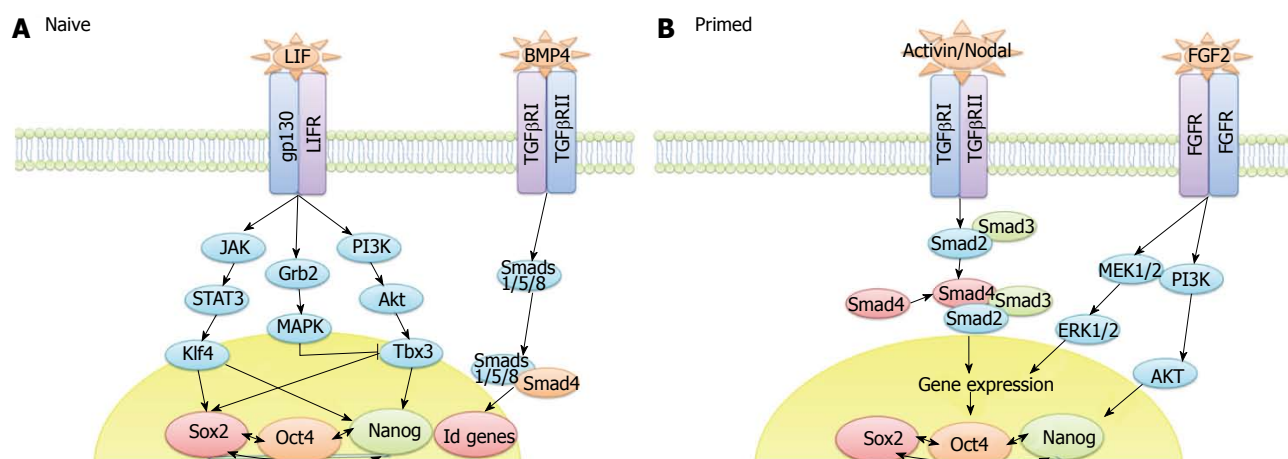


Figure 2 The core signalling networks that maintain pluripotency in (A) naive and (B) primed pluripotent cells.

phorylation to anaerobic glycolysis, pre-iPS cells assume an ES cell-like phenotype^[68]. ES cells are likely to have developed this form of metabolism as an adaptation to the hypoxic *in vivo* environment of the early embryo^[69]. Interestingly, various groups have shown that iPS cell reprogramming is enhanced by hypoxia^[70,71], likely due to the acceleration of this metabolic shift.

MATURATION

Tanabe *et al.*^[72] have recently identified the maturation stage of iPS cell reprogramming as being a major bottleneck in the process, which is likely to account for the low efficiency of the process generally. They demonstrate that LIN28, but not NANOG, shp53 or CYCLIN D1, promotes maturation of iPS cells. During maturation, epigenetic changes occur allowing expression of the first pluripotency-associated genes^[40]. These genes include *Fbxo15*, *Sall4*, *Oct4*, *Nanog* and *Esrrb*. Interestingly, *Esrrb* has been shown to be sufficient to reprogram MEFs in collaboration with *Sox2* and *Oct4*^[73].

LIF/STAT3 signalling is required for the maturation phase of mouse iPS cell reprogramming^[74]. Interestingly, pre-iPS cell colony formation has been observed in the absence of LIF, however, beyond day 6 of reprogramming these colonies detach. This is likely due to the requirement that cells undergoing the reprogramming process have for LIF signalling to maintain *cMyc* expression^[75]. In addition, Tang *et al.*^[74] demonstrate that LIF/STAT3 activation induces earlier formation of an increased number of pre-iPS cell colonies. Mechanistically, this group demonstrate that LIF/STAT3 signalling is required for demethylation of pluripotency-associated gene promoters. Specifically, STAT3 signalling was shown to directly block the action of the DNA methyltransferase DNMT1 and Histone deacetylases 2, 3 and 8.

Wnt signalling also enhances the maturation phase of mouse somatic cell reprogramming whereby exogenous stimulation of the pathway using Wnt3a between days 6 and 9 after induction of reprogramming enhances the formation of *Nanog* positive colonies^[76]. Various groups

have suggested that expression of *Nanog* is necessary for cells to advance from the maturation phase to the stabilisation stage^[39,77] and thus, Samavarchi *et al.*^[36] suggest that *Nanog* expression alone is responsible for mediating the transition from pre-iPS cells to stably reprogrammed cells. This group demonstrate that removal of the reprogramming factors from mouse iPS cells at day 9 after induction of reprogramming did not induce phenotypic reversion. Other groups, however, have reported different time points for the stabilisation stage, including day 11^[78,79] and day 16^[80], suggesting that this can vary depending on discrete protocols and culture variations. It is clear that there remains substantial information to be learned regarding this critical intermediary step but NANOG appears to play a pivotal role in iPS cell maturation.

STABILISATION

Only around 1% of cells that initiate reprogramming make it to the stabilisation stage^[72]. This can be explained by the observation made by Golipour *et al.*^[81] that not all cells are “stabilisation competent”. This group identify a gene expression signature that distinguishes stabilisation competent and stabilisation incompetent cells and show that stabilisation competent cells require transgene repression to enter this stage. Since the stabilisation stage is characterised by transgene independence, only cells that have activated endogenous pluripotency gene expression are able to maintain pluripotency at this late stage. Endogenous pluripotency gene expression is facilitated by demethylation of pluripotency gene promoters, thus explaining why various DNA and histone methyltransferase inhibitors have been shown to accelerate iPS cell reprogramming, amongst other small molecules (Table 2). This may also explain the ability of the H3K27 demethylase UTX to substitute for some of the original reprogramming factors^[82].

The end-point of iPS cell reprogramming is a matter of some controversy. For example, the stabilisation stage of mouse iPS cell reprogramming involves X

chromosome reactivation whereas human iPS cell reprogramming does not^[83]. X chromosome inactivation is a process that occurs as female embryonic cells, which have 2 active X chromosomes, commit to differentiation. This feature of human ES and human iPS cells, amongst others (reviewed in^[84]), means that they represent the primed pluripotent state. Human iPS cells generated in the presence of ACTIVIN/NODAL and FGF2 ligands are stabilised in this primed state whereas mouse iPS cells reprogrammed in the presence of LIF and BMP4 can be fully reprogrammed to the uncommitted naïve ground state (Figure 2). Interestingly, human dermal fibroblasts (HDFs) have been shown to give rise to naïve human iPS cells when reprogrammed in the presence of LIF, FGF2 and TGFβ1 plus inhibitors of c-Jun NH2-terminal kinase, p38, MAPK and glycogen synthase kinase 3 (3i)^[85], thus demonstrating that the cell signalling context is critical to the determination of naïve and primed pluripotency rather than the two states representing a species difference. The derivation of various novel stem cell lines, including intermediate epiblast stem cells which exhibit dual responsiveness to LIF and ACTIVIN/NODAL signalling^[86], has challenged the concept of 2 distinct pluripotent states, instead suggesting that a spectrum of pluripotency exists, an idea we develop in Hawkins *et al.*^[87]. Thorough investigation into this spectrum of pluripotency, and therefore the transition from pluripotent cells to differentiated cells, should accelerate the delineation of mechanisms occurring throughout the reverse process, from a somatic cell to an iPS cell.

CONCLUSION

A proposed model for the signalling networks required for the various stages of mouse and human iPS cell reprogramming can be found in Figure 1. However, this knowledge is still vastly incomplete. New technological advances are required to thoroughly interrogate the contribution of a wide range of signalling pathways to somatic cell reprogramming. One of the limitations of many current approaches is the inability to track reprogramming cell signalling in real-time since cells must be sacrificed to obtain data, for example for microarray analysis^[36], fluorescence-activated cell sorting or protein extracts^[78] at various time points. Some advances have been made to track reprogramming cells in real-time, for example, Smith *et al.*^[88] carried out time-lapse imaging with the aim of tracking single cells undergoing the reprogramming process. However, they concluded that this was virtually impossible. We are currently interrogating the role of cell signalling networks in iPS cell reprogramming using a range of GFP reporter HDF lines activated by transcription factors involved in relevant cell signalling pathways. This allows us to monitor signalling pathway activity throughout an entire iPS cell reprogramming experiment in real-time. We anticipate this will enable us to temporally map the contribution of a wide range of signalling pathways to iPS cell reprogramming, thus illu-

minating this enigmatic biological phenomenon.

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Pluripotent stem cell-derived neural stem cells: From basic research to applications

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ferentiation method, the neural stem sphere method, which we developed.

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Key words: Pluripotent stem cells; Embryonic stem cells; Neural stem cells; Neural Stem Sphere method; Cell-based therapies

Core tip: *In vitro* techniques for manipulating stem cells can enhance the development of stem cell-based therapies and effective prevention against human diseases. This review summarizes the techniques required to generate neural cells from pluripotent stem cells, as well as focusing on current research applications of a simple neuronal differentiation method, the neural stem sphere method.

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Abstract

Basic research on pluripotent stem cells is designed to enhance understanding of embryogenesis, whereas applied research is designed to develop novel therapies and prevent diseases. Attainment of these goals has been enhanced by the establishment of embryonic stem cell lines, the technological development of genomic reprogramming to generate induced-pluripotent stem cells, and improvements in *in vitro* techniques to manipulate stem cells. This review summarizes the techniques required to generate neural cells from pluripotent stem cells. In particular, this review describes current research applications of a simple neural dif-

INTRODUCTION

All somatic cells forming an individual are derived from one fertilized egg, a totipotent stem cell, which differentiates into preimplantation blastocysts that possess a pluripotent inner cell mass (ICM). Pluripotency is defined as the potential to differentiate into any somatic cell *via* three embryonic germinal layers: the endoderm, the mesoderm and the ectoderm. Mechanisms of pluripotency have been studied in embryonal carcinoma (EC) cells as *in vitro* models^[1,2]. Although EC cells have some properties similar to pluripotent ICMs, EC cells isolated from teratocarcinomas frequently have abnormal chro-

mosomes and their ability to differentiate is restricted^[3,4]. Nevertheless, studies using EC cells have provided valuable information on culture conditions and characterization criteria of pluripotent stem cells.

The strategies used to create normal pluripotent stem cells were very simple. Most important was developing methods to isolate ICM from blastocysts and to maintain the isolated pluripotent stem cells *in vitro*. Mouse embryonic stem (ES) cells from ICM of blastocysts were successfully maintained in a proliferative and undifferentiated state in serum-containing medium on a mouse embryonic fibroblast (MEF) feeder cell layer^[5,6]. In addition, leukemia inhibitory factor (LIF) was identified as a protein secreted by the feeder cells that was required to maintain mouse ES cells so that they did not differentiate spontaneously^[7,8]. Subsequently, monkey and human ES cells were established under appropriate culture conditions, which differed from those for mouse ES cells because of no effect of LIF on maintenance of the undifferentiated state of these primate cells^[9-11]. Human ES cells are cultured in the presence of basic fibroblast growth factor (FGF-2) instead of LIF^[12]. Human ES cells as normal pluripotent stem cells provide not only an effective tool to uncover novel biological knowledge related to processes of cell differentiation, but may be stable sources of donor cells for cell-based therapies. Despite these biological advantages of human ES cells, they involve enormous ethical and legal issues due to the destruction of human embryos with potential to develop into human beings.

The establishment of induced-pluripotent stem (iPS) cells has overcome the ethical problems involved in using human ES cells, as well as increasing the applications of pluripotent stem cells. For example, iPS cells established from a patient, who has already been affected by a disease, can be used to analyze the progression of that disease^[13-15]. Although iPS cells are associated with several specific problems, including their reduced efficiency of reprogramming, the integration of exogenous DNA into the host genome and the carcinogenic effects of the DNA, these problems may be overcome by various technical improvements^[16-20]. In future, pluripotent stem cells, including iPS cells and somatic cell nuclear transfer derived ES cells, will be characterized by comparison to ES cells as the gold standard and will be utilized in many aspects of basic and clinical research, depending on their features^[21,22].

NEURONAL DIFFERENTIATION OF PLURIPOTENT STEM CELLS

Stemness, an essential characteristic of a stem cell, involves properties of self-renewal and the potential to differentiate into functional somatic cells. Pluripotent stem cells, like ES and iPS cells, can proliferate infinitely in an undifferentiated state and have the potential to differentiate into any somatic cell derived from the three embryonic germ layers. In contrast, neural stem (NS) cells, defined

as stem cells committed to the neural cell lineage, have lost pluripotency and acquired multipotency, or a limited ability to differentiate into several cell types. For example, NS cells can differentiate into neural cells, such as neurons, astrocytes and oligodendrocytes. The pluripotency of cells can be experimentally analyzed by two general methods, teratoma formation *in vivo* and embryoid body (EB) formation *in vitro*^[23-25]. In the EB formation method, enzymatically digested mouse ES cells are grown in hanging drop culture in serum-containing media without LIF. These dissociated ES cells immediately form unorganized aggregates, resulting in EBs after several days. These EBs consist of endodermal, mesodermal and ectodermal cells, thus closely resembling early post-implantation embryos^[26]. Many attempts have been made to modify this method to improve the reproducibility and efficiency of EB formation^[27-29]. Some modifications alter the direction of differentiation *via* EBs, indicating that optimization of culture conditions to form EBs would efficiently bias the direction of differentiation, enabling the preparation of large numbers of desired specialized cells from pluripotent stem cells.

The criteria used to assess differentiation methods include the simplicity of the procedure, the efficiency of differentiation and versatility across animal species. Several methods of neural differentiation have been developed. EB formation is the method used most frequently to assess pluripotency, as described above. However, neural differentiation *via* EB formation is spatiotemporally unusual and not unidirectional, reducing the effective generation of neural cells. To overcome these limitations, retinoic acid, a well-known morphogenic factor, is added to culture media to promote neural differentiation^[30]. In addition, FGF-2 may be used to promote the selective proliferation of NS cells from EBs, increasing the total number of NS cells^[31]. Unfortunately, even these optimized protocols involve elaborate and time-consuming procedures to generate homogeneous populations of neural cells.

The serum-free cell suspension method is based on EB formation using chemically defined media and secreted factors, similar to those utilized for neurogenesis in embryos^[32]. In brief, treatment with Wnt and Nodal antagonists during the formation of EBs promotes the selective differentiation of dissociated mouse ES cells into neural cells. This method, in combination with cell sorting techniques, can efficiently generate central nervous system (CNS) cells, including telencephalic progenitors, retinal progenitors, photoreceptor cells and hypothalamic neurons^[32-34]. Another method, dual-SMAD inhibition protocol, is based on monolayer culture with SMAD signaling inhibitors such as noggin and SB431542, generating not only CNS cells like primitive and definitive NS cells, but also neural crest cells from human ES cells with high efficiency^[35-37]. In the case of neurogenesis of human ES cells, these methods require application of Rho-associated kinase (ROCK) inhibitor Y-27632 to improve the poor survival of human ES cells after enzymatic

dissociation^[32,35]. Recently, it has been reported that this ROCK inhibitor itself promotes neuronal differentiation of mouse ES cells, suggesting that ROCK inhibitor may promote both cell viability after dissociation and improve efficiency of neuronal differentiation of human ES cells^[38]. In contrast, these methods based on chemically defined media depend on ready-to-use products, reducing efforts to introduce these experimental methods. For example, the compositions of well-known supplements, including Knockout Serum Replacement and B-27 supplement, have been kept confidential, blocking the ability to prepare and optimize them for use in individual laboratories. In addition, these commercially available supplements vary widely in their ability to support neurons in culture^[39]. Lot-to-lot variations in these products should be monitored when using these products in neuroscience research.

UNI-DIRECTIONAL NEURONAL DIFFERENTIATION OF ES CELLS BY THE NEURAL STEM SPHERE METHOD

The neural stem sphere (NSS) method is a simple neural differentiation method using only astrocyte-conditioned medium (ACM) prepared from serum-free medium under free floating conditions^[40-42]. In brief, ES cell colonies formed on MEF feeder layers at clonal density are mechanically picked. In the absence of proteolytic digestion, these ES cell colonies maintain a compact shape, like ICM in blastocysts. These ES cell colonies are subsequently cultivated in ACM on bacteriological dishes for short periods of time. Cultivation of rodent and primate ES cell colonies for 4 and 12 d, respectively, results in the efficient development of cell spheres, designated NSSs, which mainly contain NS cells and neurons.

In addition to the ease of performance of this procedure and, its versatility across animal species, the NSS method has some characteristic properties. This method promotes the unidirectional neuronal differentiation of mouse ES cells through stepwise progression, characterized as the synchronous conversion of ES into NS cells through epiblasts as intermediates^[43]. The temporal course of this process is comparable to that of neural tube organization from blastocysts during early embryogenesis. Supplementation of ACM with epidermal growth factor (EGF) and FGF-2 accelerates both the proliferation of NS cells and the suppression of neuronal differentiation, resulting in the generation of NSSs composed of a population rich in NS cells, even during the same culture period. Furthermore, adhesion culture of these NS cell-rich NSSs with mitogens, EGF and/or FGF-2 on matrigel-coated tissue culture dishes provides large numbers of homogenous NS cells. These NS cells can be maintained on monolayer cultures with mitogens, can be preserved by freezing, and can differentiate into neurons and glia^[44]. Altogether, these findings suggest that the NSS method will provide a platform for consid-

erable biological research on neurodevelopmental processes, including the generation of neuroepithelial cells from pluripotent stem cells, postmitotic neural maturation and neural cell death.

BASIC RESEARCH AND APPLICATIONS USING NEURAL STEM SPHERES AND HOMOGENEOUS NEURALS CELLS

As described above, cell spheres formed using the NSS method mimic neural tissues during early embryogenesis, with NSSs providing homogeneous NS cells that can be maintained on monolayer cultures. Since the platform based on the NSS method will provide novel findings in many biological disciplines, several basic and applied research findings using this platform are described below.

Neural stem sphere as an in vitro model to analyze early neurodevelopment

Understanding the molecular basis underlying early neurogenesis enhances the efficiency of production of neural cells *in vitro*, as well as providing insights into the mechanisms underlying neurodevelopmental disorders. In particular, some information is available about the molecular events associated with the transition from primate ES to neural cells. A search for proteins involved in mouse and monkey neurogenesis from ES cells to NS cells and neurons using two-dimensional gel electrophoresis and peptide mass fingerprinting and NSSs as *in vitro* models have identified seven proteins in mouse and 34 in monkey, all of which specifically change during neuronal differentiation^[45-47]. In these proteomic analyses, galectin-1 is identified as a protein which transiently expresses in NS cells during neuronal differentiation of mouse ES cells. This protein is well known to interact with extracellular matrix including laminin and fibronectin, and is involved in neuronal path-finding, neurite outgrowth and axon fasciculation^[48-50]. Interestingly, the expression of galectin-1 protein does not change during the conversion of monkey ES cells to neural cells, which is reminiscent of the differences in the mechanisms of neural differentiation of mouse and monkey ES cells. Taken together, these results provide valuable insights into the molecular basis of differentiation and provide novel molecular markers to assess neural cell types during early neurogenesis.

Highly pure and homogeneous, cell populations would likely improve signal-to-noise ratio, resulting in a reliable determination of molecular functions. Although neurospheres derived from neural tissues involve NS cells amplified *in vitro* and maintain the spatiotemporal specific identities of the original tissues, cell populations of the neurospheres are likely to be heterogeneous^[51]. In contrast, neural differentiation protocols realize highly pure cell populations of neural cells, particularly NS cells as described above. The expression patterns of genes encoding three BMP/RA-inducible neural-specific pro-

teins (BRINPs) have been assayed during neuronal differentiation of mouse ES cells by the NSS method to determine the functions of these genes associated with the cell-cycle regulation of NS cells^[52]. While any *BRINP* genes, *BRINP1*, 2 and 3, express in mouse ES cells with no significant difference, *BRINP1* and 2 highly express in the mouse NSS-derived NS cells. Besides, the *BRINPs* are able to suppress cell cycle progression in NS cells. In a further study, using *BRINP1* knockout mice to clarify the physiological functions of this protein in the CNS, the absence of *BRINP1* caused the deregulation of neurogenesis and impaired neuronal differentiation in the adult hippocampal circuitry^[53].

Neural stem sphere-derived homogenous neural stem cells for biological research

The self-renewal and multipotency of NS cells are restricted dramatically as neurogenesis progresses *in vivo*^[54]. During early neurodevelopmental stages, most NS cells divide symmetrically, generating indistinguishable daughter cells. This proliferation under strict spatiotemporal control declines rapidly, and NS cells gradually produce neurons and glia by asymmetric cell divisions. However, NS cells isolated from embryonic tissue samples may not be stably handled *in vitro*, making it difficult to analyze their properties associated with “stemness” *in vitro*. In contrast, NS cells prepared from ES cells *via* the formation of NSSs stably proliferate without neural differentiation on an adhesive substrate with growth factors^[47]. Using these homogeneous mouse NS cells, we have examined the effects of the mitogens, FGF-2 and EGF^[55]. Culture with these mitogens enhances the proliferation of NS cells in dose-dependent manners. Subculture of the cells at least five times does not reduce the potential of these cells to self-renew or their multipotency. These results suggest that NS cells prepared from ES cells can actively proliferate under culture conditions containing FGF-2 or EGF. In addition, these homogeneous mouse NS cells can be differentiated almost exclusively into astrocytes solely by withdrawing growth factors from the medium, and that astrocytogenesis occurs through a default pathway^[56].

Physical stimuli, including X-irradiation, heat shock, stretch and hypoxia, induce differentiation, proliferation and apoptosis at cellular levels, causing pathogenesis in the CNS *via* ectopic neural differentiation and the degeneration of neural cells *in vivo*^[57-62]. However, the complexities of CNS make it difficult to determine whether these effects are directly due to physical stimulation. We have previously investigated the responses of mouse NS cells to X-irradiation, which causes congenital brain abnormalities^[63]. Homogeneous NS cells irradiated with X-rays at a dose of 1 Gy maintain the capacity to proliferate and differentiate, although proliferation arrests temporarily. In contrast, cells cease proliferation following irradiation with > 5 Gy, suggesting that irradiation of the fetal brain at relatively low doses may cause congenital brain abnormalities, as does irradiation at relatively high doses.

Hyperthermia during pregnancy is a significant cause

of reproductive problems, ranging from abortion to congenital defects of the CNS, including neural tube defects and microcephaly. We have tested the effects of heat shock on homogeneous proliferating mouse NS cells^[64]. After heat shock at 42 °C for 20 min, the NS cells show stable proliferation, with few changes in gene expression and cell survival and proliferation. In contrast, heat shock at 43 °C causes a variety of responses, including the up-regulation of genes encoding heat shock proteins, induction of apoptosis, temporal inhibition of cell proliferation and retardation of neural differentiation. Finally, heat shock at 44 °C results in severe effects, with almost all cells disappearing and the remaining cells losing the capacity to proliferate and differentiate. These temperature-dependent effects of heat shock on NS cells may provide insight into the mechanisms by which hyperthermia during pregnancy causes various reproductive problems.

Application of homogeneous neural stem cells to cell transplantation therapies

Primate ES cells have the potential to differentiate into various functional neurons, suggesting that these cells may provide donor cells for cell transplantation therapies in patients with incurable neurodegenerative disorders. Cells are transplanted into patients with Parkinson's disease (PD) primarily for their ability to secrete dopaminergic neurotransmitters into the putamen without functional neural circuits. In one clinical application of stem cells, NS cells derived from cynomolgus ES cells have been implanted unilaterally into the putamen of two cynomolgus monkeys with chronic PD, generated by systemic administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine^[65]. Positron emission tomography (PET) reveals significantly increased uptake of PET multitracers, isotope-labeled L-DOPA and β -CFT in the grafted putamen, demonstrating that transplantation of NS cells derived from cynomolgus monkey ES cells can restore DA function in the putamen of a primate model of PD.

In addition to PD, spinal cord injury (SCI) is another degenerative disorder which cannot be rectified by current therapies to the extent desired by patients suffering from devastating traumata. To develop a novel radical cure for SCI, astrocytes generated from mouse iPS cell-derived NSSs have been transplanted into the lesions of injured rat spinal cords^[66]. Transplant recipients lived for 8 wk without tumor formation. Although locomotive tests demonstrated no improvement compared with control rats, the cell-transplantation led to greater sensitivity to mechanical stimuli. Taken together, these results partially allay a safety concern regarding tumor formation from the transplanted astrocytes, and emphasize the need to determine optimal conditions for the transplantation, *e.g.*, type of neural cell and homogeneity of transplanted cell population.

CONCLUSION

The NSS method is a simple protocol for inducing the

unidirectional neuronal differentiation of pluripotent stem cells by using astrocyte-conditioned medium prepared from serum-free medium. Analyzing the process of this neuronal differentiation can increase the opportunity to explore novel findings during early neurogenesis. These findings deepen the understanding of both the sophisticated mechanisms underlying neurogenesis and the biological variations in neural cells among animals. This, in turn, may provide insights enabling the determination of the cellular etiologies of neurodegenerative disorders and neuropsychiatric diseases. Well-characterized and homogeneous NS cells prepared by the NSS method may act as donor cells for cell transplantation therapies. In addition, the powerful platform based on NSS method will be utilized in a high-throughput, cell fate assay system to assess the effects of innumerable chemical compounds and physical stimuli suspected of being teratogens. This may result in a potentially safer environment in the near future.

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