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Use of bone morphogenetic proteins in mesenchymal stem cell stimulation of cartilage and bone repair

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Abstract

The extracellular matrix-associated bone morphogenetic proteins (BMPs) govern a plethora of biological processes. The BMPs are members of the transforming growth factor- β protein superfamily, and they actively participate to kidney development, digit and limb formation, angiogenesis, tissue fibrosis and tumor development. Since their discovery, they have attracted attention

for their fascinating perspectives in the regenerative medicine and tissue engineering fields. BMPs have been employed in many preclinical and clinical studies exploring their chondrogenic or osteoinductive potential in several animal model defects and in human diseases. During years of research in particular two BMPs, BMP2 and BMP7 have gained the podium for their use in the treatment of various cartilage and bone defects. In particular they have been recently approved for employment in non-union fractures as adjunct therapies. On the other hand, thanks to their potentialities in biomedical applications, there is a growing interest in studying the biology of mesenchymal stem cell (MSC), the rules underneath their differentiation abilities, and to test their true abilities in tissue engineering. In fact, the specific differentiation of MSCs into targeted cell-type lineages for transplantation is a primary goal of the regenerative medicine. This review provides an overview on the current knowledge of BMP roles and signaling in MSC biology and differentiation capacities. In particular the article focuses on the potential clinical use of BMPs and MSCs concomitantly, in cartilage and bone tissue repair.

Key words: Mesenchymal stem cells; Cartilage; Bone repair; Bone morphogenetic protein

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Core tip: Since their first identification, bone morphogenetic proteins (BMPs) have attracted the attention for their potential therapeutic use in tissue engineering and biomedical regenerative therapies. In particular, BMP2 and BMP7 have been successfully used in the treatment of a number of cartilage and bone defects, although these strategies present a certain number of concerning side effects. Also in the field of mesenchymal stem cell (MSC) biology there is a continually growing interest, especially in the regulation of their differentiation, and in demonstrating their utility in tissue engineering.

The review focuses on the current knowledge of BMP physiological roles in MSC biology and differentiation capacities. In particular it highlights the potentialities of the concomitant clinical use of BMPs and MSCs in cartilage and bone tissue repair.

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INTRODUCTION

The extracellular matrix (ECM)-associated bone morphogenetic proteins (BMPs) govern a plethora of biological processes^[1]. The BMPs are members of the transforming growth factor- β (TGF- β) protein superfamily^[2], and they actively participate to kidney development, digit and limb formation, angiogenesis, tissue fibrosis and tumor development^[3]. In particular, these proteins are upregulated in the limb bud epithelium playing a crucial role in the proliferation and differentiation of resident mesodermal progenitors^[4]. Thus, the dysregulation of the BMP signaling pathway has dramatic consequences for the development in mammals. As a matter of fact, mutations in BMP receptors impairing the BMP signaling are implicated in important vascular conditions and skeletal abnormalities^[5]. On the other hand, since BMPs are important morphogens in embryogenesis and development, and also regulate the maintenance of adult tissue homeostasis, their mutations lead to a wide spectrum of both skeletal and extraskeletal abnormalities^[3,6]. First of all BMP2 and 4 null mice are embryonic lethal demonstrating the fundamental role of these proteins in the early development. In general, mutations affecting BMPs are associated to various skeletal defects such as the short ear phenotype (BMP5), polydactyly (BMP4 and 7), abnormalities in rib formation (BMP7), smaller long bones (BMP6), chondrodysplasia (BMP14), bone fusions (BMP13) spontaneous fractures (BMP2 and 5) and osteogenesis imperfecta (BMP1)^[3]. For what concerns extraskeletal abnormalities many BMPs are involved in the development of the brain (BMP2, 4, 5 and 11), while BMP4 defects lead to various organ abnormalities. Mutations in BMP7 lead to severe defects in kidney and eye development; BMP6 and BMP8 are associated to decreased fertility and BMP9 to an abnormal lymphatic development^[6]. Because of these diverse functions in all organ systems, it has been suggested that BMPs deserve to be called body morphogenetic proteins^[7].

BMPs can upregulate growth factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor and insulin-like growth factor 1 (IGF1)^[8]. In particular, the expression of specific BMPs is induced during early recruitment of mesodermal progenitors,

namely mesenchymal stem cells (MSCs) and is sustained throughout osteogenic and chondrogenic differentiation until formation of woven bone^[4,9]. MSCs are multipotent cells resident in many tissues such as bone marrow, adipose tissue and periosteum^[10]. Thanks to their potential biomedical applications there is a growing interest in studying MSC biology, mainly their differentiation capacities, and in testing their true abilities in tissue engineering^[11,12]. In fact, the specific differentiation of MSCs into targeted cell-type lineages for transplantation into sites of injury is a primary goal of the regenerative medicine^[12,13].

This review summarizes the current knowledge of BMP roles in MSC biology and lineage differentiation focusing in particular on the potential clinical use of BMPs and MSCs in cartilage and bone tissue repair.

BMPs

BMPs were originally shown to induce cartilage formation and ectopic bone growth *in vivo*^[14] and are known to set up, foster and support chondrogenesis and osteogenesis^[15,16].

Approximately 20 members of the BMP family are known^[17,18]. In particular they have been grouped into several subfamilies which members are often redundant: The bona fide BMP subfamily (from BMP1 to BMP15), the osteogenic protein (OP) subfamily (OP1, OP2 and OP3 alias BMP7, BMP8, and BMP8b, respectively), the growth differentiation factor subfamily (GDF1, GDF2/BMP9, GDF3, GDF5/BMP14, GDF6/BMP13, GDF7/BMP12, GDF8, GDF9, GDF10 and GDF11/BMP11) and finally the cartilage-derived morphogenetic proteins (CDMP1 and CDMP2 alias BMP14 and BMP13, respectively)^[3,19]. BMPs are synthesized as large inactive precursors containing a N-terminal signal peptide followed by a prodomain controlling appropriate folding and a C-terminal mature polypeptide^[20].

Once secreted, BMPs mainly act as homodimers^[21] and they can be recognized by homodimeric antagonists like gremlin and noggin, which in turn restrict their biological activity^[22].

BMPs bind to two types of serine/threonine kinase receptors, namely type I (BMPR- I) and type II receptors (BMPR- II)^[23]. BMPs preferentially engage three different type II receptors and also three different type I receptors^[24]. Once bound to a BMPR- I , the ligand/receptor complex recruits BMPR- II , which in turn phosphorylates the BMPR- I on its cytoplasmic domain containing a glycine/serine rich domain (GS domain)^[5]. Upon ligand binding, the BMP signal is transduced to target genes through the Smad-dependent (canonical pathway) or the Smad-independent pathways (Figure 1). The Smad proteins are homologues of *D. melanogaster* mothers against decapentaplegic and related *C. elegans* *Sma* gene^[25]. They can be distinguished upon their functions or their activators. In particular, Smad1/5/8 (R-Smads) are so-called receptor Smads

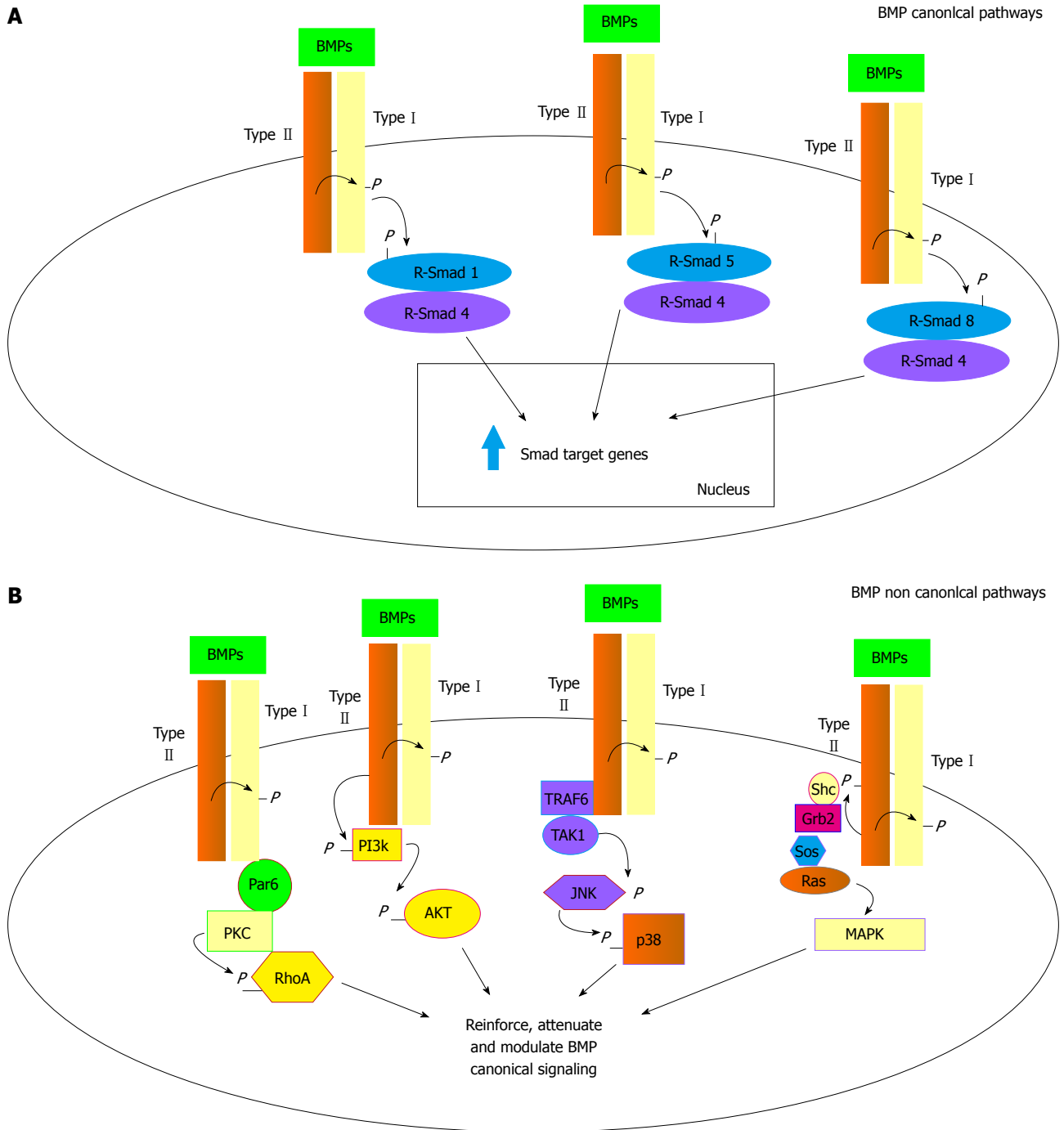


Figure 1 Canonical and non-canonical bone morphogenetic protein signal transduction. A: BMP canonical pathway: Upon type I and type II receptor dimerization the R-Smads 1, 5 and 8 can be phosphorylated by activated type I receptor leading to coupling with the common R-Smad4 coactivator. The heterodimers then translocate to the nucleus promoting expression of target genes; B: BMP non canonical pathways: activation of type I and type II receptors after dimerization can lead to stimulation of various intracellular transduction signals other than the R-Smads dependent ones. From left to right: Activation of the PKC/RhoA pathway through par6 recruitment by type I activated receptor; activation of the PI3K/AKT pathway through direct PI3K phosphorylation by activated type II receptor; activation of JNK/p38 pathway through TRAF6/TAK1 recruitment by activated type II receptor and finally activation of the MAPK/ERK pathway through Shc/Grb2/Sos/Ras recruitment by type II activated receptor. BMP: Bone morphogenetic protein; Par6: Partitioning defective protein 6; PKC: Protein kinase C; PI3K: Phosphoinositide-3 kinase; AKT: V-akt murine thymoma viral oncogene homolog 1; TRAF6: TNF receptor associated factor 6; TAK1: TGF-beta activated kinase 1; JNK: C-Jun N-terminal kinase; Shc: Src homology 2 domain containing; Grb2: Growth factor receptor bound protein 2; Sos: Son-of-sevenless; RAS: Rat sarcoma viral oncogene homolog; MAPK: Mitogen activated protein kinase.

and are triggered by BMPs *via* BMPR- I recruitment and activation. Once the R-Smads have been phosphorylated they form a DNA binding heterodimer with the mediator Smad4^[26,27] (Figure 1A). In the nucleus, the active dimer promotes the transcription of BMP target genes

through recognition of Smad-binding sequences or GC-rich elements present in the promoters of such genes^[5]. This specific transduction pathway is finely regulated both by extracellular and intracellular mediators and signals. Intracellular signals encompass proteasome-

Table 1 The use of bone morphogenetic proteins in the induction of chondrogenesis and osteogenesis in mesenchymal stem cells *in vitro*

BMP type and conditions	Cell type	Ref.
Chondrogenesis		
BMP2 (micromass + TGF- β 3)	BM MSCs	[41,45,46]
BMP2 (3D alginate beads)	BM MSCs	[37]
BMP4 (micromass + TGF- β 3)	BM MSCs	[41]
BMP6 (micromass + TGF- β 3)	BM MSCs	[41]
BMP7 (micromass + TGF- β 3)	BM MSCs	[45,46]
BMP9 (3D alginate beads)	BM MSCs	[37]
BMP2 (3D agarose)	Synovial explant MSCs	[43]
BMP7 (3D agarose)	Synovial explant MSCs	[43]
BMP7 (monolayer)	Adipose derived MSCs	[44]
BMP7 (monolayer + Asc)	C3H10T1/2 (multipotent fibroblasts)	[54]
BMP2 (monolayer)	MC615 (chondrocyte precursors)	[53]
BMP4 (monolayer)	MC615 (chondrocyte precursors)	[53]
Osteogenesis		
BMP2 (monolayer)	Adipose derived MSCs	[44]
BMP2 (monolayer + Dexa and Asc)	BM MSCs	[56,62]
BMP3 (monolayer + Dexa and Asc)	BM MSCs	[62]
BMP4 (monolayer + Dexa and Asc)	BM MSCs	[62]
BMP5 (monolayer + Dexa and Asc)	BM MSCs	[62]
BMP6 (monolayer + Dexa and Asc)	BM MSCs	[62]
BMP6 (monolayer)	BM MSCs	[58]
BMP7 (monolayer + Dexa and Asc)	BM MSCs	[62]
BMP8a (monolayer + Dexa and Asc)	BM MSCs	[62]
BMP7 (monolayer + Asc)	C3H10T1/2 (multipotent fibroblasts)	[55]
BMP7 (monolayer + Asc)	MC3T3-E1 (committed osteoblasts)	[54]

MSC: Mesenchymal stem cell; BMP: Bone morphogenetic protein; Asc: Ascorbic acid; TGF: Transforming growth factor.

promoted degradation^[28], inhibition by Smad6 and 7 factors which impair Smad4 mediator binding, and protein phosphorylation/dephosphorylation processes^[29]. The BMP/Smad signaling pathway can be also strictly regulated by a group of extracellular protein antagonists that directly bind to the BMPs and prevent the interaction with their receptors such as gremlin, chordin, noggin and follistatin^[1,17,30]. In most cases BMP antagonist expression is finely regulated in a temporospatial manner during the development. Their fundamental role as antagonists in BMP signaling is attested by a number of severe or lethal defects occurring in experimental animals lacking one of these proteins^[1]. In addition to Smad canonical pathway several non canonical pathways have been described so far. Through their type I and type II receptors, the TGF family members have been shown to activate the JNK/p38, the MAPK/ERK, the PI3K/AKT and the PKC/RhoA transduction pathways (Figure 1B)^[6,31]. The PI3K/AKT pathway seems to be recruited by the direct phosphorylation of a PI3K serine residue by a type II activated receptor. Alternatively the MAPK pathway can be activated through the docking of the Shc and Grb2 MAPK mediators recognizing three sites of rare autophosphorylated tyrosines on the type II activated receptors. Activated type II receptor can also bind to TRAF6 protein triggering the TGF- β -activated kinase 1 and leading to the JNK/p38 pathway activation. And finally an activated type I receptor can bind to the par6 scaffold protein able to recruit the PKC and RhoA proteins^[6,31]. These pathways in addition to

Smad signaling have been demonstrated to alternatively reinforce, attenuate or otherwise modulate downstream BMP cellular responses^[32,33].

BMPs IN BONE FORMATION

In vertebrates, bone formation can be achieved by direct differentiation of osteoblasts in membranous ossification, or starting from differentiation of chondrocytes in endochondral ossification^[34,35]. These two processes are directed by BMPs, with BMP2 and BMP4 acting as the master differentiation triggers of osteoblast and chondrocyte phenotypes leading to bone and cartilage formation^[35].

BMP2 and BMP4 drive bone formation through the Smad1/5/8 signaling pathway described earlier. This pathway is common to osteoblasts and chondrocytes and its precursors and is strictly regulated in these cells^[1]. BMPs are released in a mature form from osteoblasts and may interact with their cell surface receptors or bind to proteins of the ECM. In the latter case the ECM acts as a "reservoir" of BMPs for future paracrine signaling^[5]. In regard to this, a number of transcription factors necessary to cartilage and bone formation have been acknowledged regulating downstream BMP signaling^[35].

The chondrogenic potential of different BMPs has been tested because of the eventual clinical applications in cartilage repair tissue engineering (Table 1). BMP2, 4, 6, 7 and 9 have been reported to induce *in vitro* chondrogenesis of human MSCs^[36-42].

In human bone marrow-derived (BM) MSCs, BMP2 (in the presence of TGF- β 3) was the most efficient inducer of chondrogenesis with the production of a proteoglycan rich cartilage over BMP4 and BMP6^[41] while in synovial explants, BMP7 was a more effective trigger of chondrocyte differentiation than BMP2^[43]. BMP7 could also stimulate chondrogenic differentiation in adipose tissue-derived stem cells^[44] while in other studies Shen *et al*^[45,46] demonstrated that both BMP2 and BMP7 enhance TGF- β 3-mediated chondrogenic phenotype of BM MSCs *in vitro*. In another study, BMP9 and BMP2 used separately and in absence of TGF- β stimulation enhanced the expression of cartilage transcription factor Sox-9 followed by induction of type II collagen, aggrecan and cartilage oligomeric matrix protein in BM MSCs^[37]. In addition, BMP13 and 14, also called CDMP2 and 1 respectively, demonstrated to be necessary for stimulation of early chondrogenesis and chondrocyte differentiation (BMP14/CDMP1) as well as in the terminal differentiation of chondrocytes in the final stage of hypertrophy and mineralization *in vivo*^[19]. *In vivo*, BMP7 has also shown a marked anabolic activity in cartilage and bone^[47,48] and it has demonstrated to act synergistically with microfractures to boost cartilage repair^[49]. Related to this, Mishima and Lotz^[50] have more recently demonstrated that BMP4 and 7 elicit a significant chemotactic *in vitro* response from human MSCs suggesting that the use of these factors *in vivo* promotes directed cell migration in sites of injury for cartilage repair in transplanted engineered tissues.

For what concerns osteoinduction, studies of pre-natal bone development as well as of fracture repair^[9,51,52] showed the expression of a plethora of BMP genes with temporospatial variability. In particular, early experiments using human recombinant BMP2, BMP4 BMP6 or BMP7 demonstrated that such proteins are able to individually stimulate osteoblastic (or chondrogenic) phenotypes in a variety of mesenchymal precursor cell lines (Table 1)^[53-58]. However, differently from *in vitro* studies, *in vivo* investigations indicate that BMPs work in a coordinated fashion^[52,59]. In particular, BMP2 can be described as a necessary constituent orchestrating the signaling pathway that regulates fracture repair^[60-62]. Differently, BMP7 is undetectable in the MSC differentiating system, but when exogenously added may play the same function of one of the endogenous BMPs physiologically produced by the cells^[62].

As a matter of fact, both BMP2 and BMP7 are now approved in clinics for the treatment of non-union fractures as adjunct therapies^[63]. In particular human recombinant BMP2 is sold from Medtronic (Minneapolis, MN, United States) with the acronym of In FUSE®, while hrBMP7 is sold from Stryker (Kalamazoo, MI, United States) with the acronym OP-1.

Although the use of these molecules in fracture healing has been welcomed by physicians with great enthusiasm, it must be emphasized that several, clinically relevant, adverse effects have been reported especially at BMP high dosages. The most frequently

described effect is the development of antibodies against BMPs even if this event does not seem to have real adverse consequences^[64]. Differently, serious concerns raised from the observation that application of BMPs to a fracture site could result in increased bone resorption as a primary event. As a consequence a higher nonunion rate has been observed in a number of patients leading to termination of BMP use in several clinical settings^[65]. Furthermore, local inflammatory responses have also been reported at several anatomical sites, with different degrees of severity^[66]. Finally BMP use has also been associated to wound healing complications^[66], hematoma formation^[67] and several cases of heterotopic bone formation^[67]. Thus, we can conclude that the dosage of these powerful molecules needs to be finely calibrated in each clinical setting and in any case reserved to patients in which the risks associated to BMP use are clearly outweighed by the higher risks of fracture healing failure.

MSCS

Repair of adult bone involves BM MSCs which serve as a source of osteochondral progenitors able to invade the fracture site, proliferate and differentiate into cartilage and bone. MSCs are multipotent adult cells that have the ability to self-renew and differentiate into multiple lineages^[10] that were discovered in 1980^[68] but only fully recognized in 1994^[69]. MSCs have recently gained increasing attention for their potential in the regenerative medicine. The main reasons for this interest are the relative ease of isolation from several adult tissues and suitable expansion in culture and the high degree of plasticity of these cells. Currently, at least 198 registered MSC clinical trials are ongoing (www.clinicaltrials.gov), as well as autologous and allogeneic MSC products accepted for use in bone repair in a number of international jurisdictions (Mesoblast_Media_Release by Mesoblast Ltd., Melbourne, Australia; Osteocel by Osiris therapeutics Inc., Columbia, MD, United States)^[70]. Despite their apparent therapeutic potential, clinical applications of MSCs have been restricted due to the limited understanding of the factors that regulate their fate and activity. Another limiting factor is the lack of knowledge of the complex interplay between these cells and the components of their niche or immediate microenvironment. Due to the disposition of MSC to differentiate into osteoblasts and chondrocytes, and their attested clinical potential in bone tissue engineering, a great amount of research has been centered on the identification of the factors governing osteogenesis *in vitro* and *in vivo* (*i.e.*, TGF- β 1, 2 and 3, BMPs and PDGF)^[71,72].

MSCS IN CHONDROGENIC AND OSTEOGENIC DIFFERENTIATION

The chondrogenic differentiation occurs when MSCs are

seeded in serum-free, 3D culture format in the presence of one or more TGF- β superfamily members^[73]. In this asset, cells abandon the typical fibroblastic morphology and start producing cartilage-specific matrix components. *In vitro* chondrogenesis is usually obtained by the micromass pellet culture system, allowing the necessary cell-cell interactions which resemble what occurs in pre-chondrogenic condensations in the embryonic development^[74]. In these conditions cells usually differentiate in no more than 2-3 wk into chondrocyte-like cells secreting proteoglycans. Pellets are bordered by a narrow capsule of connective tissue, almost cell-free and rich in type II A collagen. The advancement to terminal differentiation is attested by accumulation of type X collagen and matrix mineralization^[75]. When BMPs are added in this experimental setting, namely MSCs in micromass culture and in the presence TGF- β , they enhance chondrogenic differentiation and cartilage formation significantly (see Table 1 for the various BMP employed). The 3D culture and the concomitant presence of TGF- β seem to be necessary to attain a real chondrocytic phenotype. Thus, it is possible that in the mesenchymal precursor chondrocyte differentiation occurs only when strict cell-cell interactions are established and when the parallel activation of different R-Smad pathways is achieved by different members of the TGF- β superfamily. In particular, the TGF- β members activating the Smad2/3 and the BMP members activating the Smad1/5/8 (see Figure 1A).

Differently, MSCs undergo an osteogenic differentiation when cultured with the opportune osteoinduction factors on two dimensional substrates. In this case, osteogenesis is promoted by a large spread area, while in the same conditions the reduction of the spread area induces adipogenesis^[76,77]. In this experimental setting, namely MSCs in 2D wide spread areas, several BMPs used alone or in the presence of ascorbic acid have demonstrated to promote significant osteoblast differentiation (see Table 1 for the various BMP employed). In the presence of BMPs, progenitor cells achieve an osteoblastic phenotype expressing several bone-characterizing ECM proteins. In particular they express type I collagen, osteopontin, osteocalcin and bone sialoprotein, and produce high levels of the alkaline phosphatase (ALP) ecto-enzyme. Sustained expression of ALP is required for mineralization of skeletal tissues^[78,79], and is induced early during osteoblast differentiation^[80,81].

Several studies have explored the use of MSCs encapsulated in osteoinductive scaffolds or morphogenic biomaterials to enhance the natural healing process of bone and cartilage *in vivo*^[82-86]. They overall suggest that these multipotent cells seem both able to differentiate themselves within the scaffolds as well as to secrete factors attracting neighboring autologous progenitors. This behavior can accomplish fracture healing faster and with a superior quality of the resulting new bone respect to the osteoinductive or chondrogenic scaffolds used alone^[13]. Thus, these promising results have prompted

the accomplishment of several studies exploring the concomitant use of MSCs and of the most promising members of the BMP family. Namely BMP2 and 7, embedded in suitable scaffolds or carriers, have been used to heal several cartilage defects and bone fractures in experimental animal models hopefully soon to be transferred to human beings.

USE OF BMPS AND MSCS IN CARTILAGE REPAIR

Cartilage defects such as degeneration of intervertebral discs and knee joints are ordinary causes of joint disabilities able to affect the quality of life of many people all over the world^[87]. It is well known that articular cartilage has a limited capacity of spontaneous repair after damage^[88]. Treatments for articular surface lesions usually encompass various clinical approaches like conservation therapies as well as invasive surgery comprising abrasion, debridement and perichondral grafting^[87,89]. In recent times also autologous chondrocyte regeneration has been used. Grafting of autologous chondrocytes to promote cartilage resurfacing has some benefits over allogeneic chondrocyte or solid tissue grafting and other procedures^[90]. Unfortunately, its application is hindered by chondrocyte de-differentiation during *in vitro* expansion and the necessity of large amounts of cartilage samples^[91]. Recently, the appearance of MSCs in the landscape of the cellular sources for cartilage repair available in quite large quantities raised a great interest and optimism for the treatment of these defects by tissue engineering and cell therapy approaches^[92]. As already mentioned, both BMP2 and BMP7 have plenty demonstrated the ability to enhance cartilage repair *in vivo* as well as the capacity to promote chondrogenic differentiation of MSCs cultured in appropriate inducing media *in vitro*. Although the outcome of the combined use of precursor cells and BMPs in suitable scaffolds for cartilage repair could be a research field actively persecuted in these years, to date a limited number of studies are present in the literature (Table 2). In particular, one of the major unresolved problems is a durable integration between cartilage and the scaffold^[93]. Thus, the presence of chondrogenic precursors releasing chemoattractant factors and of appropriate BMPs stimulating said precursors could ensure the ultimate scaffold remodeling with new cartilaginous tissue formation. Furthermore, both MSCs and BMPs seem able to stimulate endogenous cells to migrate and colonize the artificial graft further promoting the final healing.

In early studies Grande *et al.*^[94] transfected MSCs from periosteum with human BMP7 or sonic hedgehog and then seeded them on bioresorbable polymer scaffolds. These implants were used to fill full-thickness osteochondral defects created in the mid-trochlear region of New Zealand white rabbits. The authors observed that, for both genes, their addition significantly

Table 2 Use of mesenchymal stem cells and bone morphogenetic proteins in cartilage and bone defects *in vivo*

Conditions	Scaffold	Ref.
MSCs and BMPs in cartilage defects		
MSCs transfected with BMP7	Bioresorbable polymer scaffold	[94]
MSCs transfected with BMP7 and TGF- β 1	Bilayered osteochondral scaffold	[95]
MSCs + TGF- β 1, PDGF and BMP2	Bilayer scaffold with platelet rich plasma	[96]
MSCs + TGF- β 1, TGF- β 3, BMP2, 4 and 7	Osteochondral allograft with extracellular matrix proteins	[98]
MSCs and BMPs in bone defects		
MSCs transfected with BMP2	Animal models of ectopic and orthotopic bone formation	[103-110]
MSCs transfected with BMP2	Alginate or type I collagen hydrogels	[111]
MSCs transfected with BMP2	Injectable chitosan biopolymer and inorganic phosphate	[112]
MSCs + BMP2	Macroporous β -tricalcium phosphate deposited by robocasting	[72]
MSCs + BMP7	Natural bone mineral particles	[113]
MSCs + BMP7	3D collagen nanofiber implant	[114]

MSC: Mesenchymal stem cell; BMP: Bone morphogenetic protein; TGF: Transforming growth factor.

enhanced the quality of the repaired tissue, also noticing that the subchondral compartment in the animal group receiving the BMP7-transfected cells seemed to remodel with bone much faster than the sonic hedgehog group.

In another study Chen *et al.*^[95] formulated a bilayered gene-activated osteochondral scaffold containing a TGF- β 1 plasmid for the chondrogenic layer and a BMP2 plasmid for the osteogenic layer. MSCs seeded in each layer were able to differentiate to chondrocytes and osteoblasts both *in vitro* and *in vivo* supporting the articular cartilage and subchondral bone regeneration in the rabbit knee osteochondral defect model.

In a recent study Seo *et al.*^[96] investigated the use of bilayer scaffolds embedded with MSCs and platelet rich plasma (PRP) containing TGF- β 1 and PDGF for the chondrogenic layer, and MSCs and BMP2, for the osteogenic layer, on the osteochondral defect in an equine model. The defects were produced at the lateral trochlear ridge of the talus, where osteochondrosis is commonly found, and bilayered scaffolds were inserted. Tissue repair was then evaluated showing that implantation of the scaffolds significantly improved osteochondral tissue regeneration respect to controls. Differently, non-ameliorative results were obtained by Gulotta *et al.*^[97] testing the use of BMP13-expressing MSCs to improve regeneration of the tendon-bone insertion site in a rat rotator cuff repair model. This study was prompted by the observation that BMP13 has been implicated in tendon and cartilage repair and thus may augment rotator cuff repair. The results showed that new cartilage formation and collagen fiber deposition was observable in both experimental groups (MSCs expressing or non-expressing BMP13) with no significant differences between the two.

Finally, a recently published study from Geraghty *et al.*^[98] describes a novel, viable osteochondral allograft containing ECM proteins and chondrogenic growth factors (*i.e.*, TGF- β 1 and 3, BMP2, 4, 7, bFGF and IGF1) able to stimulate MSC migration and chondrocyte differentiation *in vitro* as well as cartilage repair *in vivo* in a goat microfracture model.

Taken together all the above mentioned studies

show that the use of BMPs associated to MSCs to promote articular cartilage repair has brought limited favorable results. Differently, the use of other chondrogenic induction factors such as TGF- β proteins, or heterogeneous cocktails of factors such as PRP or the ones embedded in the new osteochondral allograft described by Geraghty *et al.*^[98] have demonstrated more positive outcomes. This has happened likely because these cocktails of factors, also containing BMPs, hold more promising results than the use of BMPs alone in cartilage repair. Indeed, BMPs together with MSCs have shown a higher osteoinductive ability *in vivo* more than chondrogenic.

USE OF BMPS AND MSCS IN BONE TISSUE REPAIR

Over one million surgical procedures, and in the United States only, each year deal with bone replacement^[99]. Skeletal diseases, tumor resection, trauma and congenital malformations are the main reasons for bone defects requiring bone reconstruction. For decades, autologous bone graft has been the gold standard for treatment of bone defects in clinic. Due to limited availability of autologous bone grafts and morbidity of donor sites, stem cell-based tissue engineering strategies are very promising as an alternative therapeutic approach. The use of allogeneic transplantation is restricted due to immunological rejection, premature resorption and possible transmission of infections. Bone generated from human recombinant BMPs alone^[100], or embedded in a demineralized bone powder^[101], has a limited volume. In addition, biocompatible bone substitutes^[102] are subjected to infection and require osteoinductive molecules or tissues for large bone defects. Recently, the use of progenitor MSCs embedded in biocompatible and biodegradable scaffolds, possibly in the presence of growth or osteoinductive factors, has allowed the creation of functional tissues (Table 2).

In early studies a number of researchers showed that autologous or allogeneic MSCs engineered with BMP2 were capable of differentiating into the osteoblast

lineage and inducing bone formation in several animal models in both ectopic and orthotopic sites in mice, rats, rabbits and pigs^[103-107]. In all these systems the authors concluded that combining MSC implantation with *BMP2* gene transfer more effectively induced bone formation than MSC implantation alone.

With similar results, but using a different modular expression system approach Moutsatsos *et al*^[108] used a tetracycline-regulated expression vector encoding human *BMP2* to transfect a MSC cell line. With such expression system the authors were able to demonstrate that doxycycline controlled *BMP2* expression and thus controlled MSC osteogenic differentiation both *in vitro* and *in vivo* in a mouse ectopic bone model. Moreover, they showed increased angiogenesis accompanied by bone formation whenever genetically engineered MSCs were induced to express *BMP2 in vivo*.

In other studies, Chang *et al*^[109] demonstrated the usefulness of *BMP2*-expressing MSCs in bone repair of large cranial defect in two different animal models: The rabbit model and the swine model^[110]. The authors clearly demonstrated near-complete repair of the large cranial defects by the tissue engineered bone containing *BMP2*-expressing MSCs in the three months of the experiment both in the rabbit^[109] and in the swine^[110] with respect to the controls.

Thus, the use *BMP2* together with MSCs in bone repair, either exogenously added to cells either enabling cells to directly express the protein, has been in the years thoroughly validated by the above mentioned studies. Consequently, the attention has been focused on the use of different scaffolds able to support the MSC colonization and differentiation as well as the temporospatially controlled delivery of the BMPs to quicken bone reconstruction and healing. Thus, in this contest were alternatively tested: (1) alginate or type I collagen hydrogels as scaffolds loaded with MSCs expressing *BMP2* for bone regeneration in a large cranial defect repair in the swine demonstrating the superiority of *BMP2*-MSC/collagen type I construct over the alginate counterpart^[111]; (2) an injectable biopolymer of chitosan and inorganic phosphate seeded with MSCs and *BMP2* in a rat calvarial critical size defect demonstrating the superiority of the MSC/*BMP2* coupling over the controls^[112]; and (3) a macroporous β -tricalcium phosphate (β -TCP) system fabricated by robocasting loaded with MSCs and with *BMP2* embedded in microspheres to provide a prolonged *BMP* release in a critical rat calvarial defect^[72]. In the latter case only a minor synergistic effect was demonstrated in the *BMP2*-MSC group with respect to the *BMP2* group alone.

Alternative to these studies only a limited number of works have focused on the concomitant use of *BMP7* and MSCs in bone repair (Table 2). In particular Bura-stero *et al*^[113] used the association of human MSCs and *BMP7*, with natural bone mineral particles as a scaffold to fill the bone loss, to improve bone regeneration in a rat model of critical size segmental bone defect. Indeed a significantly higher score in bone regeneration was

observed in the rats treated with MSCs and *BMP7* compared to controls, receiving either MSCs or *BMP-7*. The data indicated that the association of the two provided a better osteoinductive graft compared to MSCs or *BMP7* alone. Finally, Schiavi *et al*^[114] tested a novel 3D collagen nanofiber implant functionalized with *BMP7* nanoreservoirs and equipped with human MSC microtissues. The implant was optimized for cell colonization, differentiation and growth. The group clearly demonstrated an acceleration of ectopic bone growth *in vivo* of the coupled *BMP7*/MSC microtissues respect to the controls using either *BMP7* or MSC microtissues alone.

CONCLUSION

Since their first identification, BMPs have demonstrated great potentialities in the regenerative medicine and tissue engineering fields. They have been tested in numerous preclinical and clinical studies exploring their chondrogenic or osteoinductive potential in several animal model defects and in human diseases. During the years two BMP members in particular, *BMP2* and *BMP7*, have been thoroughly used in the treatment of a number of cartilage and bone defects and have been recently approved for employment in protocols of nonunion fractures as adjunct therapies.

On the other hand, to date the scientific literature provides extensive *in vitro* evidence of the improvement of the osteoblastic and chondrogenic potential of MSCs, now obtained from many tissues, by treatment with BMPs. Thus, it was just a matter of time for the two, BMPs and MSCs, to be investigated together hopefully to finally achieve the goal of producing the ideal graft for bone replacement. Besides, recently the grafts have evolved including more and more sophisticated scaffolds, appropriate cell precursors and optimal differentiating factors. As outlined in this review, the growing literature in this field and the promising results in recent years suggest that this goal indeed can be achieved and that both BMPs and MSCs in the future will take part to the production of successful avant-garde implants especially designed for bone tissue engineering.

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Targeting head and neck tumoral stem cells: From biological aspects to therapeutic perspectives

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Abstract

Head and neck squamous cell cancer (HNSCC) is the sixth most common cancer in the world. Effective therapeutic modalities such as surgery, radiation, chemotherapy and combinations of each are used in the management of the disease. In most cases, treatment fails to obtain total cancer cure. In recent years, it appears that one of the key determinants of treatment failure may be the presence of cancer stem cells (CSCs) that escape currently available therapies. CSCs form a small portion of the total tumor burden but may play a disproportionately important role in determining outcomes. CSCs have stem features such as self-renewal, high migration capacity, drug resistance, high proliferation abilities. A large body of evidence points to the fact that CSCs are particularly resistant to radiotherapy and chemotherapy. In HNSCC, CSCs have been increasingly shown to have an integral role in tumor initiation, disease progression, metastasis and treatment resistance. In the light of such observations, the present review summarizes biological characteristics of CSCs in HNSCC, outlines targeted strategies for the successful eradication of CSCs in HNSCC including targeting the self-renewal controlling pathways, blocking epithelial mesenchymal transition, niche targeting, immunotherapy approaches and highlights the need to better understand CSCs biology for new treatments modalities.

Key words: Biology; Head and neck neoplasms; Oral cancer; Neoplastic stem cells; Molecular targeted therapy; Radiation therapy; Chemotherapy

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Core tip: The cancer stem cells (CSCs) theory offers an insight into why currently available therapies for head and neck cancer fail so often. Eradication of cancers may

require the targeting and elimination of CSCs, especially for head and neck squamous cell cancer (HNSCC). This represents a challenge because many pathways, such as those involved in self-renewal, are shared by CSCs and their normal counterparts and might lead to major toxicities. Developing radio sensitizing strategies is investigated and appears to eliminate CSCs. Overcoming chemo resistance, radio resistance and immune evasion mechanisms of CSCs remains a cornerstone of novel adjuvant therapies specifically targeting CSCs in HNSCC.

Méry B, Guy JB, Espenel S, Wozny AS, Simonet S, Vallard A, Alphonse G, Ardail D, Rodriguez-Lafrasse C, Magné N. Targeting head and neck tumoral stem cells: From biological aspects to therapeutic perspectives. *World J Stem Cells* 2016; 8(1): 13-21 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v8/i1/13.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v8.i1.13>

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) remains a major health problem throughout the world, with an estimated 500000 new cases diagnosed yearly^[1]. HNSCC refers to a group of cancers that originate in the epithelium of the oral cavity, pharynx and larynx. Currently, therapeutic strategies for HNSCC include surgery, radiotherapy, chemotherapy, concurrent chemoradiation and monoclonal antibodies. Despite progress in the field of oncology, the overall 5-year survival rate of HNSCC is below 50%, unchanged in the last 30 years^[2]. Local recurrence affects about 60% of patients and metastases develop in 20% of cases. Locoregional failure is linked to unfavorable outcome^[3,4]. A new, more strategic approach is needed for the treatment of recurrent head and neck squamous cell carcinoma, as most cases cannot be cured with current therapeutic modalities. The presence of a peculiar subpopulation of cells has been identified in several tumors, including HNSCC: This small population of cancer cells possesses the capability to self-renewal, is highly tumorigenic, and behaves as tumor progenitor cells. Such characteristics are consistent with the features of cancer stem cells (CSCs)^[5,6]. The role of these cells in HNSCC progression and metastasis is a significant point to be further emphasized on for eliminating the disease. Indeed, in addition to their ability for self-renewal, differentiation, and regeneration, CSCs possess significant resistance to radiochemotherapy^[7,8]. Furthermore, by being able to do epithelial mesenchymal transition (EMT), which is a key step in embryogenesis, CSCs might facilitate the metastatic characteristics of tumors^[9-11]. Therefore, targeted elimination of these CSCs could define new therapeutic strategies for head and neck cancer treatment. If the most common method for identifying CSCs relies on the expression of specific cell surface antigens that enrich for cells with CSC properties, their detection within the total tumor

bulk remains a challenge. Indeed, the development of new CSC targeting therapeutic strategies is currently obstructed by the lack of trustworthy markers for the identification of CSCs^[12-14]. Besides, molecular mechanisms at the basis of CSCs origin are yet not fully understood. Nonetheless, targeting self-renewal pathways in CSCs, such as the Wnt, Notch, and Hedgehog pathways, or specific CSC markers, such as CD133, CXCR1, and CD44 may offer therapeutic benefits to head and neck cancer therapy^[13]. In addition to CSC biomarkers, micro environmental factors, such as niche-specific properties constitute obvious potential targets in order to eradicate high-risk HNSCC cells; to abolish the crosstalk between endothelial cells and CSCs in a targeted manner might be relevant for the treatment of head and neck cancer patients^[15]. This review discusses the properties of head and neck tumoral stem cells, outlines initial targeted therapeutic strategies against them, and presents challenges for the future (Figure 1).

CSCS IN HSNCC: IDENTIFICATION, CHARACTERIZATION AND PROPERTIES

Role of stem cell molecular markers

HNSCC are solid tumors with heterogeneous content. Indeed, into the tumor, not all cells possess the capacity for self-renewal and unlimited growth. In tumor architecture, it is widely agreed that CSCs are held accountable for tumor growth whereas differentiated cells usually contribute to the tumor bulk^[5]. CSC populations are defined by four key features: Only a small portion of intratumoral cancer cells can form a new tumor in an *in vivo* xenograft assay, particular cell surface markers allow to identify CSC populations from non-CSC populations, the ability to generate endless copies of themselves through self-renewal, and the potential to give rise to differentiated non-stem cell cancer progeny^[16]. As all chemotherapy regimens often damage normal, rapidly dividing cells, CSC-like populations, with low turnover and infrequent cell cycling, may escape treatment^[17]. Thus, there is an urgent need for early detection of CSCs in the tumor cell population. Identification of CSCs based on increased expression of certain markers in cancerous tissue is the basis of the target therapy which is described later in this review. It is more clear that the development of novel therapeutic strategies will come about through identification of HNSCC CSC populations that regulate tumor growth, metastasis, and treatment resistance. Thanks to the development of immunofluorescence tools, it is possible to more easily isolate CSCs using their surface proteins. The main molecular markers implicated in HNSCC CSC detection are summarized in Table 1.

Role of the CD44 marker

One of the first studies of CSCs in HNSCC using an immunodeficient mouse as model demonstrated that a minor population of CD44+ cancer cells, which account

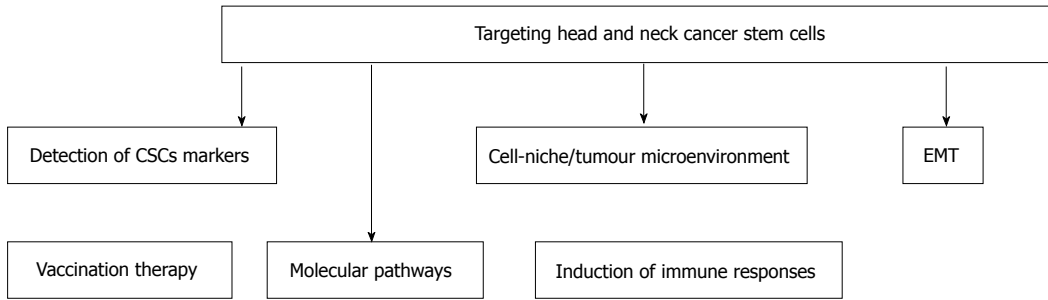


Figure 1 Therapeutic perspectives through a multistrategic approach. CSCs: Cancer stem cells; EMT: Epithelial mesenchymal transition.

Table 1 Main molecular markers implicated in head and neck squamous cell cancer cancer stem cell detection

Ref.	Stem cell marker	Cancer cell lines studied
Prince <i>et al</i> ^[5]	CD44, BMI 1	HNSCC generated in immunodeficient mouse model
Wei <i>et al</i> ^[25]	CD133	HNSCC cell lines (hep-2)
Chen <i>et al</i> ^[21]	ALDH 1	Immunodeficient mouse model
Krishnamurthy <i>et al</i> ^[15]	ALDH	Head and neck squamous cell carcinoma

HNSCC: Head and neck squamous cell cancer; ALDH: Aldehyde dehydrogenase.

for less than 10% of the cells in a HNSCC primary tumor could give rise to new tumors *in vivo* and displayed the ability of self-renewal and differentiation. The CD44 protein is a cell surface glycoprotein that is responsible for cell adhesion, migration and homing. It is a receptor for hyaluronic acid and can also interact with other ligands such as collagen species and matrix metalloproteases^[5]. Takahashi *et al*^[18] demonstrated that cell-cell dissociation and actin remodeling in tumor necrosis factor-induced EMT were mediated by specific interaction between CD44 and hyaluronan; another result was an enhanced motility. CD44+CD24-CSCs play a critical role in tumor progression and metastasis^[19]. Some of HNSCC with CD44s (standard form) and CD44 v6 (alternative splice variant) expressions are associated with a poorer disease-free survival, in laryngeal cancers particularly^[20]. Also, high levels of nuclear BMI-1 were found in CD44+CD24- cells of the tumor population. BMI-1 is a stem cell-related gene involved in the mechanisms of carcinogenesis in head and neck cancers^[5]. By simultaneous evaluating both CD44 and BMI-1, it could lead to precise characterization of the CSC population within the tumor cellular architecture.

Aldehyde dehydrogenase activity

Aldehyde dehydrogenase (ALDH) has also been considered to be a marker for identifying HNSCC CSCs. The ALDH family, of which ALDH1 is a member, is a family of cytosolic isoenzymes, which are highly expressed in many stem and progenitor cells. These enzymes are responsible for oxidizing intracellular aldehydes and contribute to the oxidation of retinol to retinoic acid, in stem cell differentiation notably; moreover, ALDH1 is involved in the resistance of progenitor

cells to chemotherapeutic agents. Many studies have proved the role of ALDH1+ cells in tumorigenesis, metastasis and chemo resistance in HNSCC. For instance, Chen *et al*^[21] showed that ALDH1+ CD44+ cells resist radiotherapy and maintain CSC-like properties in HNSCC cells which allow them to promote tumor propagation^[22]. Recently, Krishnamurthy *et al*^[15] found that the combined use of ALDH1 and CD44 is more relevant for identifying CSC-like populations as it is more selective than any other marker used alone. It is clear that only one marker is not sufficient to identify a pure CSC population in HSNCC. The best chance of developing targeted identification and treatment goes through a panel of markers with a more narrowly definition of CSCs.

Other markers and role of side population cells

Several studies evidenced the abilities of CD133+ stem-like cells: They possess higher clonogenicity, higher tumorigenic potential and are more invasive, in comparison with CD133- cells. CD133+ cells play a crucial role in the resistance to standard chemotherapy with paclitaxel^[23]. CD133 antigen also known as prominin-1 is a glycoprotein that is encoded by the *PROM1* gene. It is a member of pentaspan transmembrane glycoproteins (5-transmembrane, 5-TM), which specifically localize to cellular protrusions. If it was initially considered as a marker for hematopoietic stem cells^[24], it has been then identified as a CSC marker in several cancers and particularly in the laryngeal cancer, using the Hep-2 cell line. Indeed, in an *in vivo* study, CD133+ cells sorted from the Hep-2 cell line had higher tumorigenic potential than CD133- cells^[25]. Higher CD133 levels are found in CD44+ cancer stem-like cells in comparison with CD44- cells in HNSCC, which support the putative

Table 2 Main determinants of cancer stem cell radioresistance

Molecular determinants of radioresistance	Mechanism
Intrinsic determinants	Enhanced DNA repair capability Protection from oxidative DNA damage Activation of the cell survival pathways (PI3K/Akt, WNT/ β -catenin, notch) Expression of drug efflux pumps
Extrinsic determinants	Hypoxic environment

role of CD133+ as a CSC marker. Using CD133 might serve to identify head and neck cancer patients that are resistant to conventional chemotherapy^[26]. Furthermore, side population cells have shown to express stem cell properties when isolated from cancer samples. Their identification does not rely on the relative binding of antibodies but is based on their ability to efflux a fluorescent dye that binds to DNA^[27,28]. Side population cells are more tumorigenic, chemoresistant and have displayed self-renewal *in vivo*. Besides, side population cells show a more aggressive schema of tumour growth (*in vitro*)^[29]. New strategies to target these cells need to be designed. Above all, further research on the exact role of side population cells and their implication in tumorigenesis is required as the exact mechanisms are not yet fully understood.

MOLECULAR STRATEGIES TO TARGET CSCS IN HNSCC

CSCs and therapeutic resistance

CSCs have important implications regarding cancer treatment and may lead to new perspectives on therapeutic strategies with a rethink of actual treatment paradigm. Indeed, indiscriminate cytoreduction is the aim of current chemotherapy and radiation treatment for HNSCC whereas the CSC hypothesis suggests that the elimination of CSCs is the only way to treat cancer effectively. Thus, significant reductions in the tumor volume are not enough to prevent tumor recurrence in HNSCC. Moreover, evidence suggests that CSCs have inherent drug and radiation resistance, rendering most conventional therapies ineffective. Radio resistance of CSCs has been attributed to their self-renewal capacity, DNA repair capacity, free-radical scavenging, upregulation of cell cycle control mechanisms and specific interactions with the stromal microenvironment. Chemotherapy resistance is frequently related to accelerated drug transport and to drug metabolism^[30,31]. Bmi-1 and CD44 knockdowns have led to an improvement of CSCs chemosensitivity in HNSCC. In particular, knockdown of CD44 increased the sensitivity of HNSCC cells to cisplatin, underlying the crucial of CSCs in the response to chemotherapy^[32]. Concerning Bmi-1, a stem-cell-related gene, which participates in the self-renewal of hematopoietic and neuronal stem cells, and has been implicated in the tumorigenesis of various malignancies the experiment showed that that knockdown

of Bmi-1 increased the effectiveness of radiotherapy and resulted in inhibition of tumor growth in nude mice transplanted with ALDH1+ CSCs^[33]. Moreover, Chen *et al*^[32] focused on the Snail superfamily of zinc-finger transcription factors, implicated in the regulation of EMT during embryonic development. The importance of SNAIL1 in the growth of cancer cells and their metastatic potential has been shown in various malignancies^[34]. Chen *et al*^[32] found that the endogenous co-expression of ALDH1+ and Snail resulted in decreased ALDH1 expression, inhibition of CSC-like properties, and decreased tumorigenesis in ALDH1+ CD44+ cells. By regulating the EMT, Snail is a key factor in maintaining CSC properties, and could be used as a therapeutic measure for the treatment of HNSCC. Besides, Snail small interfering RNA could reduce resistance to chemo radiotherapy in ALDH1+ cells^[32]. Ultimately, the expression of drug efflux pumps by CSCs, another mechanism of chemo resistance remains to be explored in HNSCC. A better understanding of resistance mechanisms in HNSCC CSCs will require future studies and constitutes a prerequisite for improving therapy and possibly preventing tumor spread or recurrence. The main determinants of CSC radioresistance are summarized in Table 2.

Targeting stem cell niches

Beyond intrinsic factors, the unique CSC microenvironment could play a crucial role in the radio resistance of CSCs. Indeed, it has been showed that stromal environment and CSC niche play a vital role in the behavior of cancer cells. As the vast majority of the stem cells are found within a 100 μ m-radius of a blood vessel in HNSCC, the existence of a perivascular niche was suggested. Using the SCID mouse model of human tumor angiogenesis, it was observed that specific ablation of tumor-associated endothelial cells with an inducible Caspase-9 result in the decrease of the fraction of head and neck CSCs^[15]. Thus, targeting the stem cell niche directly can weaken the source of nutrition and change the essential signals needed by CSCs to proliferate. Therapeutic strategies as suggested by Tang *et al*^[35] included targeting candidate CSCs and their microenvironment niche, which contributes to self-renewal of these cells along with the reactive oxygen species status of these cells, and tweaking their intracellular milieu to facilitate apoptotic death signals over proliferative effects may facilitate a new prospective towards target therapy in HNSCC. Similarly,

Krishnamurthy and al showed that targeting CSCs either directly or *via* their niche could lead to a more durable response in HNSCC, hence the emergence of a new concept using both conventional chemotherapy and CSC-targeted therapy^[36]. The niche provides the soil for CSC self-renewal and maintenance, stimulating essential signaling pathways in CSCs and leading to secretion of factors that promote angiogenesis and long-term growth of CSCs. Hence, the role of targeting “vascular niche” in treatment of HNSCC cannot be neglected. The use of anti-angiogenic agents, such as bevacizumab, could be a therapeutic strategy in HNSCC; if it mediates CSC depletion in gliomas it could prove useful in reducing the proportion of HNSCC CSCs. Exploiting the functional interdependence of CSCs and vascular endothelial cannot be neglected in order to reduce the rate of HNSCC recurrence and metastasis^[37-46].

EMT and molecular pathways

EMT is the process that allows a polarized epithelial cell to assume a mesenchymal cell phenotype, which is characterized by enhanced motility and invasiveness. The crosstalk between HNSCC cells and other cells of the tumor microenvironment could lead to EMT, which enhances the motility of carcinoma cells and endows them with stem cell properties. The invasive phenotype of cells that have undergone EMT allows them to penetrate the lymphatic and/or angiogenic vasculature. Blocking the crosstalk between tumor and stromal cells, and thus inhibiting EMT might be a therapeutic strategy in HNSCC. The activation of the EMT program has been shown in HNSCC populations thanks to microarray analysis; moreover, in these cells, the molecular characterization of gene expression also allowed to show the activation of Wnt/beta-catenin signaling pathway, usually involved in the maintenance of pluripotency, differentiation and proliferation. Inhibitors of this pathway are in clinical trials in several cancers^[47-49]. Numerous molecules targeting the Wnt pathway are either in the discovery stage or early phase 1 trials directed variously against Wnt/Receptor interactions and cytosolic and nuclear signaling^[50,51]. Furthermore, others implicated molecular pathways are still under investigation in HNSCC, including the promising JAK/STAT pathway. In HNSCC-CD44+ALDH1+ transplanted immunodeficient mice, an inhibitor of STAT3 combined with radiotherapy significantly suppressed tumorigenesis and improved the survival rate^[52]. Other drugs have been formulated to target other pathways in CSC formation such as Notch or Hedgehog but the ability of these drugs to selectively target CSCs while preserving normal stem cells remains a challenge. In nasopharyngeal carcinomas, targeting beta-catenin signaling pathway through E-cadherin repressor ZEB2 by using miR200a, allowed to induce stem-like traits, including CD133+ side population, sphere formation capacity, increased Oct4 and ALDH expression in tumor spheres, and tumorigenicity *in vivo*^[53]. TrkB, a 145-KDa receptor tyrosine kinase is supposed to be both involved

in EMT and invasion process of cancer cells in HNSCC. Studies showed that downregulation of TrkB, suppressed tumor growth^[54]. Ultimately, recent studies have reported the role of hypoxia or overexpression of HIF-1 α in the induction of EMT and metastasis in head and neck cancer cells. HIF-1- α regulates the expression of Twist by binding to the hypoxia-response element. Co-expression of HIF-1- α , Twist in human head and neck tumors correlates with metastasis and poor prognosis^[55]. It is undeniable that EMT is a central process in the acquisition of stem-like properties and ultimately contributes to local invasion and metastatic spread frequently observed in patients with head and neck cancer.

IMMUNOTHERAPEUTIC APPROACHES

TARGETING HEAD AND NECK TUMORAL STEM CELLS

CSC-induced immune responses

Beyond chemo resistance and radio resistance, emerging CSC targeted therapies in HNSCC have to overcome another major hindrance: Immune-escape-mechanisms of CSC. Indeed, current immunotherapy is mainly based on antigens presented to effector T cells by dendritic cells. Or, generally, these antigens are selected and derived from bulk tumor cells; they are not derived of CSCs that may not express immunogenic differentiation antigens^[38]. CSCs also may be defective in antigen presentation due to the downregulation of human leukocyte antigen (HLA) surface expression^[39]. Therefore, in a heterogeneous tumor entity, CSCs may lead to a treatment failure and disease progression, escaping from the attack of current immunotherapy. Concerning HNSCC, a better knowledge of the crosstalk between CSCs and the immune system is crucial in order to develop specific targeted therapies, the immunogenicity of HNSCC-CSCs having been observed recently. Recently, a CD8 defined T-cell epitope of ALDH1 was identified as a potential target^[22]. Among reported CSCs markers, ALDH1 is the most specific CSC marker used to identify highly tumorigenic cells present in HNSCC^[21]. ALDH1 has been recognized as an antigen-source eliciting a humoral immune response in HNSCC. Visus *et al*^[22] showed that ALDH1A1 peptide was an HLA-A2-restricted, naturally presented, CD8+ T cell-defined tumor-antigen. ALDH1 peptide-specific CD8+ T cells could only recognize HLA-A2+ HNSCC cell lines overexpressing ALDH1 but not a human fibroblast cell line. Moreover, the data presented by Liao *et al*^[40] have shown that the host immune system is able to recognize and distinguish CSCs with ALDH1 phenotype from non-CSC cells. In addition to ALDH1, other cancer antigens were found to be preferentially expressed in CSCs: Cyclin A1 was reported in leukemic stem cells of acute myeloid leukemia whereas DNAJB8 was identified as novel cancer antigen in renal CSCs^[56,57]. This specific expression of cancer antigens may enable us to target

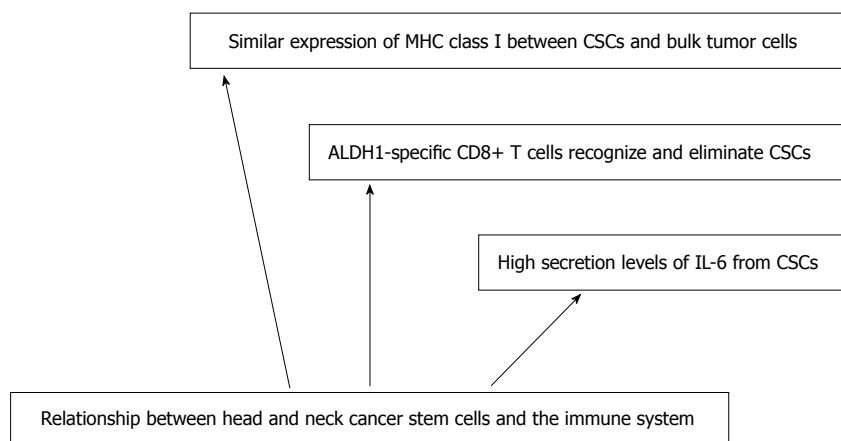


Figure 2 Immunotherapeutic approaches. CSCs: Cancer stem cells; ALDH: Aldehyde dehydrogenase.

CSCs specifically. Moreover, development of ALDH1A1 peptide-based vaccines for therapy represents a novel area for future research in HNSCC.

ALDH1A1: A potential target for vaccination therapy

Another attractive approach to target CSCs is to develop antitumor T-cell vaccines. Studies on vaccination against antigen ALDH1A1+ of CSCs have been performed and have achieved significant progress. Visus *et al.*^[41] have demonstrated the ability *in vivo* of generated ALDH1A1-specific cytotoxic T lymphocytes to eliminate ALDH (bright) cells present in HLA-A2+ HNSCC carcinoma cell lines. They also found antitumor activity by adoptive immunotherapy with ALDH1A1-specific cytotoxic T lymphocytes *in vivo*. The elimination of ALDH(bright) cells thanks to ALDH1A1-specific CD8+ T cells could inhibit tumor growth and metastases^[41]. Ning *et al.*^[42] investigated immunogenicity induced by murine ALDH (high) CSC used as a source of antigen to prime derived-cells as a vaccine for malignant squamous cell carcinoma in immunocompetent mice used as hosts. High immunogenicity was found among ALDH(high) CSCs with a most effective role as an antigen source in comparison with unselected tumor cells. A high level of IgG produced by splenocytes subjected to CSC-tumor-lysate-pulsed derived-cells and the binding of the antibody from CSC-vaccinated murine hosts to CSCs which resulted in the CSCs lysis *via* complement-dependent cytotoxicity have been observed. Studies showed that cytotoxic T lymphocytes generated from peripheral blood mononuclear cells or splenocytes harvested from CSC-vaccinated hosts had the ability to kill CSCs *in vitro*^[42]. Consistent with the findings of Ning group, Duarte *et al.*^[43] first demonstrated an ALDH(high) CSC-based vaccine could drastically reduce both tumor volume and occurrence in a rat colon carcinoma syngeneic model: 50% of the CSC-based vaccinated animals became resistant to tumor development and a 99.5% reduction in tumor volume compared to the control group occurred. Beyond the fact that these studies provide a greater view of the immune biology of

CSCs, vaccination with CSCs has proved to be effective in killing head and neck CSCs specifically, reducing tumor volume and preventing tumor recurrence.

Immune suppressive role of CSCs

Immunotherapeutic approaches for HNSCC are complicated due to the deep immune suppression induced by this disease. Mechanisms such as increased apoptosis of tumor-specific CD8+ T-cells and increased tumor-infiltrating T regulatory cells in peripheral blood and at the tumor site have been demonstrated^[58]. Krishnamurthy *et al.*^[15] showed that the location of CSCs was in close proximity to blood vessels. Clinically, patients with recurrent HNSCC showed an increased concentration of IL-6 in serum in comparison with patients with primary HNSCC^[44]. Elevated IL-6 levels could independently predict tumor recurrence, poor survival, and tumor metastasis^[45]. Yu *et al.*^[44] demonstrated that secretion levels of IL-6 from CSCs were crucial to maintain the self-renewal and tumorigenic properties of CSCs in HNSCC. On the one hand, CSCs can be recognized and inhibited in their outgrowth by the immune system and on the other hand, CSCs can promote tumor progression either by immunoediting for CSCs that are more suitable to survive in an immunocompetent host or by establishing conditions that facilitate tumor outgrowth within the tumor immune-microenvironment. Tumor associated macrophages may play a critical role in tumor progression by interacting with the tumor microenvironment and tregs are thought to promote tumor progression^[59]. In a study concerning primary human gliomas, the distribution of TAM at the invasive tumor front was correlated with the presence of CD133+ glioma CSCs. Tumor associated macrophages could significantly enhance the invasive capability of glioma stem cells through paracrine production of TGF-β1^[60]. The role of tumor associated macrophages in the regulation of CSCs drug resistance has been identified by Jinsuhi *et al.*^[61] They found a large amount of tumor associated macrophages in CD44+ ALDH+ colon tumor and CD133+ ALDH+ lung cancer cells: Those

macrophages allow activating Sonic Hedgehog pathways in CSCs in cooperation with IL-6. Targeting tumor associated macrophages by inhibiting either the myeloid cell receptors colony-stimulating factor-1 receptor or chemokine receptor improves chemotherapeutic efficacy, inhibits metastasis and increases antitumor T cell responses in pancreatic ductal adenocarcinoma^[62]. All these findings validate the interplay between CSCs and the tumor immune microenvironment. Therefore, specific targeting of head and neck tumoral stem cells by immunotherapeutic approaches may lead to more efficacious and lasting therapeutic results in the future. Nonetheless, it seems necessary to address several points before immunotherapeutic approaches targeting CSCs can be brought into clinical trials. These include the effective isolation of CSCs from bulk tumor mass to measure potential immunotherapeutic effects on CSC, to determine the antigen-profile presented on CSCs specifically to identify specific CSC targets as well as the induction and enhancement of antigen processing and presentation of CSC epitopes. A lot of work remains to be done to get a better understanding of the immune suppressive role of CSCs in HNSCC. The various immunotherapeutic approaches are displayed in Figure 2.

CONCLUSION

The CSC theory provides new opening for the treatment of HNSCC. This theory also helps to explain why currently available therapies for head and neck cancer so often fail. Eradication of cancers may require the targeting and elimination of CSCs, especially for HNSCC and thus, there is an urgent need to alter the current paradigm in drug development. Efforts are still advocated to determine specific markers and methods to specifically target these cells, towards a more specific tumor treatment. To date, no antibody selectively targeting CSC has been described in HNSCC yet, but candidates are under investigation. For instance, CD44v6 antibodies either radiolabeled or coupled with a cytotoxic drug entered phase I clinical testing in patients with HNSCC. In a phase I dose escalation study, the treatment with a radiolabeled antibody showed promising anti-tumor effects^[63]. Clearly, huge variety of approaches to eradicate CSCs is being explored, and particularly *in vitro* assays; there still remains the issue of how to avoid unwanted toxicity *in vivo*. Developing radio sensitizing strategies is also being investigated and appears to eliminate CSCs. Overcoming chemo resistance, radio resistance and immune evasion mechanisms of CSCs remains a cornerstone of novel adjuvant therapies specifically targeting CSCs in HNSCC. Bertrand *et al.*^[64] demonstrated that the combination of UCN-01 (a checkpoint kinase inhibitor) and ATRA (all-trans retinoic acid) with irradiation decreased the survival fraction of CSCs and could be used as a powerful radio sensitizing strategy in HNSCC. Furthermore, advances in nanotechnology could allow a better understanding of the regulatory mechanisms that govern CSC biology *in*

vivo.

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Insights into kidney stem cell development and regeneration using zebrafish

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Abstract

Kidney disease is an escalating global health problem, for which the formulation of therapeutic approaches using stem cells has received increasing research attention. The complexity of kidney anatomy and function, which includes the diversity of renal cell types, poses formidable challenges in the identification of methods to generate replacement structures. Recent work using the zebrafish has revealed their high capacity to regenerate the integral working units of the kidney, known as nephrons, following acute injury. Here, we discuss these findings and explore the ways that zebrafish can be further utilized to gain a deeper molecular appreciation of renal stem cell biology, which may uncover important clues for regenerative medicine.

Key words: Kidney; Renal stem cell; Renal progenitor; Regeneration; Nephron; Blood filter; Renal corpuscle; Tubule

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Core tip: The emergence of regeneration-based options for kidney disease has the potential to reduce the growing worldwide health burden of these heterogeneous conditions. Research into the mechanisms of renal regeneration in vertebrates like the zebrafish may provide knowledge about fundamental principles that could be useful for cellular reprogramming or endogenous modulation of kidney cells in humans.

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INTRODUCTION

Kidneys are organs that are responsible for excreting metabolic wastes and maintaining fluid homeostasis in the body. The functional subunit of the kidney is the nephron, which is composed of different segments of epithelial cells that can be characterized by their expression profile of unique solute transporters^[1,2]. Each human kidney contains approximately 1 million nephrons that are situated in an arborized fashion around a centralized collecting duct system (Figure 1). The nephron segments constitute three major functional regions within each nephron, and these regions act as a blood filter, a tubule that reabsorbs and secretes solutes, and a duct that fine-tunes water and electrolyte levels (Figure 1).

In the event of injury, the mammalian nephron has limited potential for self-repair^[3]. After a nephron dies, fibrosis occurs which can cause a chain reaction of additional nephron stress and death, which eventually decreases organ functionality^[4-6]. This is the case in acute kidney injury (AKI), chronic kidney disease, and end stage renal disease as the kidney suffers an initial injury, advanced fibrosis, and total loss of function, respectively^[7-10]. Finding treatments for these conditions is no easy task for regenerative medicine due to the intricate architecture of the kidney and its rich diversity of cell types^[11-14]. This has caused researchers to look for answers to renal regeneration queries in less obvious places^[15,16]. One of those places is in the zebrafish, a vertebrate model organism with the uncanny ability to regenerate entire nephrons throughout its lifetime^[17].

THE ZEBRAFISH: A MODEL FOR KIDNEY RESEARCH

Danio rerio, commonly known as zebrafish, are small, tropical freshwater fish that have been used in biological research since the 1960s^[18,19] (Figure 2). They spawn large clutches of transparent eggs that allow the growing embryo to be studied non-invasively *in vivo* and phenotypically sorted^[20]. They are also logistically favorable for experimental studies as they undergo rapid development *ex utero*, and the adults can be kept in high densities, which is space and cost effective^[20-22]. Most importantly, modern molecular technology is applicable to experiments with zebrafish and extensive genetic mapping has shown they exhibit considerable conservation with other higher vertebrates, including humans^[21,22].

It is no surprise that since zebrafish are an excellent animal model they have also been utilized in studying the kidney^[23-25]. To date, zebrafish have been used to advance our knowledge of renal development as well as aspects of several kidney disease phenotypes^[26-32]. In particular, mutagenesis screens in zebrafish have been used to identify genes that cause kidney malformations, which led to the discovery of ciliogenesis genes that cause renal cyst formation when disrupted^[33-38]. Zebrafish have also

been useful in chemical screens, such as the application of candidate and market-based pharmaceuticals to embryos, to examine influences to nephrogenesis and identify methods to alter disease states^[39-41].

These various avenues of research have significance because of the fundamental similarities in the development and morphology of zebrafish nephrons compared to their mammalian counterparts. The embryonic zebrafish renal progenitor cell field is derived from the intermediate mesoderm, as in mammals^[42,43], and gives rise to the first kidney iteration known as the pronephros^[34]. The mesenchymal renal progenitors can be identified during ontogeny based on their gene expression profile using whole mount *in situ* hybridization (WISH) with markers such as *lhx1a*, *osr1*, *pax2*, and *pax8*^[34,44-48]. Analogous to other vertebrates, these precursors in zebrafish are arranged in bilateral fields^[34]. These renal progenitors undergo convergence and extension movements during late somitogenesis to form a pronephros composed of two nephrons, which involves a mesenchymal to epithelial transition to form the tubule and duct regions of the nephron^[49,50]. The nephrons become fully functional after morphogenesis of the blood filter during the first few days of development^[51-57]. Approximately 11-13 d after fertilization, clusters of basophilic cells appear on the pronephros that give rise to additional nephrons that make up a more advanced kidney, the mesonephros^[58]. Mesonephros formation continues through the larval stages, with the penultimate organ becoming comprised of several hundred nephrons in total that are centralized into a single adult organ located on the dorsal wall of the abdominal cavity^[59]. The mesonephros is the most mature kidney the zebrafish will have, unlike higher vertebrates that include reptiles, birds and mammals who undergo an additional stage of kidney development to make the metanephros, an elaborate kidney form comprised of thousands to millions of nephrons in different species^[60]. Another interesting distinction is that the pronephros in higher vertebrates is a vestigial kidney that exists for only a short time during ontogeny^[60]. Despite these differences, it has been shown that the segment pattern in the nephrons of the zebrafish pronephros^[61] and mesonephros^[62-64] is remarkably similar to the segment pattern in nephrons of other vertebrates, supporting the notion that nephron composition is the evolutionary link across species with diverse forms that inhabit a wide range of environments^[60]. This has spurred a number of segmentation studies utilizing the zebrafish pronephros^[65-70], which further suggest nephrogenesis mechanisms have been conserved during evolution^[60]. The zebrafish pronephros and mesonephros are therefore useful for studying developmental and regenerative pathways.

WHY USE ZEBRAFISH TO STUDY RENAL REGENERATION?

It has been known for the last 25 years that some fish are capable of robust renal regeneration. Two

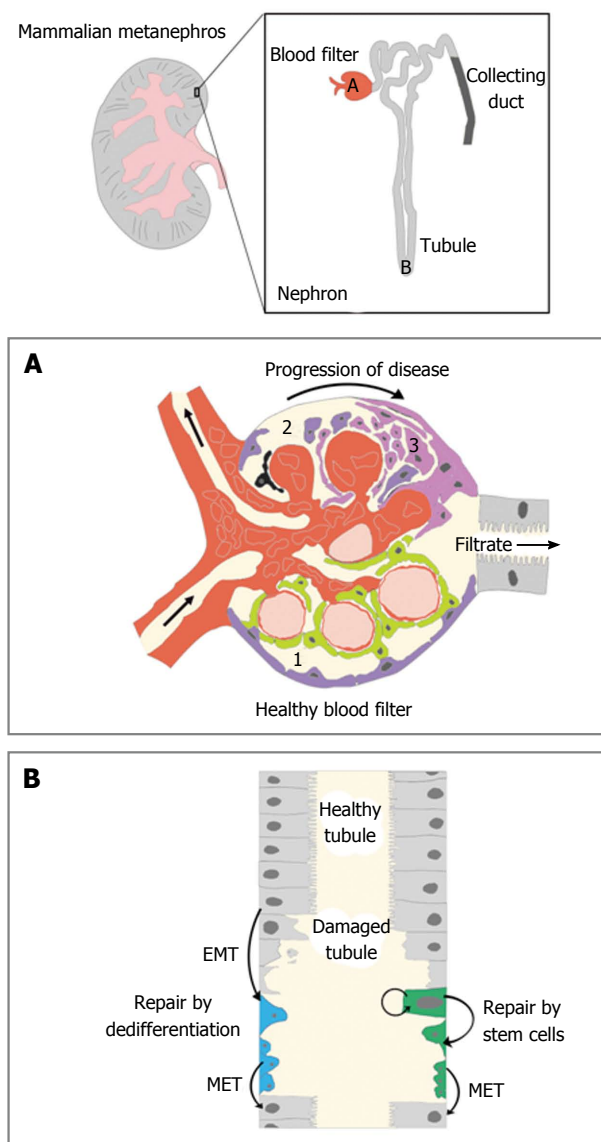


Figure 1 Kidney form, function, and regeneration. The mammalian metanephros is composed of upwards of hundreds of thousands of small, functional subunits known as nephrons. There are three main components of the nephron: The blood filter (red), the tubule (light grey) and the collecting duct (dark grey). The unique cell types in these nephron regions have variable roles in function, injury response, and regeneration. A: (1) the healthy blood filter contains intact podocytes (green) attached to capillaries to enhance the process of blood filtration. Parietal epithelial cells or PECs (purple) that line the Bowman capsule do not function in filtration; (2) after an initial physical or chemical insult, podocyte death occurs (black). This leads to a decrease in filtration selectivity and damage to other structures of the blood filter; and (3) upon podocyte death, scar formation can transpire, which obscures filtration. Excessive scar tissue creates a fibrotic matrix that can render the blood filter useless and destroy the remainder of the nephron; B: If epithelial cells of the tubule become damaged, there are two hypothesized pathways of repair. In the dedifferentiation model, a neighboring epithelial cell senses the damage and undergoes a temporary transition to a proliferating mesenchymal cell to repopulate the tubule epithelia. The stem cell model asserts that a residential pool of self-renewing stem cells exists in the tubule to create proliferating mesenchymal progeny for upkeep and repair. EMT: Epithelial to mesenchymal transition; MET: Mesenchymal to epithelial transition; PECs: Parietal epithelial cells.

phenomena have been observed: (1) replacement of epithelial populations within existing nephrons; and (2) the formation of new nephrons, also known as neo-

nephrogenesis, during adult growth or in response to catastrophic organ damage^[15]. The latter phenomenon was first observed when goldfish that had been treated with the nephrotoxin hexachlorobutadiene formed new nephrons after several weeks^[15,71-73]. This was observed by an increase in DNA replication, as indicated by the incorporation of the nucleotide analog bromodeoxyuridine, followed by an increase in the percentage of volume of the kidneys of injured goldfish^[15,71-73]. By comparison in mammals, if the basement membrane of the nephron remains intact, renal regeneration occurs by just the first phenomenon listed above, through the formation of a wave of flattened mesenchymal cells that differentiate into the required specialized epithelia^[15]. Thus, mammals only exhibit nephron genesis during gestation and sometimes during early post-natal stages (the latter being a trait which varies across species), and never form new nephrons during adulthood^[14,15]. However, both of these forms of renal regeneration have now been reported in a variety of other fish that include the zebrafish, catfish, trout, medaka and tilapia^[15,62,63,74-76].

Amongst these possible animal models, the advancement of sophisticated genetic tools and methodologies in the zebrafish offers a particularly appealing avenue to delineate the mechanisms of renal regeneration events^[77,78]. Indeed, a bevy of recent studies have provided an initial molecular framework to track and decipher such phenomena^[78,79]. For example, in zebrafish new nephrons first appear as clusters of renal progenitors near existing mesonephros tubules^[62,63]. These clusters express early markers of renal development such as *lhx1a*, *pax2a*, *wt1b* and *pax8*, which suggests that developmental pathways mirror pathways of regeneration^[62,63,79]. These clusters then expand into S-shaped bodies that mature into nephrons that fuse with preexisting nephrons^[62,63,79]. Understanding the molecular attributes of these cells is an attractive option to identify cell features that could allow for similar events to be induced in humans. In the following sections we further explore the utility of zebrafish for regeneration research in different renal cell types.

REGENERATION STUDIES IN THE NEPHRON BLOOD FILTER

The first segment in the nephron is the blood filter, a structure also known as the renal corpuscle^[77] (Figure 1A). The main components of the corpuscle are the glomerulus, a ball of capillaries that contain circulating blood, is surrounded by a space where the filtrate is collected, known as Bowman's capsule, that enables liquid to be passed into the proximal tubule of the nephron^[77] (Figure 1A). The integrity of the blood filter remains relatively constant in humans despite clinical observations that cells from this structure can be detected in the urine of healthy individuals^[80]. Though long-term homeostasis has been largely attributed to hypertrophy of resident blood filter cells^[80-82], there has also been a series of

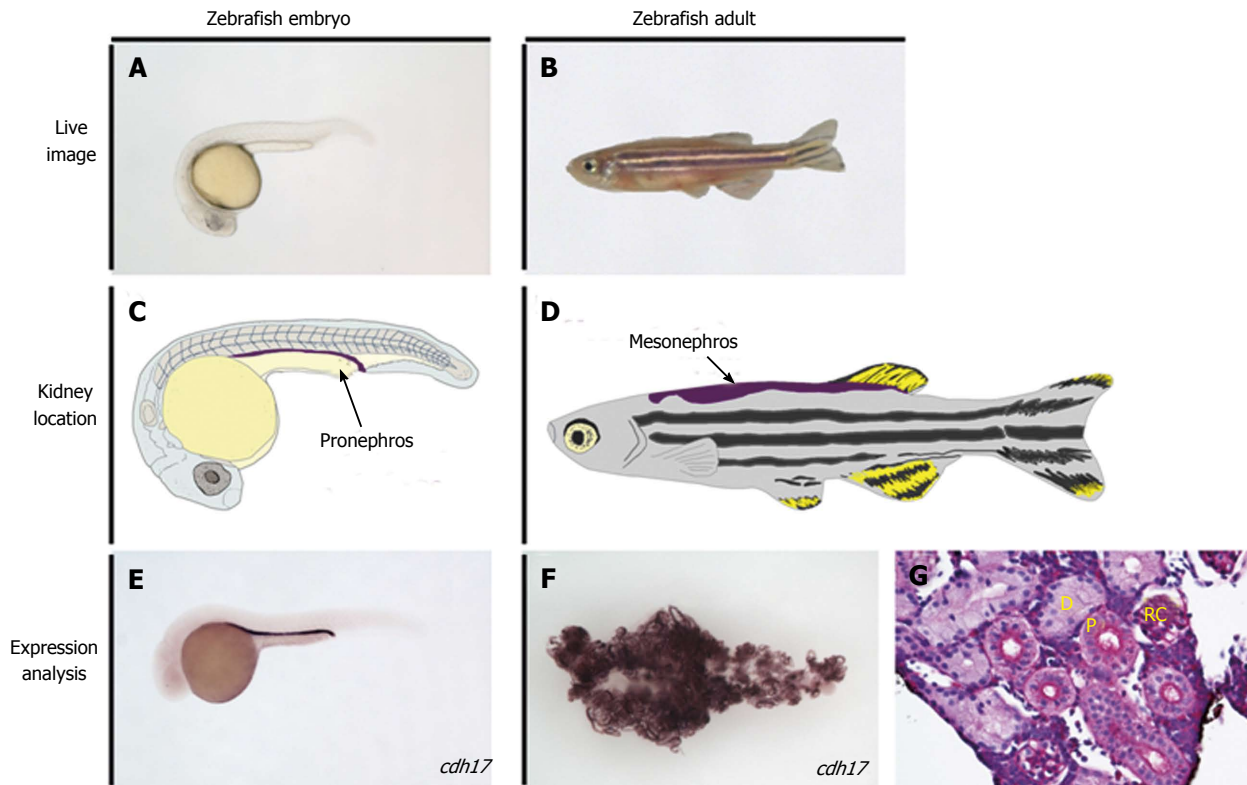


Figure 2 The zebrafish kidney in the embryo and adult. A, B: Live image of an embryonic zebrafish at 24 h post fertilization (hpf) and at the adult stage, respectively; C: Illustration of a zebrafish at the 24 hpf stage, with the location of the embryonic kidney, the pronephros, indicated in purple; D: Illustration of an adult zebrafish with the location of the mature kidney, the mesonephros, indicated in purple; E, F: Whole mount *in situ* hybridization performed on the zebrafish embryo and adult kidney, respectively, to mark the expression of the tubule and duct marker gene *cdh17*; G: Histology of a healthy adult zebrafish kidney, stained with periodic-acid Schiff to observe nephron structures. D: Distal tubule; P: Proximal tubule; RC: Renal corpuscle; *cdh17*: *cadherin17*.

investigations into the regenerative nature of these cell types.

One of the cell types that make up the glomerulus are podocytes. Podocytes are crucial for the effectiveness of the glomerulus because they form an intricate sieve network with specialized extensions known as foot processes that wrap around the capillary bundle^[77]. Low numbers of podocyte cells are a common cause and effect of glomerular diseases^[80,81]. Whether podocytes can be replaced in the mature mammalian metanephros has received substantial research attention. One theory was that another glomerular cell type called parietal epithelial cells (PECs) act as a progenitor to replace lost podocytes^[83-86]. This was suggested after observations in studies that PECs were positive for stem-cell markers CD24 and CD133, showed the capacity for self-renewal and directed differentiation in culture, contributed to damaged tubules and restored kidney function in SCID mice, and contributed to damaged glomeruli, tubules and restored kidney function in mice with adriamycin nephropathy^[83-85]. It was also posed that PECs, acting as progenitors that over-proliferate after injury, could be the cause of glomerular lesions and scars^[86]. The first theory lost traction after a subsequent study showed that adult mice with genetically labeled PECs did not produce genetically labeled podocytes in the context of injury^[87]. These studies highlight that there is still much

to be learned about the mechanisms behind glomerular disease and injury in mammals.

Contrary to the mammalian system, there are multiple studies that have shown that zebrafish are able to generate new podocytes. Zhou *et al.*^[88] created a conditional podocyte ablation transgenic line to observe podocyte regeneration. This line was created by inserting a bacterial nitroreductase (NTR) gene into the genome, which encodes a product that is converted into a cytotoxin when the drug metronidazole is added to the system. To induce podocyte-specific ablation, the NTR was subcloned downstream of the promoter for the gene *podocin*, and to visualize NTR+ podocytes, they were also engineered to simultaneously co-express the red fluorescent protein mCherry. This made the cytotoxic damage podocyte specific and allowed the injury to be viewed efficiently. A novel method of measuring proteinuria was also utilized in this study by marking vitamin D-binding protein (a similar protein to mammalian albumin which is how proteinuria is typically measured) with green fluorescent protein. After metronidazole treatment, there was evident edema, apoptosis and proteinuria in both the embryonic pronephros and adult mesonephros. In addition, the expression level of *wt1b*, which marks the developing nephron^[62], increased and extended into the Bowman's capsule. Huang *et al.*^[89] (2013) independently created a similar NTR-podocyte line

and also reported observations of podocyte replacement after injury as seen by a recovery in the expression levels of podocyte markers podocin and nephrin. Collectively, this indicates that there could be podocyte-specific repair processes that occur in zebrafish. This gives hope to the notion that parallel processes might exist in the adult mammalian kidney, or could be exacerbated with the appropriate stimuli, though such possibilities have yet to be discovered.

REGENERATION OF THE NEPHRON

TUBULE

The most extensive portion of the nephron is the tubule, which is composed of numerous discrete epithelial segments^[1,2]. After the blood has been filtered by the glomerulus, the resulting filtrate is passed through the segments of the tubule^[1,2]. These segments are uniquely identified by their expression profiles of distinct solute transporters that function to reabsorb and/or secrete molecules during urine production^[2]. The epithelial cells of the tubule are sensitive to chemical damage, particularly in the proximal segments that perform bulk reabsorption of amino acids and sugars^[78] (Figure 1B). Administering the antibiotic gentamicin in zebrafish embryos creates tubule damage that mimics AKI in humans, but leads to embryonic lethality^[90]. In analogous studies performed in the zebrafish mesonephros, gentamicin injury was shown to cause a significant drop in expression of the proximal tubule marker *slc20a1a* in existing nephrons and a decrease in kidney functionality as indicated by an inability to uptake dextran^[63,79]. However, the expression of *slc20a1a* is returned to near normal levels after 15 d post injection^[63,79], while the reabsorptive functionality is broadly restored after approximately 3 wk^[79]. Since these reports, laser ablation is another method of targeted injury that has also been used in zebrafish larvae to study renal regeneration. The laser ablation methodology provides the benefit of injuring a small region within the nephron tubule, which can be non-invasively imaged in real time to capture regenerative events^[91-93].

While it is clear that regeneration occurs in the pre-existing nephron tubules of both the zebrafish mesonephros and the pronephros, the source of these cells has not been resolved (Figure 1B). Based on parallel studies in mammals, it is likely that intratubular cells replenish damaged regions, however the cellular mechanism remains controversial^[14]. The two competing models are currently the dedifferentiation model and the stem/progenitor model^[14]. In the dedifferentiation model, neighboring cells that are left intact in a damaged tubule undergo an epithelial to mesenchymal transformation to become immature, replicating cells that can replace the lost cells. After a sufficient number of cells have been produced, the mesenchymal cells then convert into differentiated epithelial cells to reconstitute the nephron. The alternative theory is that a residential, self-sustaining group of stem cells is responsible for replacing damaged epithelial cells.

In contrast to the controversy about the mechanics of nephron epithelial regeneration, the process of neonephrogenesis has been attributed to renal progenitors within the adult kidney organ^[62,63]. A study by Zhou *et al.*^[62] first characterized the process of neonephrogenesis after gentamicin-induced AKI in adult zebrafish. They found that *wt1b* expression increased as soon as 48 h after injury followed by the appearance of *wt1b* aggregates within 4 d that gave rise to new nephrons. In a subsequent study, Diep *et al.*^[63] provided evidence for the extensive proliferative potential of these amazing renal progenitors in zebrafish. A transplantation assay was conducted by moving whole-kidney marrow cells expressing either red or green fluorescent protein into fish that had been injected with gentamicin. They found that 100% of recipient fish had grown fluorescent, donor-derived nephrons within three weeks after transplantation. Evidence that these nephrons were functional came from dextran uptake assays in which the nephrons were shown to be capable of absorbing sugar moieties circulating in the bloodstream. Interestingly, when an equal number of red fluorescent and green fluorescent donor cells were transplanted into an injured fish, mosaic nephrons containing both cell types were found which demonstrated that more than one nephron progenitor can contribute to the formation of a single nephron. When cells from these donor-derived nephrons were transplanted into a second and third fish, the progenitor cells showed continued proliferative potential.

Another major aim of the Diep *et al.*^[63] study involved the characterization of cellular aggregates that give rise to nephron tubules. In injected fish, there were multiple types of aggregates that formed from three to four fluorescent donor cells. Some were groups of 10-30 cells that were positive for the renal progenitor marker *lhx1a* and others were larger aggregates that expressed *wt1b*. These aggregates were positive for the expression of early acting renal markers and negative for mature nephron markers. Furthermore, only cells expressing the *lhx1a* reporter, when transplanted in aggregate form, had the potential to form new nephrons. These cells were found to express *six2a* and *wt1a*, similar to mammalian cells that possess nephrogenic potential during metanephros development. Overall, these and other studies suggested that *wt1b* is a marker for developing nephrons, and that *lhx1a* is a marker for renal progenitor cells^[62,63]. Future research is needed to uncover the origins and developmental regulation of these potent renal progenitors in the zebrafish.

INNOVATIVE APPROACHES IN ZEBRAFISH: UTILITY OF CHEMICAL GENETICS

The knowledge that has been gained about renal regeneration in zebrafish has been shown to be useful and translatable to experiments using the mammalian model. A study by de Groh *et al.*^[40] used zebrafish

embryos to screen thousands of commercially available pharmaceuticals to see how they impacted the renal progenitor field. The expression of markers *pax2a*, *pax8*, and *lhx1a* were examined in drug treated 14-h-old embryos using WISH and *Tg:(lhx1a:GFP)* transgenic embryos. It was found that the histone deacetylase inhibitor PTBA increases the number of cells expressing *lhx1a*, *pax2*, and *pax8*. Although there was significant edema and curvature in drug treated embryos at 48 hpf, the expression of *cdh17* appeared to be thicker, which marks the entire nephron. Furthermore, the expression of segment markers *wt1a*, *slc4a4*, *slc12a1* were also increased. This indicated that increasing the number of renal progenitors could lead to an increase in the number of differentiated cells in the nephron.

A follow-up to this study by Cosentino *et al.*^[41] administered PTBA analog m4PTB to adult zebrafish after gentamicin-induced injury. Using the nucleotide 5-ethynyl-2'-deoxyuridine to assess the proliferation index, the authors found that proliferation was increased in chemically injured fish treated with m4PTB compared to fish that had been chemically injured alone. Treatment with m4PTB also caused increased survival after gentamicin injections. m4PTB was also administered to mice with induced moderate ischemia-reperfusion (IR) induced AKI. This compound improved renal function and recovery in IR-AKI mice as indicated by a decrease in levels of serum creatine and markers for renal fibrosis. There was also a decrease in the number of cells in arrest, which indicates an activation of cells participating in repair.

A more recent study by Chiba *et al.*^[94] is another example of how chemical genetic studies in the zebrafish kidney are translatable to the mammalian model. In this study, the group first used transgenic retinoic reporter zebrafish and mice to establish that retinoic acid (RA) signaling increases in response to kidney injury as visualized by an elevation of fluorescently labeled RA expressing cells compared to controls. They found that this was most likely due to an increase of RA-synthesizing macrophages that are recruited to the tubules in the first few days after injury. Inhibiting RA signaling before and after injury increased the extremity of injury and reduced the effectiveness of repair mechanisms. The activation of RA signaling in mice using the compound all-trans-RA reduced levels of serum creatinine, M1 macrophage expression and fibrosis. These studies are proof-of-concept that compounds that improve renal regeneration and repair in zebrafish could also be useful to mammals.

CONCLUSION

Kidney diseases continue to be a considerable medical problem in our society with few avenues of treatment. Regenerative medicine aims to expand these avenues by directing the human body to repair itself. As zebrafish are already masters at regeneration in the kidney, understanding the mechanisms behind these processes may hold the key to understanding it in humans.

Moving forward, there are several essential areas of research. One major aspect of zebrafish kidney regeneration to study is the developmental origin of the renal progenitors that drive neonephrogenesis. Several reports have now provided substantial support to the notion that there is a residential pool of renal progenitors that exists in the mature kidney, which are likely to be self-renewing^[62,63,79], but their developmental origins are obscure. Genetic fate mapping is necessary to appreciate where these cells arise and to help understand their self-renewal capacity^[95]. Future studies that ascertain the molecular features of these cells may be needed to support genetic fate mapping with existing markers, such as *lhx1a*.

Studies are also needed to understand the source(s) of epithelial regeneration in damaged nephrons. In the nephrology field, this has only been addressed in mammals, which has indicated that intratubular populations are vital. As discussed in this review, whether the intratubular source is a resident stem/progenitor cell or is a dedifferentiated cell will be important to establish^[96]. To further complicate this debate, the complexity of the nephron may allow for a scenario where both sources of regeneration are possible^[97].

In summary, future studies need to better characterize the cellular basis of nephron repair and nephron neogenesis, and the zebrafish model provides a genetically tractable model to pursue these topics. Recently, a transgenic zebrafish line has been created by Wang *et al.*^[98] (2014) that utilizes green fluorescent protein to specifically label cells of the proximal tubule. This is an important advance for pursuing research about nephrogenesis in this segment during development and regeneration. Additional tools and transgenic lines such as this are needed to advance our ability to visualize renal regeneration, as they could be combined with chemical genetics to identify signals that modulate regeneration and the development of renal progenitors during embryogenesis^[99]. Processes that are happening in regeneration that we cannot "see" must also be evaluated, such as renal cell signaling, gene activation, and cell fate decisions. Another mystery of kidney health that needs to be investigated in all vertebrates is why some subjects advance from initial injury to chronic conditions and even death while others fully recover. Factors that limit regenerative potential in humans may include genetic background, ethnicity, overall health, and age. While ongoing research in the reprogramming field shows promise for creating alternative sources of replacement kidney cells^[100], clues from the zebrafish show promise for defining additional factors to advance renal regenerative therapies in the years to come.

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Generation of diverse neural cell types through direct conversion

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Abstract

A characteristic of neurological disorders is the loss of critical populations of cells that the body is unable to replace, thus there has been much interest in identifying

methods of generating clinically relevant numbers of cells to replace those that have been damaged or lost. The process of neural direct conversion, in which cells of one lineage are converted into cells of a neural lineage without first inducing pluripotency, shows great potential, with evidence of the generation of a range of functional neural cell types both *in vitro* and *in vivo*, through viral and non-viral delivery of exogenous factors, as well as chemical induction methods. Induced neural cells have been proposed as an attractive alternative to neural cells derived from embryonic or induced pluripotent stem cells, with prospective roles in the investigation of neurological disorders, including neurodegenerative disease modelling, drug screening, and cellular replacement for regenerative medicine applications, however further investigations into improving the efficacy and safety of these methods need to be performed before neural direct conversion becomes a clinically viable option. In this review, we describe the generation of diverse neural cell types *via* direct conversion of somatic cells, with comparison against stem cell-based approaches, as well as discussion of their potential research and clinical applications.

Key words: Adult stem cells; Embryonic stem cells; Generation of neural cells; Induced pluripotent stem cells; *In vitro* differentiation; *In vivo* differentiation; Clinical applications; Direct conversion; Induced neural cells

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Core tip: The process of neural direct conversion, in which cells of one lineage are converted into cells of a neural lineage without first inducing pluripotency, shows great potential for the generation of a range of neural cell types, providing an attractive alternative to neural cells derived from embryonic or induced pluripotent stem cells. In this review, we describe the generation of diverse neural cell types *via* direct conversion of somatic cells, with comparison against stem cell-based

approaches, as well as discussion of their potential research and clinical applications.

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INTRODUCTION

While the ability of the mammalian peripheral nervous system to undergo axonal regeneration following injury has been well documented^[1-3], the mammalian central nervous system is largely incapable of regeneration and repair^[4-6]. A variety of factors are believed to contribute to this lack of recovery, including limited and location restricted neurogenesis, cell death, astrocytic glial scarring, oligodendrocytic myelin inhibition, insufficient growth factor support, and lack of substrates suitable for axonal growth^[7-11]. Combined with a lack of effective treatments, these factors lead to the severity of neurological disorders, including spinal cord injury, brain damage, and neurodegenerative diseases such as Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, multiple sclerosis, and Alzheimer's disease, which often result in major disability^[12].

Neurological disorders often result from the loss of critical populations of cells that the body is unable to replace^[13], thus there has been much interest in identifying methods of generating clinically relevant numbers of functional cells to replace those that have been damaged or lost^[14]. Stem cells possess great potential for treatment of neurological disorders, providing a theoretically inexhaustible supply of cells for transplantation^[15]. Similarly, the process of neural direct conversion, in which cells of one lineage are converted into cells of a neural lineage without first inducing pluripotency^[16], also shows great promise. In this review, we describe the generation of diverse neural cell types *via* direct conversion of somatic cells, with comparison against stem cell-based approaches, as well as discussion of their potential research and clinical applications.

GENERATION OF NEURAL CELL TYPES THROUGH STEM CELL-BASED APPROACHES

Stem cell-based approaches provide a number of therapeutic advantages, through their ability to offer cellular replacement by transplantation of exogenous stem cells and stem cell-derived neural cell types, or mobilisation and induction of endogenous stem cells to generate new neural cell types, as well as their ability to release neuroprotective and inflammation modulating molecules, creating an enriched environment for minimisation of neurodegeneration^[17,18]. Current stem cell-based methods

of generating neural cell types utilise embryonic, induced pluripotent, or adult stem cells, with each exhibiting a range of advantages and disadvantages.

Embryonic stem cells

Embryonic stem cells (ESC) are pluripotent, and as such have the capacity to form all tissues in the body^[15] (Figure 1), thus they show great promise for the *in vitro* generation and subsequent study of specific cell lineages^[19], with evidence of ectodermal neural progenitor^[20,21], neuronal^[22,23], astrocytic, and oligodendrocytic^[24] cells derived from both mouse and human ESC lines. ESC also have great therapeutic potential, in particular for treatment of neurological disorders^[25]. ESC have been shown to differentiate into a range of neural cell types, with noted improvements in function following implantation, with examples in models of Parkinson's disease^[26,27], motor neuron disease^[28,29], stroke^[30,31], and spinal cord injury^[32,33].

Despite the research and clinical potential of ESC, their use is surrounded by much debate, due to technical obstacles, as well as legal and ethical issues regarding their isolation^[34]. Prior to implantation of ESC-derived differentiated cells, it is necessary to ensure that the implant consists of a pure cell population, due to the risk of teratoma formation or disruption to normal tissue function if undifferentiated ESC or inappropriate cell types are implanted^[15]. Another risk includes host rejection of allogeneic ESC-derived differentiated cells, as while immunogenicity can be contained through the use of immunosuppressive drugs, they are associated with numerous side effects that can result in patient susceptibility to infection^[15]. Furthermore, the use of ESC is highly controversial due to the fact that they are derived from pre-implantation embryos, with considerable differences in opinion in regards to their ontological and moral status^[35].

Induced pluripotent stem cells

Since the seminal discovery that ectopic expression of a set of four pluripotency reprogramming factors (Oct4, Sox2, Klf4, and c-Myc) could induce the generation of pluripotent cells from murine fibroblasts under ESC-like conditions^[36] (Figure 1), induced pluripotent stem cells (iPSC) have been proposed as a replacement for ESC, as they not only avoid the use of embryonic material, but can also be patient-derived^[37], minimising the potential for immune rejection, and allowing for the production of a variety of somatic cells with the same genetic information as the patient from which the iPSC were derived^[38]. iPSC have been utilised in the investigation of a variety of diseases of the central and peripheral nervous systems, including Parkinson's disease^[39], amyotrophic lateral sclerosis^[40], schizophrenia^[41], and Huntington's disease^[42]. iPSC have also been utilised in toxicology and drug screening studies, with examples of iPSC-derived models of familial dysautonomia^[43], Rett syndrome^[44], and Alzheimer's disease^[45]. Additionally, a number of studies have investigated the therapeutic potential of

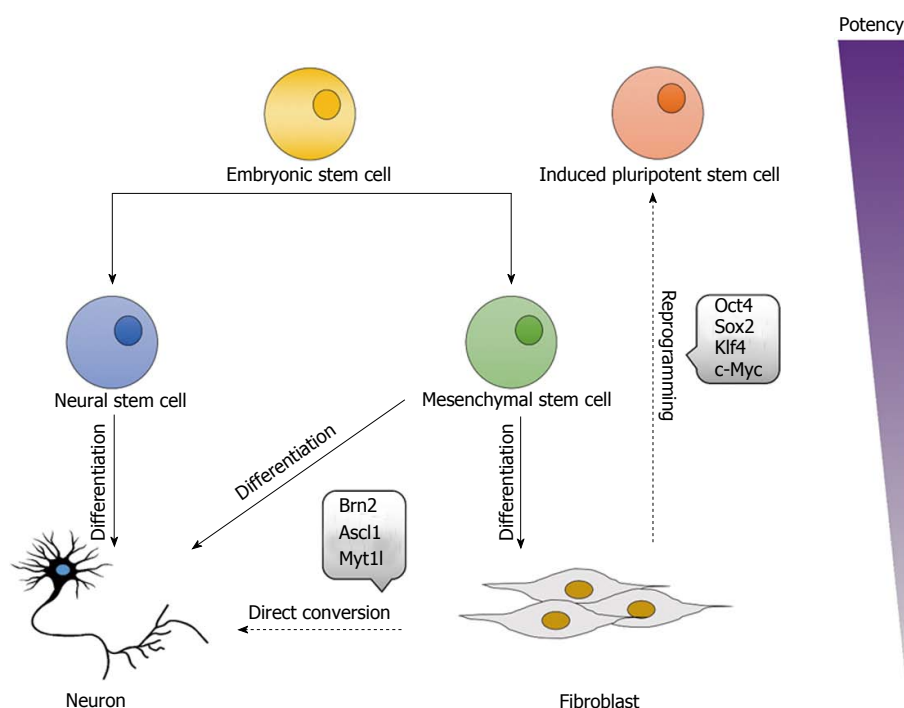


Figure 1 Overview of cellular differentiation, direct conversion, and reprogramming. Embryonic stem cells are pluripotent, and thus capable of differentiating into cells of any lineage. Reprogramming reverses this process, with forced expression of Oct4, Sox2, Klf4, and c-Myc shown to induce pluripotency in fibroblasts, generating induced pluripotent stem cells. Cells are also capable of switching lineages during direct conversion, with forced expression of Brn2, Ascl1, and Myt11 shown to convert fibroblasts into induced neuronal cells.

iPSC in animal models of neurological disorders, with evidence of locomotor function recovery in an injured mouse spinal cord^[46], functional peripheral nerve regeneration in transected rat sciatic nerves^[47], and improved motor behaviour in rat models of Parkinson's disease^[48,49]. Significantly, the first therapeutic use of iPSC has been approved for human trials in Japan, with cells from the skin of a patient suffering from age-related macular degeneration reprogrammed into iPSC and subsequently differentiated into retinal pigment epithelium cells, prior to implantation into the eye^[50].

Despite the successful therapeutic applications and reduced ethical concerns regarding their use, iPSC are similarly associated with a number of issues, from technical obstacles to safety concerns such as potential tumourigenicity^[51]. Technically, the process of reprogramming somatic cells into iPSC can be quite lengthy, taking between 10 d^[52] to 8 wk^[53], without accounting for extra time required for subsequent differentiation of iPSC into the desired somatic cell type. Reprogramming also occurs at extremely low efficiencies, which can make it difficult to generate sufficient iPSC when working with small cell numbers from the source^[54]. There have also been reported differences between iPSC based on their cell source, with gene expression profile studies showing persistent gene expression of donor cell specific markers following reprogramming into iPSC^[55,56], and further studies demonstrating not only distinct transcriptional and epigenetic differences, but also that cell source influences their *in vitro* differentiation potential, suggesting a retained epigenetic memory of their somatic cell of origin, however these differences did appear to be attenuated following continuous pass-

aging^[57,58]. Additionally, the reprogramming factors Klf4 and c-Myc are known oncogenes, thus their residual expression has the potential to induce cancer^[51], with evidence of tumour formation due to c-Myc reactivation following transplantation of mouse fibroblast-derived iPSC into nude mice^[59].

Adult stem cells

The numerous limitations associated with the use of ESC and iPSC has led to investigations into alternative sources of stem cells, such as progenitor cells residing within the adult organism^[34], with reports of mesenchymal stem cells differentiating into neural-like cells under specific experimental conditions (Figure 1), such as supplementation with a range of chemicals including β -mercaptoethanol, butylated hydroxyanisole, dimethylsulphoxide, isobutylmethylxanthine, dibutyl cyclic AMP, epidermal growth factor, and brain-derived neurotrophic factor^[60-66]. However, assessment of neuronal functionality is varied between studies, with some reporting a lack of action potential generating voltage-gated ion channels in induced neuronal cells^[65], and others demonstrating formation of synaptic vesicles, with electrophysiological evidence of functional synaptic transmission^[66], thus further investigations are required before the use of adult stem cells can become a viable alternative.

GENERATION OF NEURAL CELL TYPES VIA DIRECT CONVERSION

Lineage restriction was once one of the core principles of

developmental biology, with the concept that cells cannot cross germ layer boundaries and are thus restricted in their ability to differentiate into cells of only the germ layer from which they originate^[12]. However, these principles have since been challenged, with evidence that forced expression of specific transcription factors could directly convert cells of one lineage into another without first inducing pluripotency, in a process known as direct conversion^[67]. Early studies demonstrated the neural direct conversion of astrocytes into neuron-like cells *via* forced expression of the neurogenic transcription factors Pax6, Ascl1, Ngn2, and Dlx2^[68-70], however investigations into neural direct conversion really gained momentum following reports by Vierbuchen *et al.*^[16] of the conversion of fibroblasts into neuron-like cells (Figure 1).

The beginnings of neural direct conversion

Based on what had previously been reported in direct conversion studies generating other cell types, Vierbuchen *et al.*^[16] hypothesised that multiple transcription factors would be required to induce direct conversion of fibroblasts into neural cells, and as such, they first identified candidate genes that were known to express in neural tissues and play key roles in neural development. Starting from a pool of 19 factors, they elucidated that forced expression of the neuronal transcription factors Brn2, Ascl1, and Myt1l (BAM) could rapidly and efficiently convert mouse embryonic and postnatal fibroblasts into neuron-like cells (termed induced neuronal cells), with a conversion efficiency of 19.5%. Induced neuronal cells demonstrated expression of the pan-neuronal markers beta III tubulin, NeuN, MAP2, and synapsin, as well as the neurotransmitter phenotype markers vGLUT1 and GABA, with the majority of induced neuronal cells described as excitatory, expressing markers of cortical identity. Furthermore, induced neuronal cells exhibited spontaneous action potential generation, ligand-gated ion channels, and the ability to synaptically integrate into pre-existing neural networks, thus indicating that induced cells were of a mature and functional phenotype^[16] (Figure 2).

Pang *et al.*^[71] subsequently furthered this work, with evidence that human foetal and postnatal fibroblasts could be directly converted into induced neuronal cells *via* forced expression of BAM in addition to the neuronal transcription factor NeuroD1, following screening of a pool of 20 additional factors. Similarly, induced neuronal cells were predominantly excitatory, demonstrating expression of the pan-neuronal markers beta III tubulin, NeuN, MAP2, NCAM, and synapsin, as well as spontaneous action potential generation, and synaptic integration into pre-existing neural networks. Compared to those derived from mouse fibroblasts^[16], human fibroblast-derived induced neuronal cells required longer culture periods to develop synaptic activity, with lower reported conversion efficiencies, ranging from 2%-4%^[71].

Generation of induced neuronal cells

Following these initial studies, neural direct conversion

was investigated with great interest. Along with the numerous studies demonstrating generation of induced neuronal cells from mouse and human fibroblasts, induced neuronal cells have also been generated from common marmoset fibroblasts using the neuronal transcription factors BAM and NeuroD1, however with a conversion efficiency of < 1%^[72]. In addition to fibroblasts, induced neuronal cells have been generated from hepatocytes^[73], cord blood-derived stem cells^[74,75], pericytes^[76], glioma cells^[77], adipocyte progenitor cells^[78], and astrocytes^[79], *via* forced expression of BAM and variations of BAM, as well as a variety of new transcription factor combinations, as summarised in Table 1.

Conversion efficiencies of various studies ranged from < 0.1% up to 85%, with the more efficient methods incorporating additional factors, such as small molecules inhibiting GSK-3 and SMAD signalling^[74], retinoic acid receptor and nuclear receptor signalling^[80], delaying transgene activation after transduction^[81], and blocking cellular senescence through depletion of p16^{Ink4a}/p19^{Arf} or expression of human telomerase reverse transcriptase^[79,82]. Other studies have also investigated the use of microRNA in conjunction with neuronal specific transcription factors, including expression of microRNA-9/9* and microRNA-124^[83,84], as well as repression of a single RNA binding polypyrimidine-tract-binding protein, a key target and negative regulator of microRNA-124^[85]. microRNA-9/9* and microRNA-124 are known to act on critical target genes that regulate neuronal differentiation and function^[83,84], with microRNA-9* and microRNA-124 found to instruct compositional changes of SWI/SNF-like BAF chromatin remodelling complexes in a process that is important for neuronal differentiation and function^[83,85]. However, this particular method of neural direct conversion appears less successful than others, with conversion efficiencies in mouse and human fibroblast studies ranging from 1.5%-14%. Throughout all reported studies, induced neuronal cell functionality has been confirmed by electrophysiological analysis. Interestingly, a recent in-depth examination of the electrophysiological profiles of human induced neuronal cells generated by lentiviral vector expression of BAM, Olig2, and Zic1 has revealed that the conversion of fibroblasts to neuron-like cells is incomplete, with passive membrane properties comparable to that of highly immature neurons^[86]. However, the induced neuronal cells used in this study were sourced from research that has since been retracted, thus questioning the validity of these results.

Generation of induced neuronal subtypes

Investigations have also expanded into generation of induced neuronal subtypes. Numerous studies have reported the generation of induced dopaminergic neurons, directly converted from both fibroblasts^[81,87-91] and astrocytes^[92] using transcription factors involved in the specification of dopaminergic neurons, such as Lmx1a, Lmx1b, Nurr1, and FoxA2. Induced dopaminergic neurons were shown to display uptake and

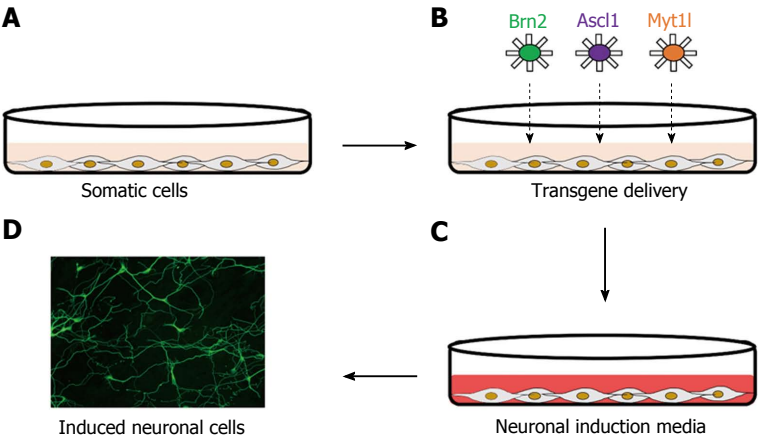


Figure 2 The process of neural direct conversion, as first described by Vierbuchen *et al.*^[16]. Somatic cells (A) are transduced with inducible lentiviral vectors expressing the neuronal transcription factors Brn2, Ascl1, and Myt1l (B), and cultured in a defined neuronal induction media (C). Following culture, induced neuronal cells can be identified by positive beta III tubulin staining (D), prior to further characterisation.

Table 1 Summary of studies demonstrating generation of functional induced neuronal cells by direct conversion

Species	Original cell	Transgenes	Method	End cell	Ref.
Mouse	Fibroblast	BAM	iLV	iN	[16]
Human	Fibroblast	BAM, NeuroD1	iLV	iN	[71]
Human	Fibroblast	miR-9/9*-124, Ascl1, Myt1l, NeuroD2	LV	iN	[83]
Human	Fibroblast	BAM	iLV	iN	[153]
Human	Fibroblast	miR-124, Brn2, Myt1l	iLV	iN	[84]
Mouse	Hepatocyte	BAM	iLV	iN	[73]
Human	Fibroblast	Ascl1, Ngn2	iLV	iN	[74]
Human	Pericyte	Ascl1, Sox2	RV	iN	[76]
Mouse	Fibroblast	Brn2, Ascl1, Ngn2	AV	iN	[144]
Human	Cord blood cell	Sox2	RV	iN	[75]
Human	Cord blood cell	Sox2, c-Myc	RV	iN	[75]
Human	Glioma cell	Brn2, Ascl1, Ngn2	LV	iN	[77]
Mouse	Fibroblast	BAM	NV	iN	[147]
Mouse	Adipocyte progenitor cell	BAM	iLV	iN	[78]
Mouse	Fibroblast	shR-PTB	LV	iN	[85]
Human	Fibroblast	Ascl1, Myt1l, Sox2	LV	iN	[154]
Common marmoset	Fibroblast	BAM, NeuroD1	iLV	iN	[72]
Human	Fibroblast	BAM	iLV	iN	[81]
Mouse	Fibroblast	Ascl1	iLV	iN	[155]
Human	Fibroblast	Ascl1	iLV	iN	[155]
Mouse	Astroglia	Ink4a/Arf ^{-/-} , Dlx2	iLV	iN	[79]
Mouse	Fibroblast	Ink4a/Arf ^{-/-} , BAM	iLV	iN	[79]
Mouse	Fibroblast	Brn2, Ascl1, Ngn2, Rarg, Nr5a2	AV	iN	[80]
Human	Fibroblast	Brn2, Ascl1, Ngn2, Rarg, Nr5a2	AV	iN	[80]
Human	Fibroblast	shR-p16-19	LV	iN	[82]
Human	Fibroblast	hTERT	LV	iN	[82]

BAM: Brn2, Ascl1, and Myt1l; miR: MicroRNA; shR: Small hairpin RNA; PTB: Polypyrimidine-tract-binding protein; iLV: Inducible lentiviral vector; LV: Lentiviral vector; RV: Retroviral vector; AV: Adenoviral vector; NV: Non-viral; iN: Induced neuron.

production of dopamine and spontaneous pacemaking activity consistent with dopaminergic neurons of the brain, as well as provide symptomatic relief in rat and mouse models of Parkinson’s disease. There have also been reports of the generation of induced motor neurons, directly converted from fibroblasts using BAM in addition to transcription factors that participate in different stages of motor neuron specification^[93], as well as the transcription factor Ngn2 supplemented with the small molecules forskolin and dorsomorphin^[94]. Induced motor neurons exhibited motor neuron-like features, such as morphology, gene expression, and mature

electrophysiological properties, as well as the formation of functional neuromuscular junctions. Similarly, recent studies have also demonstrated the generation of induced medium spiny neurons^[95], sensory neurons^[96], and astrocytes^[97], using transcription factors required for appropriate differentiation of these specific cell types, as summarised in Table 2.

Generation of induced neural stem and progenitor cells
The generation of induced neuronal cells and subtypes is often associated with low conversion efficiencies and yields, resulting in difficulties obtaining sufficient cells

Table 2 Summary of studies demonstrating generation of functional induced neuronal subtypes by direct conversion

Species	Original cell	Transgenes	Method	End cell	Ref.
Human	Fibroblast	BAM, Lmx1a, FoxA2	iLV	iDN	[87]
Mouse	Fibroblast	Ascl1, Nurr1, Lmx1a	iLV	iDN	[88]
Human	Fibroblast	Ascl1, Nurr1, Lmx1a	iLV	iDN	[88]
Mouse	Fibroblast	BAM, Lhx3, Hb9, Isl1, Ngn2	RV	iMN	[93]
Human	Fibroblast	BAM, Lhx3, Hb9, Isl1, Ngn2, NeuroD1	RV	iMN	[93]
Mouse	Fibroblast	Ascl1, Pitx3, Lmx1a, Nurr1, FoxA2, EN1	iLV	iDN	[89]
Mouse	Astrocyte	Ascl1, Lmx1b, Nurr1	iLV	iDN	[92]
Mouse	Fibroblast	Ascl1, Lmx1b, Nurr1	iLV	iDN	[92]
Mouse	Cord blood-derived stem cell	Ascl1, Lmx1b, Nurr1	iLV	iDN	[92]
Human	Fibroblast	Ascl1, Ngn2, Sox2, Nurr1, Pitx3	LV	iDN	[90]
Mouse	Fibroblast	Brn2, Ascl1, Lmx1b, Nurr1, Otx2	RV	iDN	[91]
Mouse	Fibroblast	Brn2, Ascl1, Ngn2, Pax6, Hes1, Id1, c-Myc, Klf4	RV	iNPC → iDN	[91]
Human	Fibroblast	Ngn2	RV	iMN	[94]
Human	Fibroblast	BAM, Lmx1a, Lmx1b, FoxA2, Otx2	iLV	iDN	[81]
Human	Fibroblast	Ascl1, Ngn2, Sox2, Nurr1, Pitx3, p53-DN	LV	iDN	[156]
Human	Fibroblast	miR-9/9*-124, Myt1l, Bcl11b, Dlx1, Dlx2	iLV	iMSN	[95]
Mouse	Fibroblast	Brn3a, Ngn1/2	iLV	iSN	[96]
Human	Fibroblast	Brn3a, Ngn1/2	iLV	iSN	[96]
Mouse	Fibroblast	Nfia, Nfib, Sox9	iLV	iA	[97]
Human	Fibroblast	Nfia, Nfib, Sox9	iLV	iA	[97]

BAM: Brn2, Ascl1, and Myt1l; miR: MicroRNA; shR: Small hairpin RNA; iLV: Inducible lentiviral vector; LV: Lentiviral vector; iN: Induced neuron; iNPC: Induced neural progenitor cell; iDN: Induced dopaminergic neuron; iMN: Induced motor neuron; iMSN: Induced medium spiny neuron; iSN: Induced sensory neuron; iA: Induced astrocyte.

for therapeutic applications. This may be in part due to the post-mitotic state of the target cell type (neuron-like cells), with the conversion procedure including a halt in proliferation, thus limiting the ability of these cells to expand once reprogrammed^[98-100]. In addition to determining methods of increasing conversion efficiency, studies have expanded into investigating whether similar methods could be utilised for generation of proliferative neural stem and progenitor cells, which are both expandable *in vitro* and capable of generating multiple neural cell types^[101], with initial studies demonstrating the generation of induced neural progenitor^[101] and crest^[102] cells.

Kim *et al.*^[101] first demonstrated the direct conversion of mouse embryonic fibroblasts into induced neural progenitor cells, through transient expression of the iPSC reprogramming factors Oct4, Sox2, Klf4, and c-Myc, followed by incubation in a defined neural reprogramming media. Treatment resulted in the rapid and highly efficient formation of colonies containing cells expressing the rosette neural stem cell marker PLZF and the early neural transcription factor Pax6, without transiting through a pluripotent intermediate stage. Induced cells were both proliferative and functional, capable of differentiating into functional neurons and glial cells^[101]. Similarly, Zabierowski *et al.*^[102] demonstrated the direct conversion of human melanocytes into induced neural crest cells, driving a cascade of dedifferentiation through forced expression of the intracellular domain of the transmembrane protein Notch1. Induced cells displayed biological attributes consistent with native neural crest cells, including spherical proliferation under stem cell culture conditions, expression of neural crest stem cell-related genes, and differentiation into multiple

mesenchymal and neuronal lineages, as well as *in vitro* and *in vivo* migration potential^[102].

These initial studies led to further investigation of direct conversion into neural stem and progenitor cells, with additional reports of the generation of induced neural crest^[103] and progenitor^[104] cells, as well as generation of induced neural stem cells^[105]. Furthermore, a number of studies also demonstrated the generation of induced neuroblasts^[106], and induced oligodendrocyte^[107,108] and dopaminergic neuron^[109] progenitor cells. While the majority of studies utilised fibroblasts as the starting cell type, there have also been reports of the direct conversion of astrocytes^[106,110], Sertoli cells^[111], epithelial-like cells in urine^[112], cord blood-derived stem cells^[113], bone marrow-derived stem cells^[114], liver cells, and B lymphocytes^[99] into induced neural stem and progenitor cells, as summarised in Table 3.

Generation of induced neural stem and progenitor cells has been achieved using a variety of approaches. One such approach, the cell activation and signalling-directed (CASD) method, combines transient overexpression of pluripotency reprogramming factors and/or small molecules (cell activation) with soluble lineage-specific signals (signalling-directed) to reprogram somatic cells into lineage-specific cell types while bypassing the pluripotent state^[115]. CASD induced neural stem and progenitor cells have been generated using a range of pluripotency reprogramming factors and microRNAs, such as Oct4, Sox2, Klf4, c-Myc, SV40LT, and microRNA-302-367, in conjunction with neural stem/progenitor cell permissive culture conditions^[101,109,112,116-120]. Other approaches involve overexpression of lineage-specific transcription factors, with examples of induced cells generated using individual factors, such as Oct4^[121],

Table 3 Summary of studies demonstrating generation of functional induced neural stem and progenitor cells by direct conversion

Species	Original cell	Transgenes	Method	End cell	Ref.
Mouse	Fibroblast	OSKM	iLV	iNPC	[101]
Human	Melanocyte	Notch1	LV	iNCC	[102]
Human	Astrocyte	4-Oct	LV	iNSC	[110]
Human	Astrocyte	Sox2	LV	iNSC	[110]
Human	Astrocyte	Nanog	LV	iNSC	[110]
Mouse	Fibroblast	Brn4, Sox2, Klf4, c-Myc, E47	RV	iNSC	[105]
Human	Fibroblast	Oct4, Sox2, Klf4, Zic3	RV	iNPC	[104]
Mouse	Fibroblast	Brn2, Sox2, FoxG1	iLV	iNPC	[123]
Mouse	Fibroblast	OSKM	RV	iNSC	[116]
Human	Fibroblast	OSKM	RV	iNSC	[116]
Human	Fibroblast	Sox2, Pax6	NV	iNPC	[98]
Mouse	Fibroblast	Sox2	RV	iNSC	[122]
Human	Fibroblast	Sox2	RV	iNSC	[122]
Mouse	Sertoli cell	Ascl1, Ngn2, Hes1, Id1, Pax6, Brn2, Sox2, c-Myc, Klf4	RV	iNSC	[111]
Mouse	Fibroblast	OSKM	RV/iLV	iNSC	[117]
Mouse	Fibroblast	Brn2, Nr2e1, Sox2, c-Myc, Bmi1	RV	iNPC	[157]
Human	Fibroblast	OSKM	SV	iNPC	[118]
Monkey	Fibroblast	OSKM	SV	iNPC	[118]
Mouse	Fibroblast	Sox10, Olig2, Nkx6.2	LV	iOPC	[107]
Human	Urine cells	Oct4, Sox2, Klf4, SV40LT, miR-302-367	NV	iNPC	[112]
Mouse	Fibroblast	Sox10, Olig2, Zfp536	iLV	iOPC	[108]
Rat	Fibroblast	Sox10, Olig2, Zfp536	iLV	iOPC	[108]
Human	Fibroblast	Oct3, Sox2, Klf4, c-Myc	RV	iNPC	[125]
Mouse	Fibroblasts	Brn2, Hes1, Hes3, Klf4, c-Myc, Plagl1, Notch1 (NICD), Rfx4	NV	iNSC	[99]
Mouse	Liver cells	Brn2, Hes1, Hes3, Klf4, c-Myc, Plagl1, Notch1 (NICD), Rfx4	NV	iNSC	[99]
Mouse	Blymphocytes	Brn2, Hes1, Hes3, Klf4, c-Myc, Plagl1, Notch1 (NICD), Rfx4	NV	iNSC	[99]
Mouse	Fibroblast	OSKM	iLV	iDNPC	[109]
Rat	Bone marrow-derived stem cell	Ngn2	LV	iNPC	[114]
Mouse	Fibroblast	Sox2, Klf4, c-Myc, Brn4	RV	iNSC	[158]
Human	Fibroblast	Sox10	iLV	iNCC	[103]
Human	Fibroblast	4-Oct	LV	iNPC	[121]
Pig	Fibroblast	Oct4, Sox2, Klf4, Lin28, L-Myc	NV	iNPC	[120]
Human	Fibroblast	4-Oct	LV	iNSC	[119]
Human	Fibroblast	Oct4, Sox2, Klf4, shR-p53	NV	iNSC	[119]
Human	Fibroblast	Sox2, c-Myc, Brn2	LV	iNPC	[159]
Human	Fibroblast	Sox2, c-Myc, Brn4	LV	iNPC	[159]
Human	Astrocyte	miR-302/367	LV	iNB	[106]
Human	Fibroblast	Sox2, HMGA2	RV	iNSC	[113]
Human	Cord blood-derived stem cell	Sox2, HMGA2	RV	iNSC	[113]

OSKM: Oct4, Sox2, Klf4, c-Myc; miR: MicroRNA; shR: Small hairpin RNA; NICD: Notch intracellular domain; HMGA2: High-mobility group A2; iLV: Inducible lentiviral vector; LV: Lentiviral vector; RV: Retroviral vector; SV: Sendai virus vector; NV: Non-viral; iNCC: Induced neural crest cell; iNSC: Induced neural stem cell; iNPC: Induced neural progenitor cell; iOPC: Induced oligodendrocyte progenitor cell; iDNPC: Induced dopaminergic neuronal progenitor cell; iNB: Induced neuroblast.

Sox2^[122], Sox10^[103], and Nanog^[110], as well as different sets of factors^[111,123]. Additionally, a number of studies have also incorporated the use of small molecules such as TGF- β ^[118], GSK-3^[119], MEK^[112], ROCK^[112], BMP^[112], JAK^[109], and histone deacetylase^[103,120] inhibitors to enhance direct conversion. Making comparisons between different methods of generating neural stem and precursor cells is difficult, particularly as many studies do not report conversion efficiency values, however research has shown that neural direct conversion using lineage-specific factors results in greater chromosomal stability than neural direct conversion using pluripotency reprogramming factors^[124], thus suggesting a preference towards this particular method for future clinical applications.

Significantly, Meyer *et al.*^[125] also reported the direct conversion of fibroblasts from patients with both familial and sporadic forms of amyotrophic lateral sclerosis (ALS)

into induced neural progenitor cells. Induced cells were subsequently differentiated into astrocytes, a key cell type involved in the degeneration of motor neurons in ALS, which demonstrated toxicity toward motor neurons as similarly demonstrated by autopsy spinal cord-derived astrocytes. These findings not only enable personalised modelling of ALS and potentially other neurodegenerative diseases, but could also lead to high-throughput testing of therapeutics for individual patients^[125].

Generation of induced neural cells *in vivo*

Generation of neural cell types through direct conversion has been studied extensively *in vitro*, with confirmed long-term survival and functional integration following transplantation^[126]. As such, investigations have expanded into generation of neural cells through direct conversion *in vivo*, in which cells are directly converted

within their native physiological environment^[127]. Preliminary research described the transplantation of fibroblasts and astrocytes transduced with inducible forms of neural reprogramming genes into the adult rat brain, with conversion into induced neuronal and dopaminergic neuronal cells following gene activation *in vivo*^[128]. Further studies demonstrated the direct conversion of endogenous glial cells into induced neural cells, with BAM expression converting resident astrocytes into induced neurons in the mouse striatum^[128], Fezf2 expression converting resident embryonic and early postnatal callosal projection neurons into induced corticofugal projection neurons in the mouse neocortex^[129], and Sox2 expression converting resident astrocytes into induced neuroblasts in the mouse striatum, with subsequent differentiation into mature and functional neurons^[130].

Neural direct conversion *in vivo* has also been demonstrated in a number of injury models. Ngn2 expression in addition to growth factor exposure has been shown to convert non-neuronal cells into induced neurons in the rat neocortex and striatum following stab wound injury^[131]. Similarly, induced neurons have been generated from endogenous NG2 glia by Sox2 expression in the mouse cerebral cortex following stab wound injury^[132], as well as from endogenous reactive glial cells by NeuroD1 expression in the mouse cortex following stab wound injury and in an Alzheimer's disease model^[133]. Sox2 expression has also been reported to convert resident astrocytes into induced neuroblasts in an injured mouse spinal cord, with subsequent differentiation into mature and functional neurons^[134]. While induced cells were determined to be functional throughout these studies, there was no evidence that they had any significant impact on behavioural recovery following injury, thus further investigation is required to fully elucidate the potential of endogenous cells for neurological repair.

Methods of neural direct conversion

As evident in the summaries of neural direct conversion studies (Tables 1-3), transgene delivery methods vary greatly throughout. Primarily, transgenes are delivered using viral vectors, due to their intrinsic ability to efficiently express their genome in the nucleus of target cells^[135], however safety concerns regarding clinical translation have resulted in investigations into non-viral methods of transgene delivery, as well as methods of chemically-induced neural direct conversion.

Integrating viral vector transgene delivery: The majority of neural direct conversion studies to date have utilised retroviral vectors (RV) and lentiviral vectors (LV) for transgene delivery, due to their comparatively higher efficiency and ability to integrate into the target cell genome, thus ensuring sustained transgene expression^[136]. However as a result of genomic integration by these vectors, there is an associated risk of spontaneous transgene reactivation, as well as tumour formation due

to insertional mutagenesis^[59], as previously observed with proto-oncogene activation in four out of ten patients following retrovirus-mediated gene therapy for X-linked severe combined immunodeficiency disorder^[137]. LV are often preferable to RV, as while RV require passage through mitosis for transduction, LV do not, and as such are capable of transducing both dividing and non-dividing cells^[138]. Additionally, LV are generally considered a safer alternative to RV, as they are designed without the majority of the viral genes, retaining only the *cis*-acting sequence elements necessary for nuclear export of the RNA, RNA dimerisation, packaging, and reverse transcription^[139]. Furthermore, innovations in LV design have led to the creation of self-inactivating LV, knocking out viral long terminal repeat (LTR) enhancer-promoter activity^[140], as well as non-integrating lentiviral vectors, with mutations in their integrase or LTRs to inhibit integrase binding^[141], thus reducing the risk of integration and vector-related pathologies^[139]. LV have also been used in conjunction with drug-based induction systems, in which transgene expression is dependent upon the delivery of a specific drug (e.g., tetracycline, ecdysone, mifepristone), thus allowing for tightly regulated conditional transgene expression, an appealing prospect for a number of potential gene therapy applications^[142].

Non-integrating viral vector transgene delivery:

The use of genome integrating RV and LV poses a number of limitations due to the increased risk of gene mutations and insertional mutagenesis, thus studies have investigated transgene delivery *via* non-integrating adenoviral vectors (AV) and Sendai virus vectors (SV) for safer generation of induced neural cells. Similarly to LV, AV are able to transduce both dividing and non-dividing cells, with transient expression in dividing cells, and long-term expression in non-dividing cells^[136]. Importantly, AV demonstrate little to no integration into the target cell genome, instead being maintained episomally as linear or circular DNA molecules^[139]. However, AV have been shown to induce several classes of innate immune responses, thus despite minimal genomic integration, AV still have the risk of host immune response to overcome^[143]. Furthermore, AV have been associated with a comparatively lower neuronal conversion efficiency than using LV systems^[144], and as such it is critical to identify other factors or chemical compounds to obtain neurons with a higher efficiency, as evident in the addition of Rarg and Nr5a2 to the neuronal transcription factor combination of Brn2, Ascl1, and Ngn2, with a demonstrated increase in conversion efficiency from 2.9%^[144] to 46.2%^[80]. SV are non-integrating viral vectors, capable of transient but strong gene expression in a wide range of dividing and non-dividing cells^[145]. Significantly, SV pose no potential pathogenicity towards humans, with temperature-sensitive variants of SV allowing temperature-specific activation/inactivation of gene expression, further alleviating some of the safety concerns associated with their use clinically^[146]. SV have

been utilised in the generation of highly proliferative induced neural progenitor cells from primate species, with conversion efficiencies ranging from 0.03%-0.19%, and subsequent temperature-mediated removal of viral genomes^[118]. Despite the relatively low conversion efficiency, the many favourable safety attributes of SV promotes further investigation into their use in the generation of induced neural cell types.

Non-viral methods of transgene delivery: Neural direct conversion using non-viral transgene delivery methods is becoming an increasingly attractive alternative to viral vector-based methods^[136], with a number of studies reporting generation of induced neuronal and neural stem and progenitor cells *via* non-viral methods. The first example of non-viral neural direct conversion described the generation of induced neuronal cells from mouse embryonic fibroblasts through repeated delivery of plasmids encoding BAM with a bioreducible linear poly(amido amine) polymer, resulting in mature, electrophysiologically functional neuron-like cells with a conversion efficiency of 7.6%^[147]. Following the confirmed feasibility of non-viral neural direct conversion, studies expanded into investigating non-viral methods of generating induced neural stem and progenitor cells, capable of differentiating into multiple mature and functional neuronal subtypes. Non-viral delivery of Sox2 and Pax6 by plasmid transfection or protein transduction was initially shown to convert adult human fibroblasts into induced neural progenitor cells, with a conversion efficiency of 0.05%^[98]. Following this, non-integrative episomal vectors were utilised for non-viral direct conversion, with induced neural progenitor cells generated from epithelial-like cells in human urine following episomal vector delivery of Oct4, Sox2, Klf4, SV40LT, and microRNA-302-367 in combination with a cocktail of small molecules, with a conversion efficiency of 0.2%^[112]. Similarly, episomal vector delivery of Oct4, Sox2, Klf4, Lin28, and L-Myc in combination with histone deacetylase inhibitor treatment has converted pig fibroblasts into induced neural progenitor cells^[120], and Oct4, Sox2, Klf4, and small hairpin RNA-p53 with a cocktail of small molecules has converted human fibroblasts into induced neural stem cells^[119], however no conversion efficiencies were reported for either study. Interestingly, a secondary system enabling non-viral neural direct conversion has been reported, in which fibroblasts, liver cells, and B lymphocytes were isolated from chimeric mice carrying inducible vectors expressing Brn2, Hes1, Hes3, Klf4, c-Myc, Plagl1, Notch1 (NICD), and Rfx4, with subsequent conversion into induced neural stem cells following transgene induction, however again with no reported conversion efficiencies^[99]. Overall, while some non-viral methods achieve conversion efficiencies similar to studies utilising viral vectors^[147], others achieve considerably lower conversion efficiencies^[98,112] or have not reported them^[99,119,120], thus necessitating optimisation of non-viral methods in order for them to become a viable alternative.

Neural direct conversion by chemical induction:

An attractive alternative to neural direct conversion *via* introduction of exogenous genes is chemical induction, with the discovery that iPSC could be generated by small molecules alone^[148] prompting investigations into generation of induced neural cell types using similar methods. Initial studies demonstrated the generation of induced neural progenitor cells using a defined chemical cocktail and hypoxic conditions^[149]. Induced neural progenitor cells were converted from mouse embryonic fibroblasts, mouse tail tip fibroblasts, and epithelial-like cells in human urine using a two-step induction strategy, with an initial intermediary transition of a chemical cocktail of small molecules inhibiting TGF- β , GSK-3, and histone deacetylation pathways under 5% oxygen, followed by lineage-specific induction in neural expansion media. Chemically induced neural progenitor cells resembled endogenous neural progenitor cells in terms of their proliferation, self-renewability, ability to differentiate into multiple mature and functional neuronal subtypes *in vitro* and *in vivo*, and gene expression profile, however induced cells generated from mouse fibroblasts were shown to have retained some fibroblastic epigenetic memory^[149]. Similarly, postnatal human fibroblasts have been converted into induced neuronal cells, using a specific cocktail of small molecules consisting of forskolin, and inhibitors of TGF- β , BMP, GSK-3, MEK-ERK, and p53 pathways^[150]. Chemically induced neuronal cells displayed a mature neuronal morphology, with positive immunostaining of functional neuronal markers synapsin, vGLUT1, GABA, and tyrosine hydroxylase, however no electrophysiological studies were performed to confirm functionality. Induced cells were generated with a conversion efficiency of > 80%, with efficiency reportedly unaffected by donor age and cellular senescence, thus providing a novel and efficient method of generating transgene-free induced neuronal cells with great clinical potential^[150].

Clinical applications of induced neural cell types

Induced neural cell types generated by direct conversion have long been suggested as a source of cells for clinical applications, however their true therapeutic potential has not yet been fully investigated. Studies have recently addressed this gap within the literature, reporting the restorative effects of induced neural stem cells in models of spinal cord injury and Parkinson's disease. In one study, mouse embryonic fibroblast-derived induced neural stem cells were transplanted into the contused thoracic spinal cord of rats^[151]. Following transplantation, induced neural stem cells lost their stem cell identity and differentiated into neurons, astrocytes, and oligodendrocytes, with synaptic formation observed between host and transplanted neurons. Both lesion and cavity size decreased following transplantation of induced cells, with increased myelin production and angiogenesis in the injured area, as well as promotion of axonal regeneration, motor function, and electrophysiological activity. In addition to cellular replacement, transplanted induced

cells were shown to exert their therapeutic effect through neuroprotective and immunomodulatory mechanisms, as well as promotion of endogenous regeneration, as evident by decreased expression of apoptotic and inflammatory markers^[151]. Similarly, mouse Sertoli cell-derived induced neural stem cells exogenously expressing the dopaminergic neuron-specific factor Lmx1a were transplanted into the striatum of Parkinson's disease model mice^[152]. While transplantation of induced neural stem cells was shown to improve the motor performance of mouse models, with greater tyrosine hydroxylase signal abundance in the lesioned area, only few transplanted cells survived over time, thus suggesting that the therapeutic effects may have occurred in a non-autonomous manner through enhancement of the functions of remaining endogenous cells^[152]. Interestingly, induced neural cell types generated *via* direct conversion with lineage-specific factors have been shown to possess greater chromosomal stability than neural cells derived from pluripotent or adult stem cells^[124], further promoting the clinical potential of neural cell types generated *via* direct conversion.

CONCLUSION

Neurological disorders often result from the loss of critical populations of cells that the body is unable to replace, thus methods of generating clinically relevant numbers of cells to replace those that have been damaged or lost are sought^[13,14]. The process of neural direct conversion has been demonstrated to generate a range of functional neural cell types both *in vitro* and *in vivo*, through viral and non-viral delivery of exogenous factors, as well as chemical induction methods. Induced neural cells have been proposed as an attractive alternative to neural cells derived from embryonic or induced pluripotent stem cells, with prospective roles in the investigation of neurological disorders, including neurodegenerative disease modelling, drug screening, and cellular replacement for regenerative medicine applications, however further investigations into improving the efficacy and safety of these methods need to be performed before neural direct conversion becomes a clinically viable option.

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Use of platelet lysate for bone regeneration - are we ready for clinical translation?

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Abstract

Current techniques to improve bone regeneration following trauma or tumour resection involve the use of autograft

bone or its substitutes supplemented with osteoinductive growth factors and/or osteogenic cells such as mesenchymal stem cells (MSCs). Although MSCs are most commonly grown in media containing fetal calf serum, human platelet lysate (PL) offers an effective alternative. Bone marrow - derived MSCs grown in PL-containing media display faster proliferation whilst maintaining good osteogenic differentiation capacity. Limited pre-clinical investigations using PL-expanded MSCs seeded onto osteoconductive scaffolds indicate good potential of such constructs to repair bone *in vivo*. In an alternative approach, nude PL-coated scaffolds without seeded MSCs have been proposed as novel regenerative medicine devices. Even though methods to coat scaffolds with PL vary, *in vitro* studies suggest that PL allows for MSC adhesion, migration and differentiation inside these scaffolds. Increased new bone formation and vascularisation in comparison to uncoated scaffolds have also been observed *in vivo*. This review outlines the state-of-the-art research in the field of PL for *ex vivo* MSC expansion and *in vivo* bone regeneration. To minimise inconsistency between the studies, further work is required towards standardisation of PL preparation in terms of the starting material, platelet concentration, leukocyte depletion, and the method of platelet lysis. PL quality control procedures and its "potency" assessment are urgently needed, which could include measurements of key growth and attachment factors important for MSC maintenance and differentiation. Furthermore, different PL formulations could be tailor-made for specific bone repair indications. Such measures would undoubtedly speed up clinical translation of PL-based treatments for bone regeneration.

Key words: Mesenchymal stem cells; Platelet lysate; Bone regeneration

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Core tip: Human platelet lysate (PL) offers an exciting

opportunity for expanding mesenchymal stem cells (MSCs), as well as for use in bone regeneration as a scaffold coating. In this review, we describe the state-of-the-art research in the area of bone regeneration utilising PL and MSCs and emphasise the need for standardisation of PL preparation and its quality control in order to progress further in this exciting area of research. Different PL formulations could be tailor-made for specific scaffolds and bone repair indications, both in autologous and allogenic settings. More pre-clinical and clinical work is needed to progress this research into clinical translation.

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INTRODUCTION

Bone regeneration following trauma, tumour resection or as a result of chronic or degenerative bone disease represents a serious clinical problem. The use of auto-graft bone is limited by its volume and accessibility and is known to be associated with donor site morbidity and pain^[1]. New strategies involve the use of allograft, xenograft or synthetic scaffolds, often combined with patient's own stem cells or growth factors. Multipotential stromal cells, also termed mesenchymal stem cells (MSCs) reside in the bone marrow (BM) and other connective tissues in humans and are quite abundant *in vivo* making them the preferred choice for use in bone regeneration^[2]. In the past, MSCs were derived from patient's own BM and seeded on osteoconductive scaffolds following *ex vivo* culture expansion, these clinical studies yielded promising results and good long-term safety profiles^[3,4].

However, in most of these studies MSCs were grown in media containing fetal calf or fetal bovine serum (FCS/FBS), a source of bioactive molecules required for MSC attachment and proliferation. Although FCS is a well known supplement for MSC expansion, its major drawback is a possibility for triggering immunological responses in the recipient against xenogenic antigens present in FCS. In this review, we will describe the current knowledge on the use of a potential alternative to FCS, human platelet lysate (PL), for *ex vivo* MSC expansion and *in vivo* bone regeneration, highlighting various steps in PL manufacture and their possible impact of PL bioactivity.

THE USE AND BENEFITS OF HUMAN PLATELET-RICH PLASMA AND PL AS FCS REPLACEMENTS

As mentioned above, there is an urgent need for an alter-

native to FCS as a medium supplement. The straightforward substitute is human serum (HS) and successful expansion of MSCs with the use of autologous serum has been achieved in several independent studies^[5,6]. On the other hand, the reports on the efficacy of allogenic HS have been contradictory^[7,8]. One important consideration is the age of the donor, as serum from elderly individuals seems inadequate to support MSC proliferation or differentiation^[9,10].

An alternative to HS is platelet-rich plasma (PRP), which was shown to enhance MSC proliferation in *ex vivo* culture as early as 2006^[11]. PRP is commonly produced from whole blood collected using EDTA as an anticoagulant. A low-speed centrifugation (10 min at 230-270 g) is performed first, followed by high-speed centrifugation (10 min at 2300 g or higher). The supernatant is called platelet-poor-plasma (PPP). Subsequently, 9/10th of the supernatant (PPP) volume is removed, and the platelet pellet is resuspended in 1/10th PPP volume, which is then referred to as PRP^[12]. To activate platelets and hence maximise the release of growth factors from platelets' alpha granules into the PRP, thrombin is added, the product is called tPRP^[13].

PRP as a supplement for MSC culture has several drawbacks. Firstly, not all growth and attachment factors are released from platelets without their activation^[14,15]. Secondly, the presence of fibrinogen and the formation of fibrin clots after thrombin stimulation are suggested to be the reasons for the partial loss of platelet derived growth factor BB (PDGF-BB) content. A substantial amount of released PDGF-BB was found to be trapped in fibrin glue, so the maximum amount of PDGF-BB could not be obtained^[13]. Thirdly, the storage temperature of the PRP should be no less than 4 °C. If the PRP is frozen at -20 °C or -80 °C, it could no longer be referred to as PRP as freezing releases growth factors and cytokines from the platelets. Yet some early studies have referred to platelet concentrates which have been frozen as PRP^[11].

In many ways, these drawbacks can be mitigated with the use of PL instead of PRP. Shish and Burnouf summarises the main production methods of PL materials for stem cells expansion and regenerative medicine^[12]. During PL manufacture platelets are lysed, and sometimes activated prior to their lysis, in order to release the maximum amount of bioactive molecules and reduce batch-to-batch variation. Filtering is performed to remove cellular debris and WBC contamination is minimised by leukodepletion steps. In order to use pooled PL, the blood group type being used for PL preparation is important to avoid immunogenic reaction^[16]. Although MSCs have been shown not to absorb immunogenic ABO antigens from culture media containing FCS or human substitutes such as AB serum and PRP, MSC could absorb these antigens from the fresh AB plasma used for washing and cell infusion^[17]. In order to prevent contact of MSCs to ABO blood group antigens, platelet concentrates derived from blood group O are combined with plasma from blood group AB^[12,15,18].

Recent studies have shown that MSC population doubl-

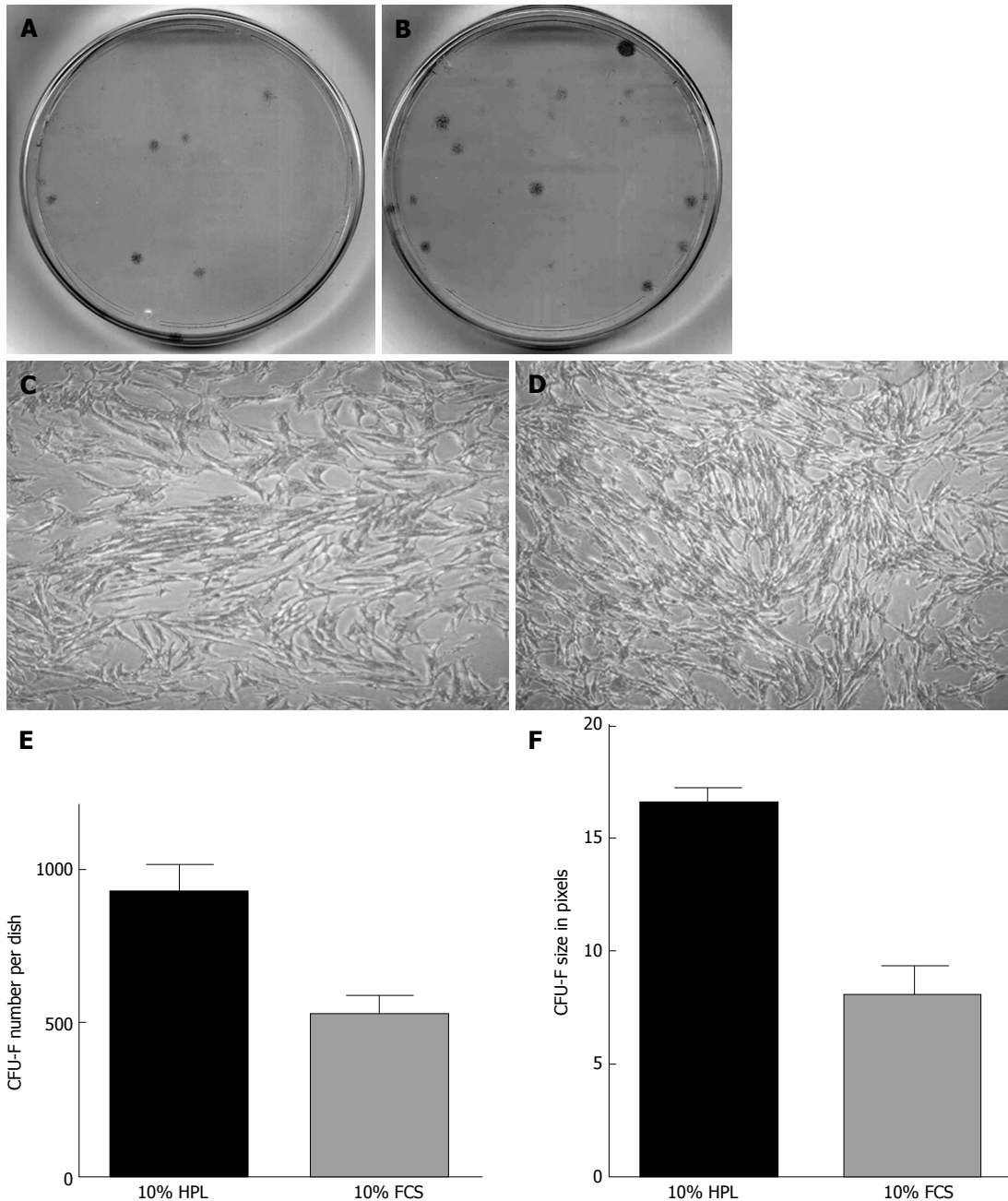


Figure 1 The enhancement of mesenchymal stem cell colony formation in platelet lysate-cultured bone marrow aspirates from a single patient. Day-14 colonies formed from 200 μ L of fresh BM aspirate seeded on plastic culture dish with either 100 mL/L FCS (A) or 100 mL/L PL (B). Higher magnification (40 \times) of individual colonies grown in 100 mL/L FCS (C) or 100 mL/L PL (D). Quantified analysis of the number of colonies (E). The average size of colonies in pixels (F). Colony number and size were analysed using NIS Elements BR Nikon software. BM: Bone marrow; PL: Platelet lysate; FCS: Fetal calf serum.

ings and expansion kinetics were significantly enhanced in PL-supplemented BM MSC cultures compared with cultures supplemented with FCS, HS, or tPRP^[18,19]. Clinically relevant numbers of MSCs could be obtained within the first to second passage in PL-supplemented cultures. This rapid increase in MSC yields could be due to increased MSC attachment (evident as increased colony numbers) as well as their faster proliferation (evident as bigger colony sizes), illustrated in Figure 1.

Several other studies have shown that media supplemented with PL shortened MSC expansion time, while preserving MSC phenotype and differentiation capaci-

ties^[20-24]. BM MSC grown with 100 mL/L PL efficiently differentiated into the adipogenic, osteogenic and chondrogenic lineages^[25-28]. Furthermore, PL was shown to be superior to FCS for BM MSC adipogenesis differentiation^[26,27]. There is also evidence that PL soluble factors can regulate osteogenesis *via* RUNX2- and Osx-independent pathways^[25,29]. The effect of PL on MSC immunoregulation capacity remains to be further explored. One study suggested that the fibrinogen depletion step was necessary in PL manufacture in order to prepare clinical-grade MSCs intended for immunomodulation^[30]. In another study, inhibition of T cell proliferation was

Table 1 Common methods of platelet lysate preparation

Ref.	Platelet collection	Leucocyte reduction	Freeze and thaw temperature (°C)	Anti-coagulation factors	PDGF level
[15]	Buffy coat	Inline filtration	-30/37	100 IU/mL H	(AA) 7424 ± 1043 pg/mL ² (BB) 4655 ± 1353 pg/mL (AB) 40458 ± 1791 pg/mL
[18]	Buffy coat	Inline filtration	-30/37	2 IU/mL H	Higher than FCS
[38] ¹	Apheresis	NR	-20/37	NR	NR
[25] ¹	Apheresis	NR	-80/NR	2 IU/mL H	NR
[36] ¹	NR	NR	-80/NR	10 IU/mL H	NR
[51]	Apheresis	NR	-80/37	2 IU/mL H	(AB) 530 ± 60 pg/μL
[37] ¹	Apheresis	NR	-80/NR	NR	(AA) 19596 pg/mL (BB) 22329 pg/mL

¹Pre clinical investigation; ²Measured in media containing 10 mL/L platelet lysate. PDGF: Platelet derived growth factor; AA, AB and BB refer to different isotypes of PDGF; H: Heparin; FCS: Fetal calf serum; NR: Not reported.

observed equally in MSCs cultured in PL and FCS^[18].

The other advantage of PL is that it can be prepared from expired platelet concentrates (older than 5 d) that are no longer suitable for use in blood transfusion. The PL produced from expired platelets has been shown to have the same impact on MSC growth and osteogenic differentiation as PL from fresh platelets in one study^[28]. Freezing and thawing seems to be the most effective process to release growth factors in platelet samples, because both WBCs and platelets are destroyed by this procedure and growth factors are not stable in plasma^[31].

GROWTH AND ATTACHMENT FACTORS PRESENT IN PL

Contained within the alpha granules of platelets are a broad spectrum of growth factors that have been shown to enhance MSC proliferation. These include PDGF-AA, PDGF-AB/BB, transforming growth factor-β1 (TGF-β1) (Table 1), epidermal growth factor, basic fibroblast growth factor (bFGF), insulin-like growth factor 1, and vascular endothelial growth factor with low batch-to-batch variability, and most of these growth factors are stable for up to 14 d^[32,33]. The higher level of these growth factors and cytokines in PL compared to FCS and HS could in part explain why PL-expanded MSCs grow faster, however, only a few studies have presented comparisons of the growth factor levels in PL compared to FCS^[15,26,34]. Interestingly, PDGF-BB was not detected in FCS^[26,34]. When PDGF-BB was low in the growth medium, BM MSC cell yield was found to be poorer in comparison to a higher PDGF-BB level media^[27]. Also, the inhibition of PDGF-BB in medium supplemented with 100 mL/L PL significantly decreased MSC proliferation^[19,33]. However, the addition of the equivalent concentration of recombinant PDGF-BB to the media did not significantly enhance MSC proliferation^[33]. Therefore, it is possible that PDGF-BB could be the main but not the only factor responsible for enhanced MSC proliferation and other growth factors, for example TGF-β1 and bFGF are involved.

The presence of the chemokines and attachment factors in the MSC growing media is equally important. In

relation to chemokines and soluble adhesion molecules, PL has been shown to contain chemokine (C-C) ligand 5 (CCL5/RANTES), CXCL1, CXCL2 and CXCL3 and, in particular, very high concentrations of soluble cluster of differentiation 40 ligand, soluble vascular cell adhesion molecule, and soluble intracellular adhesion molecule^[33]. The attachment factors are important for adhesion of MSCs to plastic surfaces whereas chemokines ensure MSC migration and equal distribution across these surfaces.

IN VIVO BONE FORMATION USING PL EXPANDED MSCS IN PRE-CLINICAL INVESTIGATIONS

As mentioned above, PL provides several advantages over FCS for MSC expansion, however, PL preparation methods remain varied. Table 1 summarises the most commonly used methods to obtain PL including those used for pre-clinical investigations^[25,35-38]. Most of the studies shown in Table 1 prepared PL *via* apheresis in a closed system using centrifugation to separate cells by weight (specific gravity), or filtration to separate cells by size, or used a combination of both, with the resulting platelet count of 1×10^9 platelet/mL. Such platelet concentrates have a larger volume (typically 200-300 mL), and their total platelet content is about 6-8 fold higher of a platelet concentrate from a single whole-blood donor^[12]. Although it is known that the apheresis procedure results in low WBC count, precise levels of WBCs present in the platelet concentrate were not reported in these studies. Alternatively, two studies used the buffy coat procedure^[15,18] to obtain a platelet concentrate and then combined it with inline filtration to reduce WBC contamination.

Only a few pre-clinical studies investigated *in vivo* bone generation using PL-expanded BM MSCs (Table 2). All of these studies have reported some success in new bone formation *in vivo*. Notably there were differences in scaffolds and animal models, making direct comparisons of bone repair outcomes difficult (Table 2). Only two of the five selected studies succeeded to present the

Table 2 Summary of pre-clinical studies with platelet lysate-expanded bone marrow mesenchymal stem cells aimed at bone regeneration

Ref.	Animal model	MSC source	MSC expansion media (supplements)	Construct type	Scaffold pre-coating before loading with MSCs	Construct <i>in vitro</i> culture before implantation (supplements)
[38]	Mouse	Iliac crest BM	50 mL/L PL	BCP + 2×10^5 MSCs	A	10^{-8} M DEX
[36]	Sheep	Iliac crest BM	200 mL/L FCS	Rat-tail-derived collagen + 4×10^7 MSCs	PL	NR
[37]	Mouse	Iliac crest BM	100 mL/L FCS	HA (65%) + β -TCP (35%) + 3×10^5 MSCs	PL for 24 h	100 mL/L FCS
[25] ¹	Mouse	Iliac crest BM	50 mL/L PL	HA (65%) + β -TCP (35%) + MSCs ²	PL for 24 h	50 mL/L PL
[35] ¹	Mouse	Iliac crest BM	50 mL/L PL	BCP + 4×10^6 MSCs	A	80 mL/L PL

¹No FCS was used at any stage of investigation; ²MSC number not reported. MSCs: Mesenchymal stem cells; BM: Bone marrow; PL: Platelet lysate; BCP: Biphasic calcium phosphate; A: Absent; DEX: Dexamethasone; FCS: Fetal calf serum; NR: Not reported; HA + β -TCP: Hydroxyapatite/beta-tricalcium phosphate.

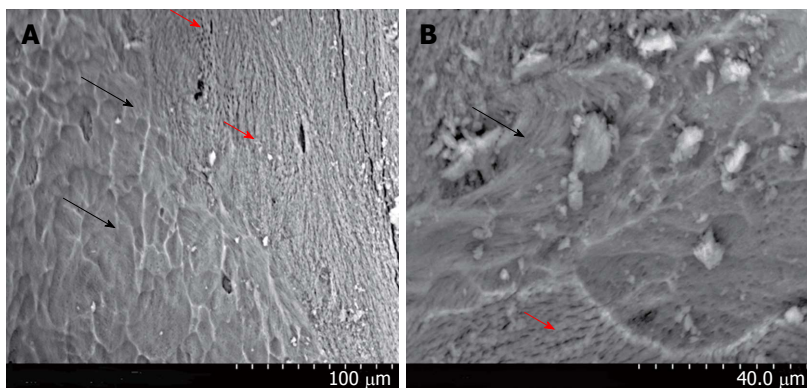


Figure 2 The attachment of platelet lysate-expanded bone marrow mesenchymal stem cells on orthoss scaffold. BM MSCs at passage 2 were incubated with the scaffold by continuous mixing at 37 °C and 5% CO₂ for 3 h with DMEM + 50 mL/L PL. The scaffold was then washed and incubated for 10 d with DMEM + 50 mL/L PL. SEM image illustrate MSCs on the scaffold (A). Higher magnification demonstrates MSC morphology on the scaffold surface (B). Black arrow: MSC cells; Red arrow: Orthoss scaffold. BM: Bone marrow; MSCs: Mesenchymal stem cells; PL: Platelet lysate.

results of *in vivo* bone formation on a scaffold seeded with MSCs grown fully with PL without any exposure to FCS^[25,35]. Other studies introduced FCS at different stages of the study - either for MSC expansion purposes^[36,37] or for *in vitro* seeding of MSCs onto the scaffold^[37]. To fully eliminate FCS-exposure for clinical translation, it is important to use PL from the beginning and throughout all stages of investigation. Pre-coating scaffolds with PL is likely to be advantageous for MSC attachment. With the use of a natural xenograft bone scaffold, it is possible to achieve good attachment and proliferation of solely PL-expanded BM MSCs by 3 h incubation of the BM MSC in DMEM supplemented with 50 mL/L PL media, as illustrated on Figure 2.

IN VITRO AND IN VIVO BONE FORMATION USING PL COATED SCAFFOLDS

Seeding exogenous MSCs onto a scaffold prior to implantation may not be necessary for repairing some types of bone defects. In this scenario, the combination of PL with a "nude" bone scaffold could be an attractive

answer to target homing, attachment and differentiation of neighbouring endogenous MSCs to the repair site. The initial *in vitro* studies investigating this varied in scaffold type and the method of combining PL with the scaffold. The simplest method involved overnight incubation of a hydroxyapatite/beta-tricalcium phosphate (HA/ β -TCP) scaffold immersed in PL^[37]. Methods utilising polymer based scaffolds involved layer by layer assembly^[39] or combining PL loaded nanoparticles with a polymer powder^[40]. Of note, "soft" PL loaded scaffolds have also been explored for use in wound healing^[41] and cartilage regeneration^[42], where methods included overnight incubation of polymer-fibrin scaffolds with a fibrinogen and PL solution or mixing PL with a polymer solution to create a hydrogel, respectively. Freeze-thaw cycles, using at least two rounds were the most common method of preparing PL from PRP.

A concern remains whether PL proteins could block the scaffolds' pores, thus preventing cell infiltration into scaffold. However, a similar scaffold pore size and 3D structure was reported after the addition of PL in two studies^[40,42]. Growth factor release from PL loaded scaffolds varied between studies, where 50 mL/L growth factor release after 1 d^[40] or 2 d^[41] was reported, with

91 mL/L release by 7 d^[41]. Importantly, the bioactivity of growth factors from loaded scaffolds was comparable to PL controls up to 7 d^[41]. When MSCs were seeded onto the scaffolds (all expanded in 100 mL/L FCS), *in vitro* cell adhesion and migration appeared to be improved by the addition of PL^[37,40,42]. Furthermore, the presence of PL appeared to induce faster osteogenic differentiation^[40]. When a non-osteoconductive polymer scaffold was used, Ca²⁺ was deposited throughout the scaffold indicating that MSCs differentiated into osteoblasts^[39].

Some studies progressed to investigate the PL coated scaffolds and membranes *in vivo*. In one study a "healing impaired" diabetic mouse model for full thickness skin wounds was used. At 15 d post injury the PL loaded scaffold group had significantly smaller open wound measurements in comparison to controls^[41]. Further histological evaluation showed higher levels of collagen deposition^[41]. In another aforementioned study (Table 2), HA/ β -TCP scaffolds, PL loaded or unloaded seeded with MSCs were inserted subcutaneously into SCID mice for 7 wk^[37]. Evidence of new bone formation and vascularisation was seen in PL coated compared to uncoated scaffold controls^[37]. PL coated scaffolds without prior MSC seeding were used as controls; however, this data was not described in the paper. In the future this type of data could provide an exciting insight into whether pre-seeding with MSCs is needed or if the PL coating is enough to encourage *in vivo* bone formation.

Based on the available literature it can be concluded that there are two types of PL incorporation into a scaffold, either simply by overnight incubation of a pre-fabricated scaffold with PL or through addition of PL into the scaffold manufacturing process. The former of which has *in vivo* evidence that it may improve bone regeneration. However, the optimal technique of PL incorporation onto a scaffold is likely to depend on the nature of the scaffold itself (chemistry, porosity, its basic architecture) as well as its intended clinical use. For example, PL-loaded scaffold intended for use as "hard" bone void filler, should aim to achieve faster MSC differentiation. On the other hand, PL loaded onto a "soft" guided bone regeneration device (a polymer or natural membrane) should speed up MSC homing and proliferation rather than to induce their differentiation. These disparate objectives could be achieved by fine-tuning PL formulations and using different methods of PL incorporation into the desired scaffold.

For orthopaedic applications, ideally "off-the-shelf" PL-loaded and packaged scaffolds could be made available to surgeons in the operating theatre, however currently no data exists on long-term storage, sterility and stability of PL-loaded scaffolds. Alternatively, commercially available scaffolds could be loaded with autologous PL during surgery. Although theoretically possible, it remains unclear whether PL preparation and loading can be achieved to fit into intra-operative time frames, so far, only overnight incubation has been evaluated pre-clinically. Further investigation into speeding up

the production of autologous PL for use in surgery, as well as whether allogenic "bottled" PL can be produced consistently in larger volumes to a controlled standard is needed for this area to progress.

FUTURE CONSIDERATIONS FOR THE USE OF PL IN BONE REPAIR

One of the advantages of PL is its relative ease of preparation (Figure 3A). Nevertheless, there are a few points that should be considered if PL is used for clinical translation for bone repair (Figure 3B). The blood collection method, WBC reduction step, platelet concentration method, freeze and thaw temperatures, and addition of the anticoagulation factors to each of these steps might affect the growth and attachment factors levels in PL that in turn influence MSC growth and differentiation.

The first step of the PL preparation is the collection of the platelet concentrate either by apheresis, buffy coat or PRP methods. Buffy coat and PRP methods are procedures to obtain platelet concentrate from whole blood by two step centrifugation, for buffy coat first with low speed spin and second with high speed spin. Conversely, the PRP method starts with high speed spin followed by a low speed spin (Figure 3A). However, study variations are expected as apheresis leads to a high platelet count and lower WBC contamination compared to the buffy coat and PRP methods^[31,43,44]. The buffy coat method tends to reduce the WBC contamination, compared to PRP^[44]. The leucocyte reduction step is vital as WBC-derived molecules could influence MSC expansion^[45]. Upon WBC lysis, matrix metalloproteinases and free oxygen radicals may be released that could lead to MSC detachment and apoptosis^[46]. Some studies tend to apply extra centrifugation steps to reduce WBCs in the platelet concentrates but a degree of WBC contamination can still be seen, which also impacts the growth factors' levels in PL^[31,47]. Therefore, it is imperative to include rigorous leucocyte depletion steps in PL preparation procedure, as centrifugation steps remain insufficient. To release growth factors from the platelet concentrates, mechanical bursts of freeze and thaw cycles are implemented^[18,25,26,31,34,37]. Most studies used -80 °C as the freeze point and 37 °C as the thaw point. However, the freeze and thaw temperatures seem to affect the yields of the growth factors in PL. In order to obtain the highest growth factors levels, one study recommended the -196 °C/4 °C cycle, as PDGF-BB levels in PL from PRP using this cycle were significantly higher than from PRP using -80 °C/4 °C cycle^[27].

Since PL contains fibrinogen and coagulation factors, adding anticoagulants is vital. Heparin is usually added to PL-media before use. However, the concentration of heparin should not exceed 0.61 IU/mL when using unfractionated heparin or 0.024 mg/mL for low-molecular-weight heparin^[48]. This is because higher concentrations of heparin may negatively affect MSC proliferation,

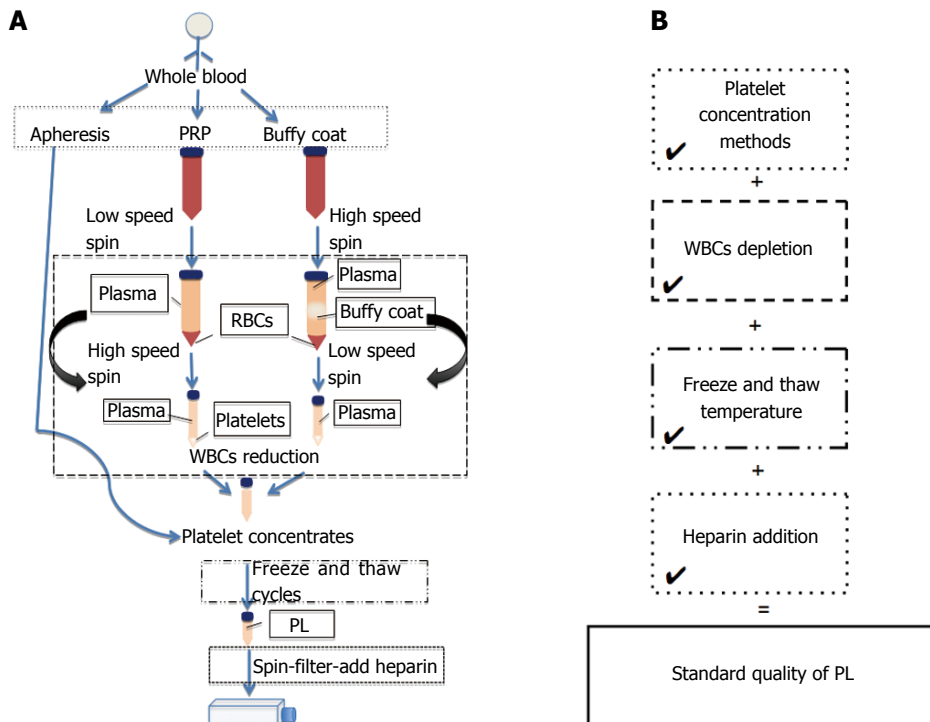


Figure 3 Summary of the current methods used for platelet lysate preparation. A: Step by step guide of the PL preparation methods; B: Suggested stages to standardise in PL preparation. PRP: Platelet rich plasma; PL: Platelet lysate.

colony formation and differentiation^[48]. Additionally, available commercial heparin, which is manufactured primarily from porcine sources, has been shown to cause adverse reactions to human tissues, despite being approved for human use^[30,49,50]. These concerns are avoided with the use of the human origin heparin or by preparing PL from serum instead of plasma^[27].

ARE WE READY FOR CLINICAL TRANSLATION?

PL represents a new and exciting opportunity for bone defect repair, either as a cell culture supplement for MSC expansion or as a coating agent for osteoconductive scaffolds and guided bone regeneration devices. PLs contain a multitude of bioactive factors that act synergistically to facilitate MSC attachment, proliferation and differentiation. Still, the exact amounts and ratios between these bioactive molecules remain to be established in order to achieve the desired and timely therapeutic effect on bone repair. For this, a consorted scientific and industrial effort is needed to establish standard protocols and quality control procedures for PL production that are tailor-made for intended therapeutic use. This can be split into two long term areas of investigation, primarily, large scale production of allogenic PL and secondly, fast small scale production of autologous PL exclusively for use in acute surgery.

Additionally, the time scale of growth factor release from scaffolds needs to be detailed to further understand the potential effect on bone regeneration. In order to

have standard PL, a minimal quality control criteria is needed. For example, a minimum of 3-4 fold increase in the platelet concentration compared to the start point, the levels of PDGF-BB and the degree of WBC contamination, could be three factors that can serve as initial quality control criteria of standard PL. Finally, while the existing pre-clinical evidence on the use of PL-expanded MSCs or PL-coated scaffolds is encouraging, clinical studies investigating the benefits of PL-based products for improving bone regeneration are still lacking. Such evidence is definitely required to progress further in this field.

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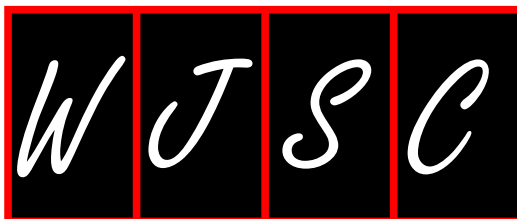
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Human pluripotent stem cells: Towards therapeutic development for the treatment of lifestyle diseases

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Abstract

There are two types of human pluripotent stem cells: Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), both of which launched themselves on

clinical trials after having taken measures to overcome problems: Blocking rejections by immunosuppressants regarding ESCs and minimizing the risk of tumorigenicity by depleting exogenous gene components regarding iPSCs. It is generally assumed that clinical applications of human pluripotent stem cells should be limited to those cases where there are no alternative measures for treatments because of the risk in transplanting those cells to living bodies. Regarding lifestyle diseases, we have already several therapeutic options, and thus, development of human pluripotent stem cell-based therapeutics tends to be avoided. Nevertheless, human pluripotent stem cells can contribute to the development of new therapeutics in this field. As we will show, there is a case where only a short-term presence of human pluripotent stem-derived cells can exert long-term therapeutic effects even after they are rejected. In those cases, immunologically rejections of ESC- or allogenic iPSC-derived cells may produce beneficial outcomes by nullifying the risk of tumorigenesis without deterioration of therapeutic effects. Another utility of human pluripotent stem cells is the provision of an innovative tool for drug discovery that are otherwise unavailable. For example, clinical specimens of human classical brown adipocytes (BAs), which has been attracting a great deal of attention as a new target of drug discovery for the treatment of metabolic disorders, are unobtainable from living individuals due to scarcity, fragility and ethical problems. However, BA can easily be produced from human pluripotent stem cells. In this review, we will contemplate potential contribution of human pluripotent stem cells to therapeutic development for lifestyle diseases.

Key words: Arteriosclerosis; Human embryonic stem cells; Glucose intolerance; Human induced pluripotent stem cells; Brown adipose tissue

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Core tip: Clinical application of human embryonic stem

cells (ESCs)/induced pluripotent stem cells (iPSCs) is currently limited to remediless diseases due to risk of tumorigenesis. However, application of these cells to therapeutic purposes and drug discovery for lifestyle diseases is promising. Because a short-term presence of human ESC/iPSC-derived vascular endothelial cells reportedly exerts long-term therapeutic effects on injured stenotic arteries, immunologically rejections can nullify risk of tumorigenesis without deteriorating therapeutic effects. Another utility is to produce high-scarcity-valued cells such as brown adipocytes, which are unobtainable from living bodies and commercially available sources, as a new tool for drug discovery for lifestyle diseases.

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INTRODUCTION

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are the only pluripotent stem cells that are applicable to therapeutic purposes (Table 1). The first clinical trial of human ESCs (hESCs) was launched in 2010 by Geron Corporation in the United States^[1-4], aiming for the safety evaluation of transplanting hESC-derived oligodendrocyte progenitor cells for the treatment of spinal cord injury. Although the program was shut down due to fund shortage in 2011, no severe side effects were reported from all four cases. Another clinical trial was started in 2010 by Ocata Therapeutics in the United States (named Advanced Cell Technology, Incorporated until 2014), aiming for the evaluation of safety and efficacy of hESC-derived retinal pigment cells for the treatment of macular degeneration^[5]. Up till now, positive results were reported from two open-label phase 1/2 studies although we have to wait for the final evaluation. Regarding human iPSCs (hiPSCs), a clinical trial was started in 2014 by RIKEN and Foundation for Biomedical Research and Innovation in Japan, aiming for the safety evaluation of hESC-derived retinal pigment cells for the treatment of macular degeneration. No severe side effects have been reported so far.

Although it was expected that the invention of hiPSCs had completely resolved the issue of immunological hurdles, it has turned out that the situation is not so simple. Up till now, two concerns have been raised. One is regarding the differentiation propensity of hiPSCs. It is known that there are marked differences in differentiation propensity among human pluripotent stem cell lines^[6], and thus, it is necessary to establish scores of hiPSC lines to obtain an appropriate line for the preparation of differentiated cells of the intended lineage. In some cases, however, an appropriate line may not be obtained and, as a result, transplantation materials would be unavailable

from patients. In addition, genetic mutations may possibly occur during the process of hiPSC establishment. In such cases, usage of mutated hiPSC lines should be avoided. Thus, there may be cases where transplantation materials are unavailable from patients themselves. This actually occurred in the second patient during the clinical trial in Japan: The authorities announced that they resigned the utilization of autologous hiPSCs but decided to use allogenic hiPSCs instead in this case. It seems, however, allogenic hiPSCs are less advantageous than hESCs from the viewpoint of safety although they have merits from the perspective of ethics and labor. The second concern is regarding an issue of possible acquisition of immunogenicity of autologous iPSCs due to spontaneous mutations in the mitochondrial DNA, which is five to ten times more prone to be mutated than the chromosomal DNA. In addition, alterations of mitochondrial DNA reportedly occur upon an induction of pluripotency in hiPSCs^[7]. Because cells that contain allogenic mitochondria are rejected by innate immune system^[8], autologous hiPSC-derived cells with mutated mitochondrial DNA may possibly be immunologically rejected, dissipating the effects of transplantation. These two concerns should be deeply reflected for the success of hiPSC-based cell therapies in near future.

Currently, the application of human pluripotent stem-derived cells is limited to such diseases that have no other therapeutic options because there is a certain level of risk including tumorigenesis in the transplantation of human pluripotent stem cells. Nevertheless, the application range will be extended if the safety is secured. Regarding lifestyle diseases such as obesity-associated metabolic disorders and ischemic diseases, we already have various therapeutic options including medications and surgeries. In addition, a large number of candidate drugs are currently in the process of research and development. Thus, development of human pluripotent stem cell-based therapies for lifestyle diseases has not been eagerly sought thus far. Nevertheless, there is ample potential for hESCs/hiPSCs to effectively be utilized towards therapeutic development in this field. In this review, we suggest two cases as examples. One is a transplantation therapy for the treatment of ischemic diseases: hESC/hiPSC-derived vascular endothelial cells (VECs) having anti-stenotic capacities, which we termed as type-II VECs^[9-11], can exert their full effects within a short time (< 1 wk) to produce long-term beneficial outcomes even after they are rejected^[11]. In those cases, risk of tumorigenesis may be nullified because hESC- or allogenic hiPSC-derived cells are promptly rejected by immune systems. The second one is utilization of human pluripotent stem cells as a novel tool to provide cells that have high scarcity value but are unavailable from living individuals. Actually, anti-stenotic VECs is an example of such high-scarcity-valued cells^[9]. As another example, we will describe human ESC/iPSC-derived classical brown adipocyte (BA), which has been much awaited as a new target of drug discovery for the treatment of obesity-

Table 1 Clinical application of human pluripotent stem cells

Human pluripotent stem cells	Sources	Ethical hurdle	Safety
ESCs	Embryos	High	Relatively high ¹
Allogenic iPSCs (with immunosuppression)	Cell banks	Low	Not yet evaluated
Allogenic iPSCs (without immunosuppression)		Low	High
Autologous iPSCs	Patient samples	Low	Under evaluation

Advantages and disadvantages of each kind of human pluripotent stem cells are described. ¹Up till now, no severe side effects have been reported. ESCs: Embryonic stem cells; iPSCs: Induced pluripotent stem cells.

associated metabolic disorders.

PROVISION OF A NOVEL TYPE OF VASCULAR ENDOTHELIAL CELLS WITH ANTI-STENOTIC PROPERTY: TOWARDS DEVELOPMENT OF A NEW TRANSPLANTATION THERAPY FOR ISCHEMIC DISEASES

According to the report by World Health Organization, the top two leading causes of death in the world in 2012 are ischemic heart disease and stroke, both which are considered as lifestyle diseases. Ischemia is caused by narrowing of arteries (*i.e.*, arteriostenosis), whose pathological basis is hyperproliferation of vascular smooth muscle cells (VSMCs). Stent revascularization is one of the most effective therapies, where a meshed tube made of shape-memory alloy is inserted into the affected artery (*i.e.*, the coronary artery for ischemic heart disease and the carotid artery for stroke) to mechanically expand the stenotic region. Nevertheless, a comparative study in India in 2010 reported that 23.1% of patients with drug-eluting stents and 48.8% of patients with bare metal stents developed restenosis^[12]. Therefore, development of new therapeutics is required for the control of ischemic diseases.

Regarding the etiology of arteriostenosis, involvements of VSMCs and macrophages are well understood. By contrast, roles for VECs remained controversial for long time. Recently, we have clarified that there are two types of human VECs: Pro-stenotic VECs (type- I) and anti-stenotic VECs (type- II)^[9-11]. We also showed that the vast majority of human VECs that are obtainable from commercially available sources such as biopsy samples and bone marrow- or umbilical cord blood-derived endothelial progenitor cells (EPCs) belong to type- I VECs, which promote VSMC proliferation and exacerbate the development of stenosis in injured arteries^[9,11]. By contrast, type- II VECs, which suppress VSMC proliferation and prevent arteriostenosis^[9,11], are rarely obtained from commercially available sources. Because type- II VECs are convert into type- I VECs by oxidative stress and aging^[9], it seems that type- I VECs are in a generative state. Intriguingly, hESCs/hiPSCs easily

produce type- II VECs, although they convert to type- I VECs after repetitive subcultures. Thus, hESCs/hiPSCs provide an excellent tool to produce high scarcity-valued cells that are otherwise unavailable.

There is still another merit in utilizing hESC/hiPSC-derived type- II VECs as a transplantation material: They can generate beneficial outcomes by their anti-stenotic effects although they are immunologically rejected shortly after the transplantation (< 1 wk)^[11]. A transient existence of hESC/hiPSC-derived type- II VECs on the luminal surface of the injured artery effectively blocks injury-associated VSMC hyperproliferation. After immunological rejection of hESC/hiPSC-derived type- II VECs, host VECs take over the role of hESC/hiPSC-derived type- II VECs^[11]. If hESC/hiPSC-derived type- I VECs cover the injured luminal surface, development of arteriostenosis is highly accelerated and, in most cases, injured arteries undergo total stenosis^[11]. Thus, the critical point that determines the fate of injured arteries is which type of VECs, type- I or type- II , covers the luminal surface immediately after the arterial injury. Because hESCs/hiPSCs can steadily provide type- II anti-stenotic VECs, which are extremely unobtainable from commercially available sources or clinical samples of patients, hESC/hiPSC-derived type- II VECs will make a large contribution of therapeutic development of ischemic diseases (Figure 1). It should be remembered that any surgical operations which mechanically dilate stenotic arteries would more or less injure endothelial layers, causing the injury-mediated stenosis. In this sense, endothelial cell-transplanting therapies may become an indispensable mean for the control of ischemic diseases.

PROVISION OF HUMAN CLASSICAL BAS, WHICH ARE HIGH-SCARCITY-VALUED CELLS AND HAVE LONG BEEN AWAITED AS A NEW TARGET OF DRUG DISCOVERY FOR METABOLIC DISORDERS

Brown adipose tissue (BAT) is a unique adipose tissue that has high calorogenic capacities, thus contributing thermogenesis under cold environments. It is distributed in specific areas including interscapular spaces (mice

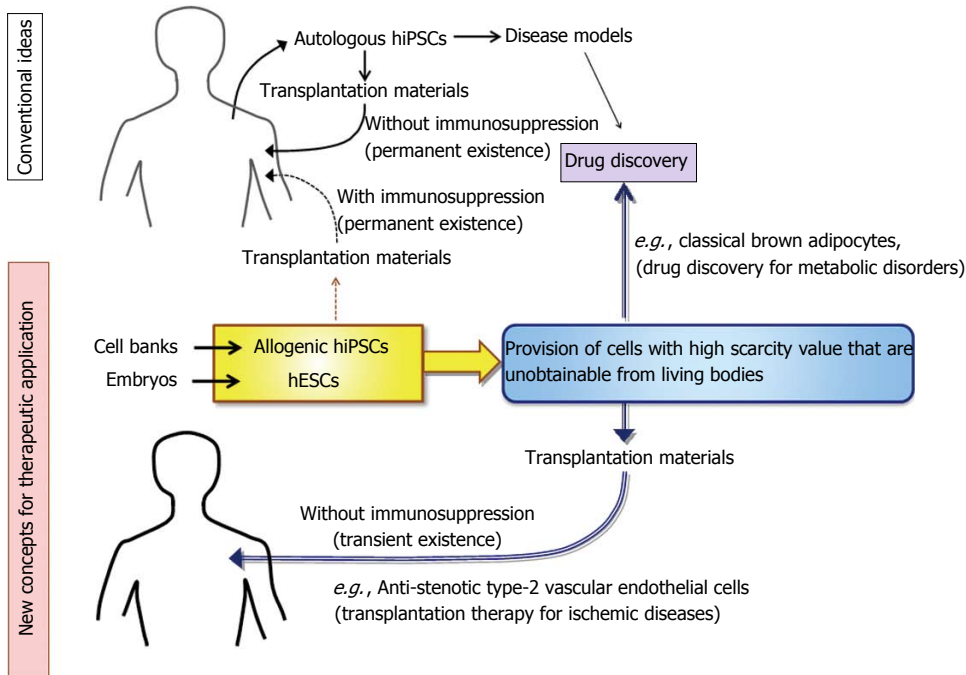


Figure 1 Application of human embryonic stem cells and allogenic human induced pluripotent stem cells to therapeutic development for the treatment of lifestyle diseases. hESCs: Human embryonic stem cells; hiPSCs: Human induced pluripotent stem cells.

and newborn humans) and deep neck regions (mice and humans). BA is derived from *myf5*-positive myoblast^[13] although the developmental process prior to the myoblast stage remains elusive. It is also known that BA-like cells called beige cells emerge in white adipose tissue under cold-acclimated conditions. To distinguish BA from beige cells, it is also called as classical BA. In addition to heat production, BAT plays crucial roles in metabolic regulation as demonstrated by murine studies: It contributes to prevention of obesity^[14,15] and improvements of glucose^[16-18] and lipid^[16,19] metabolisms.

The existence of classical BAT in humans was first reported in 2009^[20-23]. After a minor dispute in 2012^[24,25], the presence of classical BAT in adult humans was reconfirmed in 2013^[26]. Clinical studies have supported that the findings obtained from murine studies are also the case with humans^[27-30]. Thus, human classical BA is attracting great attention as a new therapeutic target for obesity-associated lifestyle diseases. However, it is hardly possible to obtain high-quality human BA samples because of economical, technical and ethical problems. First, visualization of BA-distributing sites requires an expensive medical apparatus called positron emission and computer tomography (PET/CT). Secondly, PET/CT examinations impose gamma ray irradiations on young individuals (approximately early twenties), whose BATs are visualized by PET/CT at a high probability. Thirdly, biopsy-mediated removal of BAT, whose amount is

assumed to be less than 150 g/body^[31], may possibly increase the risk of obesity-associated lifestyle diseases. Fourthly, BAT is known as a very fragile tissue to handle. Indeed, BioGPS database^[32] shows that murine BAT expresses *RNase1* and various chymotrypsin family peptidase genes at high levels. Therefore, it is extremely difficult to obtain high-quality BA samples even from mice, which have abundant BATs. Lastly, techniques for long-term cultures, expansions and frozen storage of BA do not currently exist.

All those problems have been overcome by the establishment of a method for a directed differentiation of hESCs/hiPSCs into classical BA^[33,34]. hESC/hiPSC-derived BAs possess high capacities to improve glucose/lipid metabolisms *in vivo* as proven by transplantation experiments^[33]. Moreover, this technique correctly reproduces *in vivo* developmental process of BAT because hESCs/hiPSCs were differentiated into classical BAs *via* myoblast stage^[33]. This innovative method has opened an avenue to the implementation of BA-based drug discovery (Figure 1). Moreover, it provides a groundbreaking system for basic studies to strip BAT of its aura of mystery. Although the developmental process of BA prior to the myoblast stage is currently unknown, it will be elucidated by using the method for the differentiation of hESC/hiPSC into classical BA (Figure 2). The elucidation of an early BA process will even provide new molecular targets for the drug discovery of obesity-

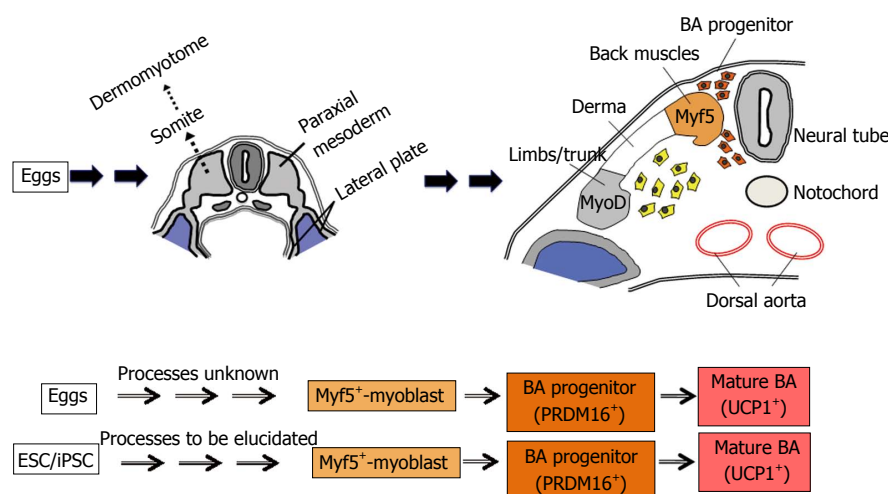


Figure 2 Developmental process of classical brown adipose tissue. Classical BA is derived from somite-derived myf5-positive populations. The schemas were drawn referring the illustrations by Sadler *et al.*^[35] and the findings by Atit *et al.*^[36]. BA: Brown adipocytes; ESCs: Embryonic stem cells; iPSCs: Induced pluripotent stem cells.

associated lifestyle diseases.

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Role of nuclear receptors in breast cancer stem cells

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Abstract

The recapitulation of primary tumour heterogeneity and the existence of a minor sub-population of cancer cells, capable of initiating tumour growth in xenografts on serial passages, led to the hypothesis that cancer stem cells (CSCs) exist. CSCs are present in many tumours, among which is breast cancer. Breast CSCs (BCSCs) are likely to sustain the growth of the primary tumour mass, as well

as to be responsible for disease relapse and metastatic spreading. Consequently, BCSCs represent the most significant target for new drugs in breast cancer therapy. Both the hypoxic condition in BCSCs biology and pro-inflammatory cytokine network has gained increasing importance in the recent past. Breast stromal cells are crucial components of the tumours milieu and are a major source of inflammatory mediators. Recently, the anti-inflammatory role of some nuclear receptors ligands has emerged in several diseases, including breast cancer. Therefore, the use of nuclear receptors ligands may be a valid strategy to inhibit BCSCs viability and consequently breast cancer growth and disease relapse.

Key words: Cancer stem cells; Hypoxia; Inflammation; Nuclear receptors; Retinoids; Peroxisome proliferator-activator receptors

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Core tip: This review examines the roles of breast cancer stem cells (BCSC) in the eliminate breast cancer disease. BCSCs represent the most significant target for new drugs in breast cancer therapy. The use of nuclear receptors ligands may be a valid strategy to inhibit BCSCs viability and consequently breast cancer growth and disease relapse.

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INTRODUCTION

The new hypothesis: Cancer stem cells

Several studies in the past years have shown that particular stem cells can have a significant role in cancer formation. These cells were identified in the hematopoietic

system, central nervous system and mammary glands. These cells are a rare cell population of "tumour initiators", with particular biological characteristics^[1-4]. These stem cells have the ability to self-renew and to develop into all the cells that form the tumour mass and are called cancer stem cells (CSCs)^[5]. In the CSCs hypothesis, cancer derives from normal stem cells that are transformed into tumour cells^[6]. Adult stem cells are characterized as long-living with a low proliferative rate and are exposed for prolonged periods to agents that can induce damage and can accumulate mutations that result in neoplastic transformation^[7]. Therefore, this condition implies the adoption of a new model to explain the carcinogenesis. Contrary to the "stochastic" model of tumorigenesis, for which the neoplastic transformation would result from random mutations incurred by a healthy cell that, consequently, undergoes clonal expansion. The CSCs hypothesis argues that the tumour begins from a stem cell, probably due to a dysregulation of the pathways involved in self-renewal^[6]. However, these mechanisms are not exclusive and we can consider that other mechanisms can participate in the genesis and tumour progression, contributing to the heterogeneity of the tumour.

Breast cancer stem cells

The existence of CSCs in tumours of the mammary gland has been widely demonstrated by several studies, based mainly on transplants. The hypothesis of the origin of the breast cancer stem cells (BCSCs) was confirmed by the finding that only a minority of human breast cancer cells have the ability to induce new tumours when transplanted into immunocompromised mice (NOD/SCID)^[8,9]. The presence of BCSCs indicates the onset of a breast tumour and they are distinguishable from other cancer cells by expression of specific membrane markers such as CD44 and an Epithelial Specific Antigen and by the absence or low expression of CD24 protein (CD44 and CD24 are adhesion molecules). Therefore, BCSCs are isolated from the tumour mass as CD44⁺/CD24⁻ by FACS analysis. Approximately 200 cells characterized by this phenotype induced tumour growth in NOD/SCID mice while 20000 cells with a different phenotype did not have this capability. CD44⁺/CD24⁻ breast cancer cells can generate cells of the same phenotype and cells phenotypically different, so that the tumour from which they develop in mice repeats the entire heterogeneity of its initial cancer^[4]. The mammary gland epithelial components are thought to arise from stem cells that undergo both self-renewal and differentiation. Self-renewal has been shown to be regulated by the Hedgehog, Notch, and Wnt pathways. Dereglulation of the self-renewal in stem cells/progenitors might be a key event in mammary carcinogenesis^[10]. Different combinations of cell surface markers such as CD44, CD49f, CD24, and CD29 as well as the activity of certain enzymes such as aldehyde dehydrogenase isoform 1 (ALDH1) have been used to identify BCSCs^[11].

The new BCSCs hypothesis has important therapeutic

implications. BCSCs have many similar characteristics to normal stem cells, such as apoptosis resistance, the capacity to repair DNA damage and multidrug-resistance (MDR). MDR is important to explain the capacity of breast cancer to overcome chemotherapy. BCSCs are characterized by expression of genes encoding ATP-binding cassette (ABC) proteins, which are transmembrane transporters involved in the extrusion of drugs from cancer cells, as ABCC1, ABCG2 and ABCB1. The principal MDR proteins that are expressed in BCSCs are the P-glycoprotein, the multidrug-resistance protein 1 (MDR1) and the cluster differentiation 243 (CD243). Chemotherapy drugs with anti-proliferative effects are less effective on CSCs population, because these cells divide less frequently than cancer cells^[6,7]. For these reasons chemotherapy destroy many neoplastic cells but does not affect the minority component of tumour such as BCSCs. Accordingly, BCSCs induce tumour relapse and metastases^[8,9]. So a change in chemotherapy strategy is necessary to kill also BCSCs.

The stem cell niche and CSC

Stem cells are localized in a niche that is a local tissue microenvironment. The niche has a limited area where the cells can maintain their peculiarity. Significant progress has been made by the studies on the interactions between the stem cells and the microenvironment in *Caenorhabditis elegans* and mammals^[12,13]. Comparing the stem cell niches in these systems, various common features and functions have emerged. The niche is formed by a group of cells (fibroblasts of the stroma) which have a support function for stem cells, serving as the anchor point for the stem cells and physical adhesion molecules mediate the interactions between the support cells and stem cells (as well as those between the stem cells and the extracellular matrix). The niche generates factors that control number, proliferation and differentiation of stem cells. Normally, it maintains the stem cells in a quiescent state, providing them with the signals that inhibit the growth and proliferation. Only after implementing a stimulus transient activator, stem cells are able to divide in order to participate in tissue regeneration. This suggests that control stem cell dependent signaling mechanisms, resulting from dynamic niche and maintaining the balance between the proliferative and anti-proliferative signals, are the key to the homeostatic regulation of the stem cells^[12,13]. When there is a change in the niche and growth and proliferation signals prevail, the stem cell population is exposed to an uncontrolled expansion which can lead to spread CSCs^[13].

Inflammation and breast cancer stroma

The idea that inflammation could play a role in carcinogenesis was born in 1863, when Rudolf Virchow noted the presence of leukocytes in neoplastic tissues. After this observation, more and more data have demonstrated that malignancy may begin at sites of infection or chronic inflammation and approximately 25% of all cancers are

associated with such conditions. In fact, although the inflammation represents a defensive response adaptive to infection or injury and is, under normal conditions, a self-limiting process which culminates in the repair of damaged tissues, an inadequate resolution induces chronic diseases or cancer^[14]. Chronic inflammation is involved in all stages of carcinogenesis (initiation, promotion and progression). Inflammation induces an excessive production of reactive oxygen species that could cause genomic instability and mutation and consequently a tumour^[14]. Stromal cells as fibroblasts, that are around the tumour, and inflammatory cells, as macrophage, that are infiltrated in the tumour, help to create an environment favoring the increase of inflammation^[15]. Fibroblasts are among the most abundant cell types in solid tumours and are especially important in breast, pancreas, colon and prostate cancer^[16]. In physiological conditions, fibroblasts have a low proliferative rate and have a constant production of extracellular matrix (ECM). ECM has anchoring function for the epithelial cells maintaining the integrity of structural epithelium. In the carcinoma *in situ*, the stroma is not compromised because it remains separated from the tumour cells through the basement membrane integrity. With the acquisition of infiltrating characteristics, some tumour cells manage to cross the basal lamina whose breaking mimics a traumatic insult to the tissue, causing changes in non-epithelial cell types. Fibroblasts are activated and become tumour associated fibroblasts (TAF) thus contributing to the growth and expansion of tumour in several ways: they produce proteins such as matrix metalloproteinases (MMPs) that have proteolytic activity on the components of the extracellular matrix^[17]; release high levels of stromal-derived factor-1 (SDF-1) which attracts endothelial progenitor cells in the tumour mass (thus promoting angiogenesis); directly promote the growth of cancer cells through interaction with their receptor CXCR4^[18]; release some growth factors such as epidermal growth factor (EGF) and transforming growth factor β (TGF β) and release a wide range of inflammatory cytokines^[16,19]. Macrophages resident in the stroma and monocytes that act together with tumour chemotactic factors, undergo changes that lead them to favor tumour growth. Thus, tumour associated macrophages (TAM) support tumour angiogenesis through the secretion of pro-angiogenic factors as the vascular endothelial growth factor (VEGF), the interleukin-1 β (IL1 β) and the angiogenin (Ang). TAM facilitate the migration of cancer cells through the release of tumor necrosis factor- α (TNF α), MMPs (such as the MMP9) and other proteases such as tissue plasminogen activator^[20,21]. Moreover, TAM produce factors, such as EGF, that directly promote the growth of cancer cells^[22] and have a role in facilitating the invasion of neoplastic cells^[23]. Finally, TAF can activate macrophages that produce cytokines to maintain an inflamed microenvironment^[24,25]. Inflammatory cytokines, including SDF-1, interleukin-1 (IL-1), IL-6 and IL-8, may affect tumour growth by regulating of CSCs population^[26]. In particular, it has been demonstrated that IL-6 can induce the acquisition of malignant characteristics in multicellular spheroids called mammosphere (MS), formed from stem cells and progenitors of the mammary gland;

such aggregates were obtained *in vitro* in conditions of non-adherence from MCF-7 breast cancer cell line (MCF-MS) or obtained from breast surgical specimens (normal and tumour, N-MS and T-MS respectively)^[27]. High levels of IL-6 mRNA were detected in T-MS, however IL-6 can stimulate the growth and self-renewal in both T-MS and N-MS. In particular, IL-6 induce overexpression of Notch-3 and of its ligand Jagged1, both implicated in the maintenance of stem cells in an undifferentiated state. It has been demonstrated that the pathway IL-6/Notch-3 increases the expression of the protein carbonic anhydrase IX (CAIX), from which depends the survival of MCF7-MS in hypoxic environment, as well as an increase of their invasive potential. IL-6 acting through different signal transduction pathways involving protein kinases such as mitogen activated protein kinase (MAPK) or the phosphatidylinositol-triphosphate kinase (PI3K) and having the ability to directly activate STAT transcription factors (such as STAT3) *via* the kinase JAK2 which is associated its receptor, leads to a number of responses that favor the proliferation, inhibits apoptosis and increases the invasive capacity of tumour cells^[28,29]. IL-6 induces the activation of the transcription factor NF- κ B that can code for several cytokines^[27]. Other studies show that the TNF α , the major inducer of NF- κ B, involves an increase in the formation of MCF-7-MS cells through up-regulation of the *Slug* gene, a regulator of stem mammary tumour phenotype^[30]. TNF α induces, after 10 d of treatment, the acquisition of typical characteristics of BCSCs (CD44⁺/CD24⁻) in not transformed mammary epithelial cell line MCF-10A; this effect is accompanied by the reduction of the E-cadherin expression and an increase of mesenchymal markers expression such as vimentin and smooth-muscle actin- α (α SMA)^[31,32]. These considerations lead to the hypothesis that the survival of the BCSCs is dependent on the activation of NF- κ B, in turn resulting from the stimulation exerted, for example, by pro-inflammatory cytokines, as confirmed by the effect of inhibition of the proliferation of MCF7-MS due to the use of selective inhibitors of the NF- κ B, as parthenolide^[33].

Hypoxia and BCSCs

Hypoxia plays a key role in carcinogenesis. Solid tumours are characterized by poorly vascularized regions and can progress under hypoxic conditions. Hypoxia is a condition that generally is found within the stem cell niche, which requires low concentrations of oxygen in order to minimize the damage that the eventual oxidation of the DNA could generate^[34]. Hypoxia is also involved in the maintenance of an undifferentiated cell, thus playing a crucial factor in the stem cells condition and for this reason; could potentially contribute to the generation and or to support the CSCs. In hypoxic condition, there is an induction of the octamer-binding transcription factor 4 (Oct4) and Notch1 expression in CSCs, two proteins that are involved in the self-renewal and differentiation pathways^[35,36]. Hypoxia induces *Shc* gene expression in BCSCs, a gene that coding for p66Shc protein, involved in cellular response to oxidative stress, which induce the up-regulation of Notch-3 and its ligand Jagged-1. Interestingly, there is a

correlation of Snail expression with histological grade and lymph node status in breast carcinomas^[37]. Snail coding for CAIX protein is a molecule that is overexpressed in hypoxic condition and in BCSCs^[38]. Cancer cells in the hypoxic tumour niche overexpressed the hypoxic inducible factor (HIF). HIF is a heterodimeric transcription factor consisting of an α -subunit (HIF-1 α or HIF-2 α), and a β -subunit (HIF-1 β), expressed constitutively. HIF-1 affects a variety of malignant features, such as hypoxic cancer cell survival, *via* the regulation of a large number of genes, including CAIX^[27]. The oxygen-dependent HIF activity is mediated by a series of enzymes containing iron (Fe²⁺), belonging to the superfamily of 2-oxoglutarate-dependent dioxygenase that are oxygen sensitive. Members of this family are the prolyl hydroxylase domain-containing protein (PHD), as PHD1, PHD2, PHD3 and the factor inhibiting HIF. In normoxic conditions, the HIF-1 α subunit is characterized by a very short half-life. In hypoxic condition, HIF-1 α translocates into the nucleus and leads to gene activation by binding to a specific sequence (59-RCGTG-39) called Hypoxic Responsive Element (HRE), through the recruitment of coactivators CBP/300^[39]. HIF-1 α causes a metabolic change that allows cancer cells to adapt to poorly oxygenated environments: It results in the use of glycolysis at the expense of oxidative phosphorylation, even in aerobic conditions, with a decrease in mitochondrial respiration and an increased lactate. This phenomenon is called "Warburg effect" and is frequently found in cancer^[40]. HIF induces a metabolic "shift" *via* transcriptional activation of genes involved in glucose turnover, including those coding for glucose transporters, glycolytic enzymes and enzymes involved in the production of lactate and in the metabolism of pyruvate^[41,42]. HIF induces the VEGF expression and reduce anti-angiogenic factors, such as thrombospondin^[43]. A recent study has shown that, in several cancer cell lines (breast, lung, cervical and ovarian), HIF-1 α increases cell invasion^[44]. Since HIF-1 α induces the transmembrane protein CAIX expression, through various mechanisms CAIX can increase the invasive potential of cancer cells^[45]. Moreover HIF controls the expression of LOX (lysyl oxidase) and the cytokine receptor CXCR4 expression that are essential for metastasis induction^[46]. Finally, HIF reduces E-cadherin expression and induces the epithelial-mesenchymal transition^[47-49].

NUCLEAR RECEPTORS

Nuclear hormone receptors (NRs) include receptors for steroid hormones such as estrogen receptors (ERs) and progesterone receptors (PRs), receptors for the thyroid hormone (TRs), receptors for vitamin D (VDRs), retinoic acid receptors (RARs), retinoid X receptors (RXRs) and a number of receptors that respond to intermediary metabolites, among which there are the peroxisome proliferator-activator receptors (PPARs) activated by fatty acids and prostaglandins^[50-52]. The members of this superfamily act as transcription factors activated by ligands and have a conserved structure^[53]. NRs are

characterized by the presence of two conserved domains: (1) A central DNA-binding domain (DBD) which interacts with the core motif, that have specific DNA sequences called "response elements" (monomeric NR recognize a single core motif, while dimeric NR complexes interact with repeated occurrences of this core motif); and (2) A C-terminal ligand-binding domain, which determines specific NRs properties and is highly variable between the different receptors. NRs are characterized by a flexible linker region between the two previous domains. NRs have a carboxy-terminal E-domain that is responsible for the ligand binding, dimerization, and contain an inducible transactivation function dependent on ligand (AF-2). Finally, the N-terminal terminal A/B-domain of the NR molecule contains a constitutive activation function independent on ligand (AF-1). NR can be activated by specific ligands that can modulate gene transcription and induce differentiation and anti-proliferative effects in cancer cells in several tumours^[54].

Retinoic acid receptors and retinoid x receptors

Nuclear receptors retinoic acid receptors (RARs) and retinoid x receptors (RXRs) mediate the effects of retinoids. Retinoids are a class of compounds that includes natural metabolites of vitamin A (retinol) and its synthetic analogues. The natural retinoids are produced *in vivo* by oxidation of retinol, a two-step process that leads to the formation of all-trans-retinaldehyde due to the action of alcohol dehydrogenase, followed by oxidized retinaldehyde due to the action of the enzyme dehydrogenase. In the reaction all-trans-retinoic acid (ATRA) is produced, which is then metabolized by CYP26 to produce hydroxylated metabolites^[51]. There are three receptor subtypes, encoded by different genes, called RAR α , β , γ and RXR α , β , γ . RARs subtypes can bind with high affinity not only ATRA as well as 9-cis retinoic acid (9cRA), the product of isomerization of ATRA, that is able to interact with RXRs, a feature that sets it apart from trans retinoic acid isoforms that do not have this possibility^[55]. Following the activation induced by the ligand, the RARs form heterodimers with RXRs (RAR-RXR) that lead to gene transcription by binding to specific DNA sequences in the promoter of target genes, those corresponding to the Retinoic Acid Response Element (RARE), while homodimers formed by RXRs (RXR-RXR) bind to sequences denominated RXRE (Retinoic X Response Element)^[52]. The RXRs are the only nuclear receptors that are capable to form both homodimers (RXR-RXR) and heterodimers (NR-RXR), constituting factors required for efficient DNA binding of many other members of the NR superfamily, including RARs and PPARs precisely^[52]. These considerations underscore the importance of RXRs ligands because they can mediate effects affecting many biological processes. The NRs partner of RXRs receptors can be "permissive", as PPARs. The heterodimer that is formed can be activated independently from agonists of one or other receptors or, synergistically, by both. RXRs may be "non-permissive", when the heterodimer cannot be activated by RXRs agonists alone, necessitating the presence of a ligand for

the receptor partners (in the case of the dimer RAR-RXR is necessary a RARs ligand as ATRA)^[51]. Various studies targeting the identification of a natural endogenous ligand for RXRs did not produce the desired results because the molecules proposed for this role (9cRA, phytanic acid, docosahexaenoic acid) have not demonstrated a selectivity only for binding to RXRs; for this reason synthetic compounds that bind only to RXRs (called rexinoids) could be essential to better understand the role of these receptors^[52].

Retinoids and breast cancer

Retinoids are widely used to treat dermatological diseases. Retinoids have recently received considerable attention for the prevention and treatment of cancer due to their role in cell differentiation and their anti-proliferative, pro-apoptotic and anti-oxidant effects^[56]. Epidemiological studies show that a low intake of vitamin A leads to a higher risk of developing cancer. Altered expression of RARs and RXRs is associated with malignant transformation both in animal tissues and in cultured cells^[57]. Furthermore, in animal models retinoids reduce cancer of skin, lung, breast, bladder, ovary and prostate. In humans, retinoids can reverse epithelial precancerous lesions, induce differentiation of myeloid cells, and have an important role in the lung, liver and breast cancer prevention^[58]. Moreover, retinoids regulate stem cell differentiation^[59]. Retinoic acid is used today in various diseases: ATRA is the principal retinoid investigated in clinical trials for the treatment of lymphoma, leukemia, melanoma, lung cancer, cervix, kidney, neuroblastoma, and glioblastoma. Its clinical use has more effect in the treatment of the acute promyelocytic leukemia (APL). Since 1995, the FDA approved ATRA to APL treatment^[60]. 9cRA differs from ATRA for its ability to activate both RAR and RXR. In addition, 9cRA activates different nuclear receptors such as PPARs, FXRs, PXR and VDRs through RXR heterodimerization. In preclinical studies, 9cRA is effective in the prevention of prostate cancer and breast cancer and was also approved by the FDA for the topical treatment of cutaneous lesions of Kaposi's sarcoma^[61]. The natural retinoid 13-cis retinoic acid (13cRA), binds both receptors RARs and RXRs, has anti-inflammatory activity and is in clinical development for different types of cancer, including cancer of the thyroid^[62]. Preclinical and clinical studies have shown the anti-tumoural effects of retinoids in breast cancer. It has been observed that 9cRA inhibits proliferation and induces differentiation and apoptosis in the breast cancer cell line MCF-7 cells. Recently, it has been demonstrated that retinoid have a role also in the regulation of BCSCs self-renewal and differentiation; ATRA reduces BCSCs proliferation demonstrated by ALDH assay^[11]. However, clinical studies have shown that natural retinoids can have side effects such as the hypervitaminosis A. It has been demonstrated that retinoids selective for RARs have chemopreventive activity with side effects, while selective RXRs retinoids (called rexinoids) suppress

mammary tumorigenesis without side effects^[63]. Since hypertriglyceridemia can be induced by rexinoids, recent research has investigated new rexinoids that have anti-tumoural effects without side effects. Among these there is (2E,4E,6Z,8Z)-8-(3',4'-Dihydro-1'(2H)-naphthalen-1'-ylidene)-3,7-dimethyl-2,3,6 octatrienoic acid (UAB30) that is currently undergoing clinical evaluation as a novel breast cancer prevention agent^[64]. Furthermore, some patients may experience relapses cancer because cancer cells become resistant to retinoids therapies. For these reasons, the synergic use of multiple molecules as NRs ligands with other molecules at lower doses might be a good strategy to block breast cancer growth, while inducing less side effects in patients. Immunotherapy with the use of retinoids and T cell has proved effective in the treatment of neuroblastoma and 13cRA+interferon- α 2a significantly increases the survival of patients with metastatic renal cell carcinoma^[65]. Lee and co-workers have shown that administration of ATRA increased the effectiveness of EGCG at a low concentration. Indeed, ATRA increased the synthesis of a EGCG molecular targets, the 67 kDa laminin receptor (LR67), which plays a key role in cell adhesion and in the breast metastatic process^[66]. ATRA is a regulator of epithelial mesenchymal transition (EMT) that is a determinant of the breast cancer cell invasion and metastatic behaviour. It has shown that in HER2-positive SKBR3 and UACC812 cells, there is an amplification of the *ERBB2* and *RARA* genes and ATRA activated a RAR α -dependent epithelial differentiation program. Moreover, ATRA blocked Notch-1 up-regulation by EGF and/or heregulin- β 1 and switches TGF β from an EMT-inducing and pro-migratory determinant to an anti-migratory mediator^[67]. ATRA can reduce the MS-forming ability of a subset of breast cancer cells, which correlates with induction of apoptosis, reducing SOX2 expression and inducing of its antagonist CDX2. The SOX2/CDX2 ratio has prognostic relevance in BCSCs^[68]. K-Ras mutant BCSCs was resistant to ATRA, which was reversed by MAPK inhibitors. Thus, ATRA can be used in combination to reduce BCSC proliferation^[68]. Interestingly, also the combination ATRA and doxorubicin can differentiate and kill the BCSCs. Differentiation of CSCs into non-CSCs can reduce their self-renewal capacity and increase their sensitivity to chemotherapy in a synergistic manner^[69].

The new rexinoid IIF can kill BCSCs

In our laboratory, we have investigated the antitumoural effects of ATRA when binded to RARs while with the RXRs ligand, we used the synthetic rexinoid 6-OH-11-O-hydroxyphenanthrene (IIF), a new derivative of retinoic acid, capable of binding selectively to RXR and mainly activating the form RXR- γ ^[70]. Several *in vitro* studies show that IIF can be used as an anticancer agent: This rexinoid showed a greater anti-proliferative effect than ATRA and 9cRA in leukemic cell line HL-60, which induces apoptosis^[71]. IIF induces differentiation in different tumour cell lines, such as colon carcinoma and neuroblastoma^[72]. In the glioblastoma mouse model IIF reduces tumour

growth and invasion through the inhibition of MMPs, such as MMP-2 and MMP-9, in combination with increased expression of their inhibitors (TIMP-1 and TIMP-2)^[73]. IIF has anti-inflammatory effects in colon cancer by suppressing the expression of cyclooxygenase-2 (COX-2), the inducible form of COX, responsible for the prostaglandin production that is overexpressed in many tumours^[73]. Recently, we have demonstrated that ATRA and IIF reduce the inflammation-dependent survival in MS generated from human tumour specimens (T-MS) and from the breast cancer cell line MCF-7 (MCF7-MS), but not in MS derived from normal mammary glands (N-MS). The effect depends on the inhibition of the inflammatory pathway NF- κ B/IL-6 which is wired in T-MS. ATRA and IIF, blocking NF- κ B axis, reduced expression of genes involved in the maintenance of a tumour stem cell phenotype (such as *Slug*, *Notch-3*, *Jagged-1*) and was accompanied by an increased expression of markers of differentiation such as ER α and keratin-18^[74]. A promising strategy is the combination of IIF with natural substances, such as Epigallocatechin-3-gallate (EGCG), that have a cytotoxic effect against breast cancer cells. In a recent study, we demonstrated that the combination of IIF and EGCG had a higher activity than the individual administration. IIF and EGCG can have a common signaling pathway that induces apoptosis by reducing epidermal growth factor receptor activation and its downstream kinase AKT-1^[75].

PPAR receptors and their agonists

RXR receptors can form heterodimers with PPARs receptors. The latter mediate the effects of many synthetic compounds called peroxisome proliferators (PPs-peroxisome proliferators). The PPs influence both the number and the size of the peroxisomes, responsible for various functions within the cell (β -oxidation of fatty acids and cholesterol metabolism). Even PPARs there exist three isoforms (α , β , γ), encoded by different genes and characterized by different tissue localization. They operate as sensors for fatty acids and their derivatives, checking therefore, important pathways concerning lipids and energy metabolism^[76]. PPAR α is expressed at high levels in organs with significant catabolism of fatty acids. PPAR β has the broadest expression pattern, and the levels of expression depend on the extent of cell proliferation and differentiation. Finally, PPAR γ is expressed as two isoforms, of which PPAR γ 2 is found in the adipose tissues, whereas PPAR γ 1 has a broader expression pattern and is expressed at high levels in cancer tissue^[76]. RXRs dimerization and the presence of coactivators are necessary for PPARs activation as transcription factor^[77,78]. There is a wide range of endogenous and exogenous ligands that can interact with PPARs, leading to have different responses. Among PPARs endogenous ligands there are arachidonic acid, eicosapentaenoic acid and prostaglandin J2, while, among exogenous ligands there are the synthetic compounds called thiazolidinediones (TZDs): Pioglitazone (PGZ), rosiglitazone and troglitazone^[54]. The TZDs are used in the treatment of type 2 diabetes because they decrease insulin resistance; they increase glucose uptake in

peripheral tissues and reduce hepatic production. Some studies show, however, that TZDs could be successfully used also against tumours. In breast cancer, for example, tumour cells often express high levels of PPARs and it was demonstrated that TZDs are able to induce differentiation and inhibit tumour proliferation both *in vivo* (nude mice) and *in vitro* (mammary tumour cell line MCF-7); these effects are increased when combined with retinoids^[79,80]. It was also noted that treatment with TZD leads to a reduction in the number of breast cancer cells in S phase and an increase of cells in phase G₀-G₁. Furthermore, TZD and retinoids induced apoptosis in 30%-40% of breast cancer cells through the inhibition of Bcl-2 expression^[80]. Among the anticancer mechanisms mediated by PPARs ligands, in addition to the induction of pro-apoptotic proteins and stabilization of cell cycle, the inhibition of the expression or activity of various cytokines and transcription factors involved in inflammatory pathways (as TNF α , IL-1, IL-4, NF- β) could help to slow the growth of transformed cells^[54]. Interfering with inflammatory pathway is an ability shown by some ligands of PPAR α ; fenofibrate and GW7647 (synthetic agonists). For example, they can significantly reduce the levels of pro-inflammatory cytokines such as IL-1, the expression of TNF α , COX-2 and an inducible form of the enzyme nitric oxide synthase in murine microglia BV-2 exposed to radiation. This effect is due to the inhibition of translocation of the NF- κ B-p65 subunit or the inhibition of phosphorylation c-jun, a subunit of the transcription factor AP-1, both involved in inflammatory mechanisms^[81]. MnSOD expression is significantly amplified in the aggressive breast carcinoma basal subtype. Interestingly, PPAR γ activation repressed MnSOD expression and increased chemosensitivity, and inhibited tumour growth in MDA-MB-231 and BT549 breast cancer cell lines^[82]. PPARs are also reported to be involved in the modulation of the EMT process in CSCs initiation and in the regulation of CSCs functions^[83]. Some data show that activation of PPAR α could induce cancer and result in the induction of inflammatory responses. If the stimulation of the PPARs, for example with the TZD, involves the inhibition of neoplastic growth and the induction of differentiation, activation of PPAR α significantly increases the proliferation of tumour cells, as demonstrated for the breast cancer cell lines MDA-MB-231 and MCF-7^[84]. This stark contrast between these isoforms of PPAR is highlighted by studies that show the effects mediated by an agonist of PPAR α , WY-14643. Chronic administration of PP in rats and mice leads to development of hepatocellular carcinoma; as a result of repeated exposure to WY-14643. Mice in which the expression of PPAR α is increased, do not develop this type of tumour, as opposed to what happens in wild-type mice for PPAR α , demonstrating that the receptor mediates the effects of carcinogenic arising by the stimulation exerted by an agonist^[85]. More recently it has been seen that WY-14643 promotes the formation of a MS-tumour (derived from cells of the mammary tumour cell line MCF-7) by stimulating the activation of the NF- κ B/IL-6 and, consequently, the expression of genes *Slug*, *Notch-3*, *Jagged-1*, whereas the silencing of PPAR α

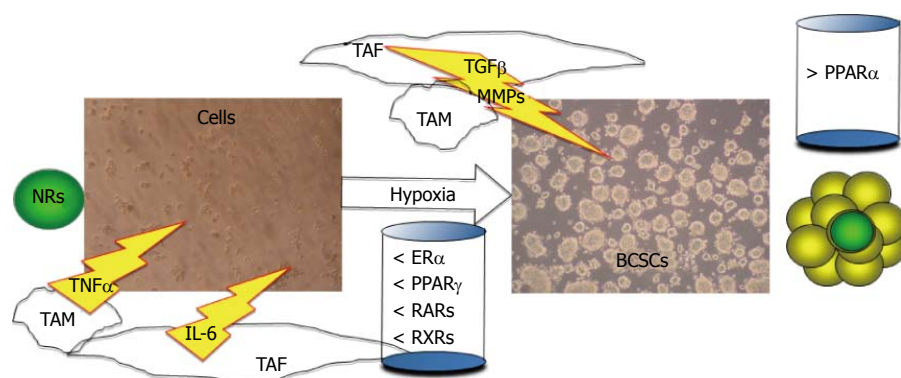


Figure 1 The nuclear receptors phenotype in breast cancer stem cells. In breast cancer, TAF and TAM promote inflammation and invasion through the secretion of cytokines, as IL6, TNF α and TGF β and the secretion of MMPs, as MMP9. In this hypoxic inflammatory niche, particular stem cells can form the BCSCs as mammospheres. BCSCs are characterized by a particular nuclear receptors phenotype: a lower level of ER α , PPAR γ , RARs, RXRs, and a higher level of PPAR α is expressed than adherent breast cancer cells. NRs: Nuclear receptors; BCSCs: Breast cancer stem cells; TAF: Tumour associated fibroblast; TAM: Tumour associated macrophage; MMPs: Metalloproteinases; IL6: Interleukin-6; ER α : Estrogen receptor- α ; PPARs: Peroxisome proliferator-activator receptors; RARs: Retinoic acid receptors; RXRs: Retinoid x receptors; TNF α : Tumor necrosis factor- α ; TGF β : Transforming growth factor- β .

with a specific siRNA reduces tumour-MS formation. Furthermore, PPAR α expression is positively correlated with the phenotype of BCSCs obtained from specimens of breast cancer patients^[86]. Finally, we have recently demonstrated that IIF potentiates the ability of PGZ to hamper the MS-forming capability of human breast tumours and MCF7 cancer cells, reducing the expression of CSCs regulatory genes (*Notch3*, *Jagged1*, *SLUG*, *IL-6*, *Apolipoprotein E*, *HIF-1 α* and *CAIX*). Notably, these effects are not observed in normal-MS obtained from human breast tissue^[87]. Recently, Wang *et al.*^[88] demonstrated that PPAR γ -binding protein upregulates several genes in the de novo fatty acid synthesis network, which is highly active in ERBB2-positive breast cancer cells. ERBB2 is a prognostic marker occurring in 30% of breast cancers and is associated with aggressive disease and poor outcomes. Inhibition of the PPAR γ pathway using PPAR γ antagonists (GW9662 and T0070907) reduces the ALDH-positive population and tumour-MS formation in ERBB2-positive breast cancer cells^[88].

Vitamin D and BSCSs

Vitamin D-3 exerts most of its cellular effects *via* its nuclear receptor, the vitamin D-3 receptor (VDR), that heterodimerizes with the RXRs. The VDR-RXR complex binds vitamin D responsive elements (VDRE) in gene promoters and regulates transcription of target genes^[89]. It has been reported in literature that vitamin D is a potential preventive/therapeutic agent against CSCs. Several proteins, such as Notch, Hedgehog, Wnt and TGF- β , are modulated by vitamin D in CSCs as well as in normal stem cell^[90]. Interestingly, MS derived from BRCA1-silenced MCF7 or MDA-MB-231 breast cancer cells were no longer sensitive to the growth inhibitory effects of vitamin D, 1 α , 25-dihydroxyvitamin D 3 (1,25D). Since, the active form of vitamin D is a potent inhibitor of BCSCs growth through the down-regulation of BRCA1 expression, which is the most frequently mutated tumour suppressor gene in breast cancer^[91]. Treatment with 1 α 25(OH)2D3 or BXL0124 (two vitamin D compounds)

repressed markers associated with the breast stem cell-like phenotype, such as CD44, CD49f, c-Notch1 and NF- κ B. Furthermore, 1 α 25(OH)2D3 and BXL0124 reduced the expression of pluripotency markers, OCT4 and KLF-4 in BCSCs^[92]. However, some authors have shown that MS were relatively insensitive to treatment with 125D compared to more differentiated breast cancer cells; instead combined treatment of 125D and DET- NONOate induce a significant decrease in the overall size of MS and reduced breast tumour volume in nude mice^[93]. Combination therapy using 125D with drugs specifically targeting key survival pathways in BCSCs could be a best strategy to overcome aggressive breast cancer.

Estrogen receptor and BCSCs

It has been reported in literature that many breast cancers express estrogen receptor- α (ER α) and are dependent on estrogens^[94]. Tamoxifen is the most widely used in endocrine therapy for ER α positive (ER $^{+}$) breast cancers during the last 30 years. Unfortunately, up to 40% of metastases from ER $^{+}$ primary breast cancer do not respond to endocrine therapy. Recent study have demonstrated that tamoxifen was effective in reducing proliferation of ER α positive (ER $^{+}$) adherent cancer cells, but not their CSCs population^[95]. Interestingly, estrogen is essential for the development of the normal breast, but adult mammary stem cells are known to be ER α negative (ER $^{-}$)^[96]. BCSCs sorted derived from ER $^{+}$ breast cancer tissue and established breast cancer cell lines, have low or absent ER expression^[74,96]. However, estrogen stimulated BCSCs activity demonstrated by increased MS-formation through the induction of EGF and Notch receptor signaling pathways^[96]. Breast cancer cells develop resistance to endocrine therapies by shifting between ER-regulated and growth factor receptor-regulated survival signaling pathways^[97]. However, the roles of BCSCs in antiestrogen resistance and the underlying molecular mechanisms have not been well established. Recent, a novel variant of ER α , called ER α 36 (molecular weight of 36 kDa) it has been investigated. ER α 36 mediates rapid antiestrogen

signaling and is highly expressed in ER⁺ breast progenitor cells. Antiestrogens increased the percentages of the BCSCs from ER⁺ breast cancer cell through stimulation of luminal epithelial lineage specific and these BCSCs are more resistant to antiestrogens than the bulk cells. Finally, ER α 36 mediated antiestrogen signaling such as the PI3K/AKT that plays an important role in antiestrogen resistance of ER⁺/BCSCs^[98].

CONCLUSION

BCSCs represent the most significant target for new anti-breast cancer drugs. In fact, BCSCs are likely to sustain the growth of the primary tumour mass, as well as to be responsible for disease relapse and metastatic spreading in breast cancer^[6-10]. The activity of NF- κ B in BCSCs and in the tumour stroma (mainly formed by fibroblasts and inflammatory cells) has been recognized to be of pivotal importance in normal and CSCs survival^[99]. It has been proposed that "NF- κ B activity addition" would make CSCs more susceptible to NF- κ B inhibitors than their normal counterparts^[26,55]. For these reasons the use of molecules, as NRs ligands, capable of inhibiting NF- κ B dependent inflammation may be the best strategy to hamper BCSCs growth^[74,86]. Recently, we demonstrated that BCSCs have a particular NRs phenotype^[86,87] (Figure 1). Therefore, the synergic use of multiple molecules (as ligands of NRs) at lower doses might be a good strategy to kill BCSCs, while inducing fewer side effects in patients. Moreover, the use of NRs ligands in combination with each other (as ligands of PPARs with ligands of RXRs) or with other substances (e.g., EGCG) may be a valid strategy to inhibit BCSCs viability.

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Homing and migration of mesenchymal stromal cells: How to improve the efficacy of cell therapy?

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Abstract

Mesenchymal stromal cells (MSCs) are currently being investigated for use in a wide variety of clinical applications. For most of these applications, systemic delivery of the cells is preferred. However, this requires the homing and migration of MSCs to a target tissue. Although MSC homing

has been described, this process does not appear to be highly efficacious because only a few cells reach the target tissue and remain there after systemic administration. This has been ascribed to low expression levels of homing molecules, the loss of expression of such molecules during expansion, and the heterogeneity of MSCs in cultures and MSC culture protocols. To overcome these limitations, different methods to improve the homing capacity of MSCs have been examined. Here, we review the current understanding of MSC homing, with a particular focus on homing to bone marrow. In addition, we summarize the strategies that have been developed to improve this process. A better understanding of MSC biology, MSC migration and homing mechanisms will allow us to prepare MSCs with optimal homing capacities. The efficacy of therapeutic applications is dependent on efficient delivery of the cells and can, therefore, only benefit from better insights into the homing mechanisms.

Key words: Mesenchymal stromal cells; Homing; Bone marrow; Homing receptors; Extravasation

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Core tip: Mesenchymal stromal cells (MSCs) are currently under investigation for use in a variety of clinical applications. In most studies, MSCs are administered systemically. This requires efficient homing and migration of the MSCs to a target tissue. However, the homing mechanisms of MSCs are not completely understood. Moreover, the *in vivo* homing and migration of MSCs does not appear to be highly efficient. Therefore, different methods have been investigated to improve homing. Here, we will review the current knowledge of bone marrow homing of MSCs, as well as the different strategies that might improve the homing capacity of these stem cells.

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INTRODUCTION

Mesenchymal stromal cells (MSCs) are non-haematopoietic cells that were first derived from the bone marrow and described approximately 40 years ago by Friedenstein *et al.*^[1]. In 2006, the International Society for Cell Therapy defined the minimal criteria to define human MSCs. They must adhere to plastic in culture and differentiate into osteocytes, chondrocytes and adipocytes. Additionally they must express CD105, CD90 and CD73 and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR surface molecules^[2].

There is great interest in using these cells in a wide variety of clinical domains, such as Neurology, Orthopaedics, Cardiology and Haematology^[3-6]. This interest arises from the following MSC characteristics: They have immunomodulatory capacities, they are multipotent and are thus possible effectors for tissue regeneration, and they tend to migrate to sites of tissue injury/inflammation^[7-11]. Additionally MSCs might escape immune recognition, although conflicting observations about this particular phenotype have been published. MSCs do not express MHC class II antigens, but the expression of these molecules can be upregulated after exposure to inflammatory cytokines or during MSC differentiation^[12]. The data from animal studies suggest that MSCs can elicit allogeneic immune responses and be rejected^[13-16]. On the other hand, there is also a report of MSCs that overcame this allogeneic immune response due to their immunomodulatory capacities^[17]. von Bahr *et al.*^[18] addressed this issue and published follow-up data of patients treated with MSCs, showing that there was no correlation between the MSC source (donor-derived or third party) and the patients' response to the MSC treatment. The clinical applications of these cells have been extensively studied in Orthopaedics, where MSCs are used to repair large bone defects, and in Haematology for the treatment of graft-vs-host disease and support for the engraftment of hematopoietic stem cells^[4,6,19]. In recent years, MSCs have been studied as vehicles to deliver anti-cancer treatments because there is evidence that MSCs home to tumour sites. They can be induced to express anti-cancer proteins [e.g., interleukin (IL) 2], to produce pro-drug activating enzymes, which ensures that the active drug will only be localized in the tumour, or to deliver oncolytic viruses^[20-23]. For these applications, the homing and persistence of MSCs in the target tissue are desirable^[24].

When MSCs are used in clinical applications, different modes of administration are possible: Systemic administration [intravenous (IV) or intra-arterial (IA) injection] or local administration [intracoronary (IC) injection or direct injection into the tissue of interest]. Of these different options, IV injection is the most widely used because it is minimally invasive, the infusions can be readily repeated

and the cells will remain close to the oxygen- and nutrient-rich vasculature after extravasation into the target tissue^[25]. However, after IV injection, the cells appear to be trapped in the lungs, and thus efficient homing to the target tissues might be compromised. IA administration requires an invasive procedure that has a higher risk of complications than IV. Although IA injections might improve tissue-specific homing compared to IV, there is a concern that microthrombi might occur as a result of trapping large MSCs in the microvasculature. One example is the concern regarding IC injections of MSCs to treat myocardial infarction^[26]. Similar concerns have been raised in studies that used MSCs to treat stroke^[27,28]. A true local injection of MSCs might require a surgical intervention, such as that used in the repair of bone defects. In this setting, the MSCs are immediately delivered to the target tissue; however, the cells' survival might be compromised due to a lack of oxygen or nutrients^[25]. Currently, haematopoietic stem cell transplantation is performed *via* an IV infusion. Intra-bone marrow transplantation is a more complex procedure, but evidence from an animal model suggests that this might improve the outcome of the treatment^[29]. Finally, some animal models of systemic administration, such as intracardiac injection, cannot readily be performed in patients.

The systemic infusion of cells for therapeutic applications implies and requires efficient migration and homing to the target site. Although there is ample evidence of MSC homing, this process appears to be inefficient because only a small percentage of the systemically administered MSCs actually reach the target tissue^[30]. The mechanisms by which the MSCs migrate and home are not yet clearly understood.

Currently, in Haematology, MSCs are mainly being tested for their ability to control graft-vs-host disease and to support haematopoiesis after haematopoietic stem cell transplantation. Chemo- and radio-therapy can damage the haematopoietic niche. MSCs are part of this niche and secrete a number of haematopoietic growth factors. To facilitate the engraftment of haematopoietic stem cells and stimulate blood formation, the MSCs should successfully home to and persist in the bone marrow^[31]. In this review, we discuss current knowledge about MSC homing, specifically focusing on bone marrow homing (based on both *in vitro* and *in vivo* data), and we review the efforts that different groups have undertaken to improve the homing efficiency of these cells.

MSC HOMING AND MIGRATION TO BONE MARROW AND OTHER TISSUES

The exact mechanisms used by MSCs to migrate and home to tissues have not been fully elucidated. It is generally assumed that these stem cells follow the same steps that were described for leukocyte homing. In the first step, the cells come into contact with the endothelium by tethering and rolling, resulting in a deceleration of the cells in the blood flow. In the second step, the cells

are activated by G-protein-coupled receptors, followed by integrin-mediated, activation-dependent arrest in the third step. Finally, in the fourth step, the cells transmigrate through the endothelium and the underlying basement membrane^[32].

The first studies addressing MSC homing examined the origin of the bone marrow MSCs after allogeneic bone marrow transplantation. Those groups all concluded that the haematopoietic cells were provided by the donor, but the stromal cells were provided by the recipient^[33-35]. However, in these studies, the patients received marrow transplants containing only a limited number of MSCs - approximately 1/250000 nucleated cells at 35 years of age - in contrast to the purified MSC product that is used in the majority of clinical trials^[36].

Since then, several studies in animal models and patients have shown that MSCs are capable of migrating and homing to a variety of tissues. Early studies of intra-uterine MSC transplantations in animal models showed that donor-derived non-haematopoietic cells were present in the bone marrow, thymus, spleen and liver^[37,38]. Devine *et al.*^[30] and Chapel *et al.*^[7] performed MSC transplantations in non-human primates and observed MSCs in a variety of tissues, with highest numbers in the gastro-intestinal tract. The percentage of MSCs in the different tissues was estimated between 0.1% and 2.7%^[7,30]. Erices *et al.*^[39] described the homing and survival of human cord blood-derived MSCs in the bone marrow of immunodeficient (nude) mice after systemic infusion^[39]. Several studies in patients have also shown MSC homing^[40-43].

A few groups have analysed the dynamics of MSC migration after systemic infusion using different techniques. Immediately after infusion, the MSCs are trapped in the lungs, and, subsequently, the cells are cleared from the lungs and distributed to other tissues^[44,45]. The cells could be injected intravenously or intra-arterially for systemic infusion. The former is the least invasive method and the easiest to perform; however, as the MSCs were trapped in the lungs, different administration routes were examined. IA injection, which is already more risky because of the arterial puncture, also appears to entail a risk of development of microvascular occlusions called passive entrapment^[27,46]. In addition, there have been reports that MSCs have a procoagulant activity^[26,47]. A few years ago, a group from the Karolinska Institute reported that MSCs, particularly those that had been subjected to extended passaging and co-culture with activated lymphocytes, exhibited increased prothrombotic capacities; this effect was dose-dependent^[47]. Gleeson *et al.*^[26] reported that MSCs express functionally active tissue factor. When MSCs were injected in the coronary arteries of a porcine myocardial infarction model, it resulted in a decreased coronary flow reserve. This effect could be reversed by the co-administration of heparin, an antithrombin agent^[26].

Kyriakou *et al.*^[48] have studied the factors influencing short-term bone marrow homing of MSCs. The stem cells were observed in the bone marrow, spleen, liver and lungs 24 h after IV injection. It was observed that homing

increased in younger animals and after irradiation but decreased with increasing passage numbers of the cells^[48]. Several other groups have also shown that MSC homing improves after irradiation^[7,8,30,49-52].

MOLECULES INVOLVED IN MSC (BONE MARROW) HOMING

The expression of molecules involved in MSC migration, homing and functionality has been widely studied.

Different molecules are involved/necessary for the different steps in the homing process. The selectins on the endothelium are primarily involved in the first step. For bone marrow homing in particular, the expression of haematopoietic cell E-/L-selectin ligand (HCELL), a specialized glycoform of CD44 on the migrating cell, is very important^[53]. Although MSCs express CD44, they do not express HCELL^[54].

The G-protein coupled receptors that are involved in the activation step are typically chemokine receptors. It has been extensively demonstrated that the CXCR4-stromal derived factor-1 (SDF-1) axis is critical for bone marrow homing^[55]. Both molecules are very physiologically important, as knock-outs are lethal due to bone marrow failure and abnormal heart and brain development^[56,57]. The expression of the chemokine receptor CXCR4 on MSCs is controversial. Some groups did not observe expression of the receptor, while other studies demonstrated that CXCR4 was expressed, albeit at low levels on the membrane, which affected migration in response to SDF-1^[58-70].

Integrins are important players in the stable activation-dependent arrest in the third step of homing. It has been shown that the inhibition of integrin $\beta 1$ can abrogate MSC homing^[71]. Integrins form dimers that bind to adhesion molecules on the endothelial cells. Integrin $\alpha 4$ and $\beta 1$ combine to form very late antigen 4 (VLA-4), which interacts with vascular cell adhesion molecule 1 (VCAM-1). It has been shown that the VCAM-1-VLA4 interaction is functionally involved in MSC homing^[72,73].

In the final step of diapedesis or transmigration through the endothelial cell layer and the underlying basement membrane, lytic enzymes, such as the matrix metalloproteinases (MMP), are required to cleave the components of the basement membrane. In particular, the gelatinases MMP-2 and MMP-9 have important roles in this step because they preferentially degrade collagen and gelatin, two of the major components of the basement membrane^[74,75]. We have shown that MSC migration is regulated by MMP-2 and tissue inhibitor of metalloproteinases 3 (TIMP-3)^[76]. Membrane type 1 MMP (MT1-MMP) has also been reported to play a role in MSC migration^[63]. MMPs are secreted as pro-enzymes. ProMMP-2 is activated by interactions with MT1-MMP and TIMP-2 and is inhibited by TIMP-1. This explains why the MMP-2, MT1-MMP or TIMP-2 knock-down decreased the invasive capacity of MSCs, and why TIMP-1 knock-down resulted in increased invasion in the study of Ries *et al.*^[77].

Table 1 gives an overview all of the migration and

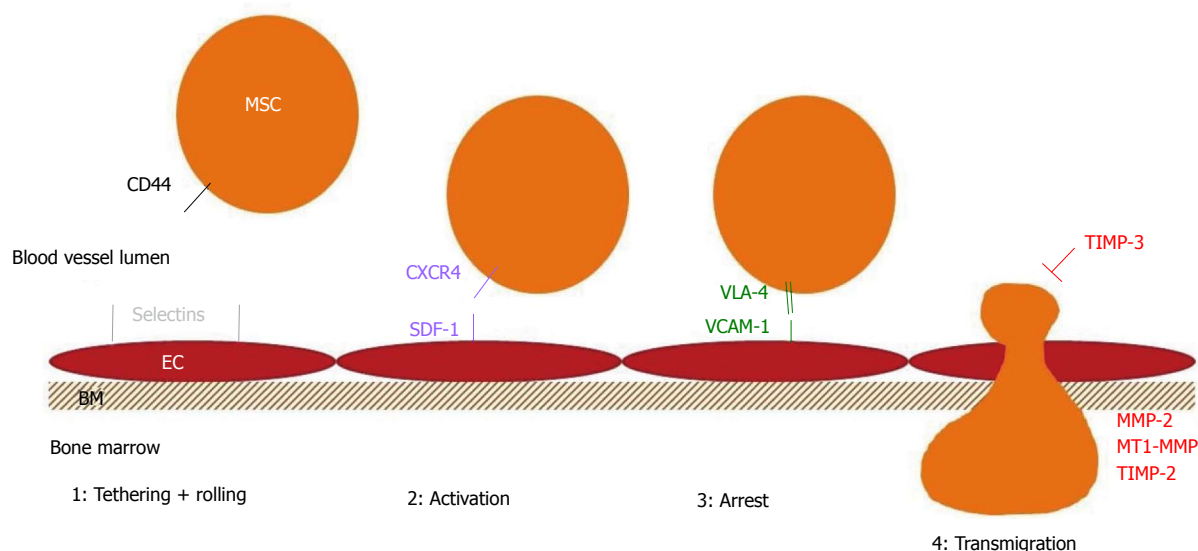


Figure 1 Overview of the homing molecules that are expressed on human mesenchymal stromal cells and known to be involved in the different steps of the bone marrow homing of mesenchymal stromal cells. EC: Endothelial cell; BM: Basement membrane; CD: Cluster of differentiation; SDF-1: Stromal cell derived factor 1; VLA-4: Very late antigen 4; VCAM-1: Vascular cell adhesion molecule 1; TIMP: Tissue inhibitor of metalloproteinases; MMP: Matrix metalloproteinase; MT1-MMP: Membrane type 1 matrix metalloproteinase; MSC: Mesenchymal stromal cell.

homing molecules that are reported to be expressed on human MSCs. Figure 1 shows a schematic overview of the molecules involved in human MSC bone marrow homing.

In addition to the expression of classic homing molecules, different groups have also described the expression of growth factor receptors on MSCs. Several studies have shown that growth factors can also induce MSC migration. For example, platelet-derived growth factor (PDGF) AB and BB can induce MSC migration *in vitro*^[68,80,91]. Another growth factor involved in MSC migration is hepatocyte growth factor (HGF), which binds to c-met^[63,68,80]. Both PDGF-BB and HGF have been loaded on gels or scaffolds as a means to improve the *in vitro* migration of MSCs^[92,93].

HOW CAN WE IMPROVE THE HOMING EFFICIENCY OF MSCs?

Several groups have demonstrated MSC homing and migration, but only a small proportion of systemically administered MSCs actually reaches and remains in the target tissue^[30]. Several factors are assumed to be involved. First, the expression of homing molecules on MSCs is limited. For example, the membrane expression of CXCR4, a critical receptor for homing to bone marrow, is very low, and some groups even claim there is no CXCR4 expression at all^[58-70]. Another concern is that the MSCs appear to lose the expression of homing molecules during *in vitro* expansion^[70,94]. Additionally, there is also heterogeneous expression of homing molecules in MSC cultures and in MSCs derived from different tissues (adipose tissue vs bone marrow), which show a different expression profile of homing molecules^[95].

Because improving the homing efficiency to and retention of MSCs in a target tissue after systemic administration would improve their therapeutic effects, many groups are investigating methods to achieve this goal. Different strategies have been developed: the mode of administration could be modified, the MSC culture conditions can be adapted to optimize the expression of homing molecules, the cell surface receptors could be engineered to improve homing or the target tissue could be modified to better attract the MSCs. Again, we will mainly focus on the strategies that might improve the bone marrow homing of MSCs. The homing molecules involved in homing to bone marrow can also be of importance in homing to other organs or sites of injury, such as the CXCR4-SDF-1 interaction for homing to the injured myocardium^[96]. However, we believe that methods that can upregulate or induce the expression of the homing molecules that are involved in bone marrow homing of MSCs are valuable. They show a potential means for improving bone marrow homing, even though the data supporting/proving this are not yet available. Figure 2 provides an overview of the methods that could be used to improve the bone marrow homing of MSCs.

Modification of the mode of administration

In vivo studies have repeatedly shown that MSCs are trapped in the lung after intravenous injection. When mice were treated with a vasodilator prior to MSC infusion, there was a clear decrease in the number of trapped MSCs in the lungs and a significant increase in MSC homing to the marrow of the long bones^[44]. Yukawa *et al.*^[97] transplanted MSCs in combination with heparin treatment and found that this strategy also significantly decreased MSC trapping

Table 1 Overview of the homing molecules expressed on human mesenchymal stromal cells

Group	Molecule	Source	Transcript	Protein	Functional assay
Chemokine receptors	CCR1 ^[70,77-82]	BM ^[70,77-79,81] WJ ^[79] AT ^[80] PB ^[82]	Yes ^[70,77,79,80]	Yes ^[70,77-82]	<i>In vitro</i> migration ^[70,77,78,80] , <i>in vivo</i> tail vein injection in mice for tissue distribution ^[77]
	CCR2 ^[68,78,81,82]	BM ^[68,78,81,82]	Yes ^[68,82]	Yes ^[68,78,81,82]	<i>In vitro</i> migration ^[68,78,82]
	CCR3 ^[68,78,81-83]	BM ^[68,78,81,83] PB ^[82]	Yes ^[68]	Yes ^[68,78,81,82,83]	<i>In vitro</i> migration ^[68,78]
	CCR4 ^[68,77,78,82]	BM ^[68,77,78,82]	Yes ^[68,77,82]	Yes ^[68,77,78] No ^[82]	<i>In vitro</i> migration ^[68,77,78,82] , <i>in vivo</i> tail vein injection in mice for tissue distribution ^[77]
	CCR5 ^[68,78,81-83]	BM ^[68,78,81,83] PB ^[82]	Yes ^[68]	Yes ^[68,78,81,82]	<i>In vitro</i> migration ^[68,78]
	CCR6 ^[78,81,83]	BM ^[78,81,83]	Yes ^[82]	Yes ^[78,82]	<i>In vitro</i> migration ^[78]
	CCR7 ^[70,78,80-83]	BM ^[70,78,81,83] AT ^[80] PB ^[82]	Yes ^[70,80,83]	Yes ^[70,78,80-83]	<i>In vitro</i> migration ^[70,78,83]
	CCR8 ^[78,82,83]	BM ^[78,83] PB ^[82]	Yes ^[82]	Yes ^[78,82,83]	<i>In vitro</i> migration ^[78]
	CCR9 ^[70,78,81-83]	BM ^[70,78,81,83] PB ^[82]	Yes ^[70,83]	Yes ^[70,78,81-83]	<i>In vitro</i> migration ^[70,78]
	CCR10 ^[77,78,81,83]	BM ^[77,78,81,83]	Yes ^[77,83]	Yes ^[77,78,81]	<i>In vitro</i> migration ^[77,78] , <i>in vivo</i> tail vein injection in mice for tissue distribution ^[77]
	CXCR1 ^[78,81,82,84]	CB ^[84] BM ^[78,81,82] PB ^[82]	Yes ^[83,84]	Yes ^[78,81,82,84]	<i>In vitro</i> migration ^[78,83,84] , <i>in vivo</i> injection in brain ^[84]
	CXCR2 ^[62,78,81-83]	BM ^[62,78,81,83] PB ^[82]	Yes ^[62,83]	Yes ^[62,78,81-83]	<i>In vitro</i> migration ^[62,78,83] , <i>in vivo</i> lung metastasis model ^[62]
	CXCR3 ^[78,81-83]	BM ^[78,81,83] PB ^[82]	Yes ^[83]	Yes ^[78,81-83]	<i>In vitro</i> migration ^[78]
	CXCR4 ^[60,62,65,66,68,70,76,78,80-83,85,90]	BM ^[60,62,68,70,76,78,81,83,85] CB ^[65,85,90] Foetal BM ^[66] AT ^[80] PB ^[82]	Yes ^[60,62,66,68,70,76,80,83,85]	Yes ^[62,65,66,68,70,76,78,80-83,85,90]	<i>In vitro</i> migration ^[60,62,65,66,68,70,76,78,80,83,85,90] , <i>in vivo</i> lung metastasis model ^[62] , tail vein injection in sublethally irradiated mice ^[66]
	CXCR5 ^[68,70,77-83]	BM ^[68,70,77,78,81,83] WJ ^[79] AT ^[80] PB ^[82]	Yes ^[68,70,77,79,80,83]	Yes ^[68,70,77-83]	<i>In vitro</i> migration ^[68,70,77,78,80] , <i>in vivo</i> tail vein injection in mice for tissue distribution ^[77]
	CXCR6 ^[70,78,80-83]	BM ^[70,78,81,83] AT ^[80] PB ^[82]	Yes ^[70,80,83]	Yes ^[70,78,80-83]	<i>In vitro</i> migration ^[70,78,80]
	CXCR7 ^[60,82]	BM ^[60] PB ^[82]	Yes ^[60]	Yes ^[82]	<i>In vitro</i> migration ^[60]
	CX3CR ^[82]	BM ^[82] PB ^[82]	Yes ^[82]	Yes ^[82]	
	XCR ^[82,82]	BM ^[82] PB ^[82]	Yes ^[82]	Yes ^[82]	
Adhesion molecules	VCAM-1 ^[74,85,86]	BM ^[74,85,86] CB ^[86] AT ^[86]	Yes ^[85]	Yes ^[85,86]	<i>In vitro</i> migration ^[74]
	ICAM-2 ^[85]	BM ^[85]	Yes ^[85]	Yes ^[85]	
	CD62 ^[11,17,54,86-89]	BM ^[11,54,86-89] CB ^[17,86,87,89] AT ^[86-89] Skin ^[87]		Yes ^[11,17,54,86-89]	<i>In vivo</i> homing in a mouse model ^[54]
	LFA-3 ^[85]	BM ^[85]	Yes ^[85]	Yes ^[85]	
	Integrin $\alpha 1$ ^[11,85,87]	BM ^[11,85,87] CB ^[87] AT ^[87] Skin ^[87]	Yes ^[85]	Yes ^[11,85,87]	
	Integrin $\alpha 2$ ^[85]	BM ^[85]	Yes ^[85]	Yes ^[85]	
	Integrin $\alpha 3$ ^[11,85]	BM ^[11,85]	Yes ^[85]	Yes ^[11,85]	
	Integrin $\alpha 5$ ^[11,85]	BM ^[11,85]	Yes ^[85]	Yes ^[11,85]	
	Integrin $\alpha 6$ ^[85]	BM ^[85]	Yes ^[85]	Yes ^[85]	

	Integrin αv ^[85]	BM ^[85]	Yes ^[85]	Yes ^[85]	
	Integrin $\beta 1$ ^[11,86-88]	BM ^[11,86-88]		Yes ^[11,86-88]	
		CB ^[86,87]			
		AT ^[86-88]			
		Skin ^[87]			
	Integrin $\beta 3$ ^[85]	BM ^[85]	Yes ^[85]	Yes ^[85]	
	Integrin $\beta 4$ ^[85]	BM ^[85]	Yes ^[85]	Yes ^[85]	
	ALCAM ^[17,87]	BM ^[87]		Yes ^[17]	
		AT ^[87]			
		CB ^[17,87]			
Proteases	MMP-1 ^[90]	BM ^[90]	Yes ^[90]	Yes ^[90]	<i>In vitro</i> migration ^[90]
	MMP-2 ^[65,68,74,76,77,85,90]	BM ^[68,76,77,85,90]	Yes ^[68,76,77,85,90]	Yes ^[65,68,76,77,85,90]	<i>In vitro</i> migration ^[65,68,74,76,77,85,90]
		CB ^[85]			
	MMP-13 ^[68,90]	BM ^[68,90]	Yes ^[68,90]	Yes ^[68,90]	<i>In vitro</i> migration ^[68,90]
Growth factor receptors	MT1-MMP ^[68,77,85]	BM ^[68,77,85]	Yes ^[68,77,85]	Yes ^[68,77,85]	<i>In vitro</i> migration ^[68,77,85]
		CB ^[85]			
	TIMP-1 ^[68,77,90]	BM ^[68,77,90]	Yes ^[68,77,90]	Yes ^[68,77,90]	<i>In vitro</i> migration ^[68,77,90]
	TIMP-2 ^[68,90]	BM ^[68,77,90]	Yes ^[68,77,90]	Yes ^[68,77,90]	<i>In vitro</i> migration ^[68,77,90]
	TIMP-3 ^[76]	BM ^[76]	Yes ^[76]	Yes ^[76]	<i>In vitro</i> migration ^[76]
	c-met (HGF-R) ^[68,80,85]	BM ^[68,85]	Yes ^[68,80,85]	Yes ^[68,85]	<i>In vitro</i> migration ^[85,68]
		CB ^[85]		No ^[80]	
		AT ^[80]			
	PDGFR α ^[68,80,87]	BM ^[68,87]	Yes ^[68,80]	Yes ^[68,80,87]	<i>In vitro</i> migration ^[68,80]
		AT ^[80,87]			
		CB ^[87]			
		Skin ^[87]			
	PDGFR β ^[68,80,87]	BM ^[68,87]	Yes ^[68,80]	Yes ^[68,80,87]	<i>In vitro</i> migration ^[68,80]
		AT ^[80,87]			
		CB ^[87]			
		Skin ^[87]			
	FGF-R1 ^[80]	AT ^[80]	Yes ^[80]	Yes ^[80]	<i>In vitro</i> migration ^[80]
	FGF-R2 ^[68]	BM ^[68]	Yes ^[68]	Yes ^[68]	<i>In vitro</i> migration ^[68]
	EGF-R ^[68,78]	BM ^[68,78]	Yes ^[68,80]	Yes ^[68,80]	<i>In vitro</i> migration ^[68,80]
		AT ^[80]			
	IGF-R1 ^[68]	BM ^[68]	Yes ^[68]	Yes ^[68]	<i>In vitro</i> migration ^[68]
	TIE-2 ^[68]	BM ^[68]	Yes ^[68]	Yes ^[68]	<i>In vitro</i> migration ^[68]
	TGFRB2 ^[80]	AT ^[80]	Yes ^[80]	Yes ^[80]	<i>In vitro</i> migration ^[80]
	TNFRSF1A ^[80]	AT ^[80]	Yes ^[80]	Yes ^[80]	<i>In vitro</i> migration ^[80]

BM: Bone marrow; CB: Cord blood; AT: Adipose tissue; WJ: Wharton's Jelly; VCAM: Vascular cell adhesion molecule; ICAM: Intercellular adhesion molecule; CD: Cluster of differentiation; LFA: Lymphocyte function associated antigen; ALCAM: Activated leukocyte cell adhesion molecule; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase; HGF: Hepatocyte growth factor; PDGFR: Platelet-derived growth factor receptor; FGF-R: Fibroblast growth factor receptor; EGF-R: Epidermal growth factor receptor; IGF-R: Insulin-like growth factor receptor; TIE: Tyrosine kinase with immunoglobulin-like and EGF-like domains; TGFR: Transforming growth factor receptor; TNFRSF: Tumour necrosis factor receptor superfamily.

in the lungs.

Pretreatment or priming of MSCs in culture or modifying the MSC culture conditions

Because MSCs appear to downregulate homing molecule expression during expansion, many groups are investigating different ways to induce or upregulate the expression of important homing molecules.

Much effort has been focused on increasing CXCR4 expression on the membrane. One way to achieve this is by adding cytokines or cytokine cocktails to the culture medium during expansion. Shi *et al.*^[66] showed that exposure to a combination of flt3 ligand, stem cell factor (SCF), IL 3, IL 6 and hepatocyte growth factor (HGF) increased both the intracellular and membrane expression of CXCR4 on cultured MSCs. More of the pretreated cells migrated towards an SDF-1 gradient, and there was no effect of the pretreatment on the function of the MSCs in supporting haematopoiesis. *In vivo* homing experiments where MSCs were intravenously injected into sublethally

irradiated mice revealed a significant increase in bone marrow homing after the cytokine treatment^[66]. Other molecules that have been shown to increase CXCR4 expression are insulin-like growth factor 1 (IGF-1), tumour necrosis factor α (TNF α), IL 1 β , interferon γ (IFN γ)^[68,98-100]. CXCR4 expression could also be upregulated by treating cultured MSCs with glycogen synthase kinase 3 β (GSK-3 β) inhibitors, resulting in an improved *in vitro* migration capacity, without affecting cell viability^[101]. Exposure to complement 1q (C1q) has been shown to increase MSC migration towards SDF-1, although there was no significant increase in CXCR4 expression. Therefore, it was postulated that C1q exposure increases the MSCs' ability to sense SDF-1 gradients^[65].

Treatments with GSK-3 β inhibitors and C1q also increase MMP expression in MSCs, which are important for the degradation of the basement membrane during extravasation^[60,101]. A combination of the haematopoietic growth factors erythropoietin (EPO) and granulocyte colony stimulating factor (G-CSF) has also been reported

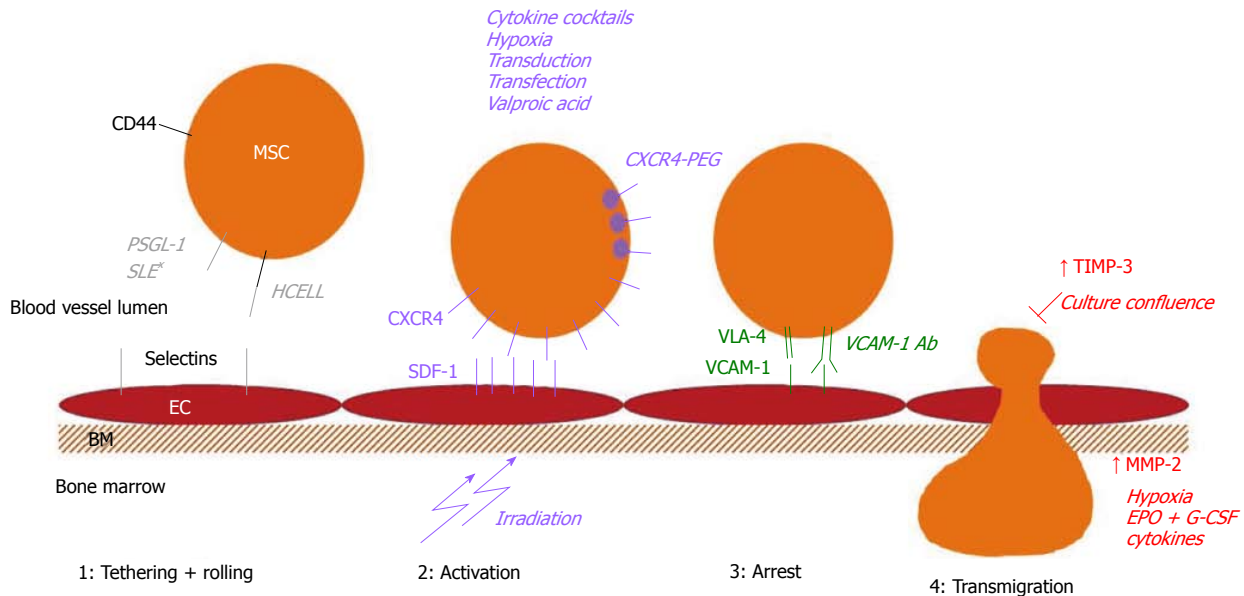


Figure 2 Schematic overview of the different strategies that can be used to improve homing in the different steps of mesenchymal stromal cell migration. CD: Cluster of differentiation; EC: Endothelial cell; BM: Basement membrane; HCELL: Hematopoietic cell E-/L-selectin ligand; PSGL-1: P-selectin glycoprotein ligand-1; SLEX: Sialyl Lewis X; SDF-1: Stromal cell derived factor 1; VLA-4: Very late antigen 4; VCAM-1: Vascular cell adhesion molecule 1; Ab: Antibody; TIMP: Tissue inhibitor of metalloproteinases; MMP: Matrix metalloproteinase; EPO: Erythropoietin; G-CSF: Granulocyte colony stimulating factor; MSC: Mesenchymal stromal cell.

to increase MMP-2 expression in MSCs and improve their motility^[102].

There is also evidence that the epigenetic modulation induced by a short-term exposure to valproic acid results in increased expression of CXCR4 and MMP-2 in cultured MSCs and an increase in their migration towards SDF-1. There was no impact of this priming on the differentiation capacity of the cells^[103].

Another approach that is under investigation is culturing MSCs under hypoxic conditions. Several groups have shown that these conditions result in increased CXCR4 expression and an improvement in MSC migration both *in vitro* and *in vivo*. This effect of hypoxia not only appears after short-term exposure but also in response to continuous culture in hypoxic conditions^[104-108]. The increase in CXCR4 expression is reported to be regulated by an increase in hypoxia inducible factor (HIF) 1 α ^[108]. Hypoxia also leads to differential expression of MMPs. For example, a decrease in MMP-2 secretion and an increase in MT1-MMP secretion and activity has been described in MSCs cultured under hypoxic conditions^[104]. However, one could be concerned that culturing MSCs under hypoxia might change their behaviour. Valorani *et al.*^[109] reported that adipose tissue-derived MSCs cultured under hypoxic conditions exhibited an increased adipogenic or osteogenic differentiation capacity^[109]. Crowder *et al.*^[110] reported that concurrent exposure to extreme hypoxia (0.5%) and a carcinogenic metal (nickel) induces carcinogenic changes in late passage MSCs. They did not observe these changes in early passage control cells^[110].

A simpler modification of culture conditions is to maintain lower confluence. Our group found that MSCs that were cultured to complete confluence had a lower migration

capacity than MSCs maintained at a low confluence. The cells cultured at higher confluence secrete more TIMP-3, an inhibitor of MMPs, which decreases migration compared to the MSCs cultured at low confluence^[76].

Finally, MSCs are a heterogeneous cell population, and a particular subset of MSCs might have better homing abilities. MSCs were separated based on their expression of Stro1 and cultured further; these cells exhibited different migration capacities in NOD/SCID transplantation experiments. The amount of Stro1⁺ MSCs was higher than the amount of Stro1⁺ MSCs in the target tissues of the mice, such as the bone marrow and spleen, after systemic administration *via* the retro-orbital plexus^[111].

Genetic modifications

As already mentioned, MSCs express low levels of CXCR4, if any at all^[58,59]. Because the CXCR4-SDF1 axis is important for bone marrow homing^[20,112], many groups have designed transfection or transduction experiments in which CXCR4 expression plasmids are either nonvirally or virally introduced into the cells. Viral transduction is the most efficient method for obtaining high and stable expression levels in the target cells. CXCR4 overexpression resulted in improved MSC homing to the bone marrow after intracardiac injection into a NOD/SCID transplant model^[112]. In a similar model, the overexpression of integrin $\alpha 4$, a subunit of VLA4 that interacts with VCAM-1, also resulted in increased bone marrow homing^[113]. However, there are some draw-backs to this technique. Most importantly, there is the concern that the use of viral vectors to introduce the plasmid DNA poses a risk of insertional oncogenesis. Techniques for site-directed integration have been developed to circumvent

this problem^[114]. Moreover, there is also a risk of adverse immune reactions and the production costs are high^[115].

Different modes of non-viral transfection of plasmid DNA have been developed. One group overexpressed CXCR4 in MSCs using mRNA nucleofection. They obtained 90% expression of the surface receptor, but cell viability was only 62% and no increase in MSC homing could be observed^[96]. Another group investigated the feasibility of inserting a short interfering RNA in MSCs using ultrasound and microbubbles to promote survival. A significant knock-down of the target (PTEN) could be obtained, but the cells were damaged after the manipulation^[116].

Different modes of chemical, non-viral transfection have been studied, including the use of lipid agents. Although these techniques are easier to scale up and less expensive than viral transduction, they come with a price. The transfection efficiencies are significantly lower because approximately 35% of the MSCs express the transfected protein compared to over 90% of the cells after viral transduction^[20].

Cell surface engineering

A method to improve homing efficiency of MSCs that has garnered interest in recent years is cell surface engineering, *i.e.*, a transient modification of the cell surface. Because transmigration through the activated endothelium takes 1-2 h, these transient alterations can be instrumental in improving MSC homing^[117]. It has been shown that these modifications do not impact cell viability, proliferation, adhesion or differentiation^[118-121]. For cell surface engineering, most groups focus on improving the first step of the homing process, tethering and rolling, by modulating the expression of adhesion molecules^[54,118,120,121]. Since the first publications, many groups have developed different techniques for the cell surface modifications of MSCs.

A seminal paper in this field was published in 2008, when Sackstein *et al.*^[54] reported that they had converted the native CD44, which is readily expressed on MSCs, into the haematopoietic cell E-selectin/L-selectin ligand (HCELL) glycoform *ex vivo*^[54]. E-selectin plays a key role in haematopoietic stem cell (HSC) homing to the bone marrow; however, MSCs do not express P-selectin glycoprotein ligand-1 (PSGL-1) or HCELL, the two E-selectin ligands that are required for HSC bone marrow homing, thus impairing their homing capacity to the bone marrow^[54,122]. MSCs natively express CD44. In this study, Sackstein *et al.*^[54] were able to alter sialofucosylation *ex vivo* and transform CD44 into the HCELL glycoform. This treatment had no effect on the viability or phenotype of the cells. *In vivo* homing experiments that injected MSCs into the tail veins of NOD/SCID mice showed that the HCELL+ MSCs homed to the bone marrow, even in the absence of CXCR4, in contrast to the unmanipulated MSCs^[54].

Sialyl Lewis X (SLE^x) is the active site of PSGL-1. Therefore, introducing this molecule into the MSC cell membrane should also lead to improved MSC homing.

Sarkar *et al.*^[118] used biotinylated microvesicles to modify the MSCs. When the vesicles were brought into contact with the MSCs, they integrated into the cell membrane, thus generating biotinylated MSCs. Using a streptavidin linker, biotinylated SLE^x could be immobilized on the cell surface. The accessibility of the lipids integrated in the cell membrane was assessed and the researchers found they could still be detected after 4 h, but the intensity had already decreased to 50% compared with that at 0 h. After 8 h, all signals were lost, confirming that the modification is indeed transient. *In vitro* tests showed that the SLE^x-expressing MSCs exhibited improved adhesion under shear stress compared to the sham-treated MSCs^[118].

Cheng *et al.*^[120] described a rapid (30 min) procedure to conjugate peptide K, an E-selectin binding peptide, to the MSC membrane. The MSC viability and proliferation rates were normal after engineering and their differentiation capacity was also maintained. In an *in vitro* model of inflamed endothelium, they subsequently demonstrated that the engineered MSCs adhered better than the control MSCs under shear stress^[120].

Lo *et al.*^[121] described yet another engineering method to improve MSC binding to selectins and facilitate tethering and rolling. The first 19 amino acids of PSGL-1 (Fc19) were combined with an IgG tail and with an SLE^x glycan to engineer a pan-selectin-binding ligand. Tests in flow chambers showed that these MSCs were indeed capable of adhesion under shear stresses^[121].

However, adhesion molecules are not the sole targets of the cell surface engineers. There is also interest in conjugating antibodies to the cell surface. Protein painting is a technique that binds antibodies to the cell surface. First, the palmitated proteins acting as docking stations for the antibodies are integrated into the cell membrane, and, subsequently, antibodies can be bound to the cell without losing affinity and with no impact on the viability and differentiation potential of the engineered cells^[123]. One example using this technique is the binding of intercellular adhesion molecule (ICAM)-1 antibodies to MSCs, which increased the binding of these cells to endothelial cells^[124]. This same protein painting technique has been applied to express VCAM-1 antibodies on MSCs, resulting in improved homing. In this study, the target tissues were the mesenteric lymph nodes and the colon. However, this technique might also be applied to improve homing to other organs, such as the bone marrow, because VCAM-1 is implicated in the bone marrow homing of MSCs^[125].

Recently, a method was also described in which recombinant CXCR4 is bound to the cell surface of MSCs using lipid-PEG. In a one-step mixture procedure, recombinant CXCR4 could be transiently expressed on MSCs, leading to migration towards SDF-1 in a concentration-dependent manner^[119].

Modification of the target tissue

Finally, MSC migration and homing can be influenced by modifying the target tissue. In early homing studies, it was

already shown that altering the target tissue by irradiation increases MSC homing^[7,8]. After chemo- and radio-therapy, there are increased levels of SDF-1 in the bone marrow, thus increasing its attraction for HSCs and MSCs^[126]. There are also reports of manipulating MSC migration with ultrasound or magnetic or electric fields^[127-129]. However, these techniques do not appear to be very practical and they need adequate expression of homing molecules. For example, application of electrical fields could induce heat and electrochemical products near the electrodes. On the other hand, ultrasound-guided delivery might be more challenging in deep organs. Finally, homing directed by a magnetic field might require the implantation of a magnet in or near the tissue of interest^[127-129].

Caveats in modifying homing molecules

In animal models and clinical studies, only limited engraftment or no engraftment at all is often observed, raising the question of whether tissue-specific homing is required for the therapeutic effect of MSCs^[30,42]. A study on the use of systemically administered MSCs for the treatment of stroke in an animal model also showed very limited migration of MSCs to the tissue of interest, the brain. However, the researchers found that MSC homing to the spleen was important and correlated with a reduced infarct size and peri-infarct inflammation. They propose that MSCs exert a beneficial effect by abrogating secondary, inflammation-related cell death^[130]. These data show that tissue-specific MSC homing is important, even though the target tissue is not the brain, as one would expect in a stroke model. Fernández-García *et al.*^[131] performed cotransplantation studies with MSCs and HSCs and found that cotransplantation improves short- and long-term haematopoietic reconstitution. This was the result of MSC and HSC interactions, and they propose that MSCs act as carriers that facilitate HSC homing to the bone marrow^[131].

Manipulating stem cells, such as MSCs, to improve their homing capacities might not only change their migratory capacities but also have other consequences. For example, Liu *et al.*^[132] claim that the CXCR4-SDF-1 axis plays an important role in MSC survival because MSCs pretreated with SDF-1 exhibited significantly improved survival and proliferation. These effects could be partially inhibited by AMD3100, an inhibitor of CXCR4^[132]. The pretreatment of MSCs with cytokines also revealed some conflicting observations. In a recently published paper, Kavanagh *et al.*^[133] report that licensing murine MSCs with inflammatory cytokines does not improve homing to the injured gut in an ischaemia/reperfusion model in their hands. More importantly, they found that while the untreated MSCs improved tissue perfusion, this effect was abrogated with the pretreated MSCs^[133]. However, another group reported positive effects of pretreatment on the biological functions of the MSCs. Szabó *et al.*^[134] found that licensing murine MSCs with pro-inflammatory cytokines resulted in a significant reduction in the variability in immunosuppressive capacities of these MSCs. This reduction in variability was due to an increased

immunosuppression of clones that were poor inhibitors of T-cell proliferation prior to licensing^[134].

The pretreatment of MSCs with different factors or conditions, *e.g.*, hypoxia and inflammatory cytokines, could also modify their response to these treatments. Naaldijk *et al.*^[135] found that the oxygen concentration (normoxia vs hypoxia) alters the response of rat and human AT MSCs. They also found that the migration of MSCs isolated from older donors (rat and human) was not significantly impaired compared with the MSCs from young donors^[135]. In contrast to this last finding, Choudery described that MSCs from aged mice exhibit diminished effectiveness and increased expression of apoptotic and senescent genes^[136].

In this review, we have described different techniques for improving MSC homing and the expression of homing molecules on MSCs. Importantly, however, the expression of homing molecules and the resulting migration, homing and biological functions of MSCs might easily be altered unintentionally. Currently, many different protocols are used to expand MSCs for *in vitro*, animal and clinical studies. These variables can have a major impact on the expression of the homing molecules and the biological functions of MSCs; we will briefly discuss this below.

MSCs were first isolated from bone marrow. Since then, MSCs have been isolated from a wide variety of tissues, including adipose tissue (AT), umbilical cord blood (CB), Wharton's jelly (WJ), *etc.*^[59,79,80,82]. Several groups have reported differences in the expression of homing molecules in human MSCs isolated from different sources; these are listed in Table 1. Additionally, the MSCs derived from different sources also exhibit differences in their biological functions. For example, AT MSCs might have better immunosuppressive capacities than bone marrow MSCs^[95]. On the other hand, bone marrow MSCs appear to be the only MSCs that are capable of forming a haematopoietic niche that can support human haematopoietic tissue in an *in vivo* model^[87].

When using MSCs for organ-specific treatments, one might choose to induce differentiation *in vitro* before transplantation. However, *in vitro* differentiation might not always result in a clinical benefit during MSC therapy. In a study using human CB MSCs in a mouse model for liver disease, the researchers found that hepatic differentiated MSCs performed worse than the undifferentiated MSCs. The differentiated MSCs showed decreased expression of the homing molecules and decreased *in vivo* migration after IV infusion. Additionally, their immunosuppressive capacity was decreased and the expression of HLA DR was increased, thus increasing their immunogenicity^[137]. Ullah *et al.*^[138] also found that chondrogenic differentiated human MSCs exhibited a significantly reduced *in vitro* migration capacity than undifferentiated MSCs. However, CCR9 expression and *in vitro* migration to its ligand, CCL25, were retained in the differentiated MSCs^[138].

Many parameters in MSC cultures vary between different research groups, including seeding density, number of passages, basal medium, and growth supplements [foetal bovine serum (FBS) vs platelet lysate (PL)]. All of these

factors might have an important impact on MSC function and migration. For example, Cholewa *et al.*^[139] found that PL increased MSC proliferation and increased the number of population doublings before senescence compared to FBS. However, they also showed that seeding MSCs at lower densities selected a highly migratory MSC population^[139]. There are also reports of MSCs losing their migratory capacity and/or expression of homing molecules after *ex vivo* expansion^[48,94]. After culture, MSCs are harvested with trypsin to detach them for passaging. Chamberlain *et al.*^[140] reported that the cell surface expression of chemokine receptors was decreased when the cells were detached with trypsin.

Future research directions

As described above, there is currently substantial variability in the isolation and expansion protocols for MSCs. Research on MSC homing and migration would clearly benefit from standardized MSC expansion protocols. What appears to be a rather minor aspect of the expansion protocol might have a significant impact on MSC function and/or migration. Thus, standardizing MSC expansion protocols would minimize unintentional modifications of the homing molecules. Of course, different culture conditions should be compared to create an optimal expansion protocol. Once this protocol is defined, it will also be easier to evaluate therapeutic efficacy of MSCs in clinical settings. It may be that different clinical applications require different expansion protocols to obtain the desired therapeutic effect.

We summarized the strategies for improving MSC homing. Many of these methods have not yet been validated *in vivo*. Before they can be translated to the clinic, the techniques with the most promising results should be first validated using *in vivo* homing models. In these experiments, the migration of engineered MSCs should be compared with the migration of untreated cells, and the therapeutic efficacy of the treated MSCs can also be assessed in animal disease models.

Although MSCs are widely studied and used in many clinical trials in a variety of clinical domains, little is known about the exact mechanisms by which MSCs exert certain therapeutic effects and their homing to certain tissues. Further studies would benefit from a better understanding of MSC biology. Understanding whether and where MSC migration or homing is necessary can help to define the optimal expansion protocols.

Finally, when transitioning to clinical trials, all conditions should be strictly defined, and, ideally, randomized controlled trials would be designed.

CONCLUSION

MSCs are interesting effector cells that can be used in a variety of therapeutic applications. Systemic administration is often the preferred route of delivery. However, this approach requires that adequate numbers of MSCs migrate and home to the target tissue(s). MSCs do not express many homing receptors, which impairs their migration

capacity and hampers their therapeutic efficacy. Studies are ongoing and are needed to further elucidate the MSC homing mechanisms. A better understanding of MSC homing, as well as the factors influencing this process, will allow researchers to optimize the migration capacities of these stem cells and their therapeutic effects in a target tissue.

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Malaria modeling: *In vitro* stem cells vs *in vivo* models

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breakthrough and novel tools were adapted and opened new frontiers for malaria research. In addition to the new *in vitro* systems, in recent years there were also significant advances in the development of new animal models that allows studying the entire cell cycle of human malaria. In this paper, we review the different protocols available to study human *Plasmodium* species either by using stem cell or alternative animal models.

Key words: Malaria; Stem cells; *In vitro* models; Animal models; Humanized mice

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Core tip: To better understand *Plasmodium* biology, researchers can whether proceed to *in vitro* studies or use *in vivo* models. Thanks to recent progresses, stem cells have been extensively employed to study *Plasmodium* liver and blood cycle *in vitro*. In parallel, the development of animal models opened new opportunities to study parasite biology *in vivo*. In this review, I go through and discuss the different available protocols using stem cells for modeling malaria *in vitro* as well as available animal models. This review has for goal to decipher which system would be the more suitable to study the parasite biology.

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Abstract

The recent development of stem cell research and the possibility of generating cells that can be stably and permanently modified in their genome open a broad horizon in the world of *in vitro* modeling. The malaria field is gaining new opportunities from this important

INTRODUCTION

Despite many years of eradication efforts, Malaria remains a major threat to humans living in endemic area, particularly in sub-Saharan Africa (WHO report 2014). In the last two decades, the knowledge on many aspects of *Plasmodium* biology advanced significantly

including mechanisms of motility and cell invasion^[1], modification of the host cell such as cytoadherence^[2], immune evasion^[3] establishment of liver infections^[4] and hypnozoites dormancy^[5]. These achievements would not be possible unless Trager *et al*^[6] were able to establish *Plasmodium* culturing *in vitro*. The ability to successfully freeze *Plasmodium* isolates^[7,8] and routinely culture laboratory-adapted strains (*i.e.*, DD2, 3D7, W2) was one of the most important steps that allowed more researchers to study malaria outside endemic areas.

In recent years, breakthroughs in stem cell research provided additional opportunities to study new aspects of the parasite biology, primarily of stages in the cell cycle in which culturing had been thus far challenging or impossible. In addition, the development of novel animal models completes the study of the entire cell cycle of human *Plasmodium* spp. and represents an appealing alternative to study host-parasite interactions with no need of human infection.

In this current paper we review and discuss the recent advances of novel procedures used to study human *Plasmodium* infection *in vitro* and *in vivo*.

STEM CELL DERIVED CELLS

Stem cell derived-erythrocytes

The lack of blood supply in blood banks that rely on constant blood donations, lead many researchers to look for alternative solutions to produce erythrocytes for transfusion^[9]. The first report of the production of human erythrocytes from hematopoietic stem cells using a liquid system was described by Fibach *et al*^[10]. These authors isolated mononuclear cells (MNC) from peripheral blood of a patient with α -thalassemia, in which a defect in the chain of hemoglobin, cause an erythropoiesis increase. Using a two-step protocol, they could observe erythroid cells when cultured in the presence of erythropoietin (EPO). However, ethical concerns of using blood from a β -thalassemia patient presenting a defect in hemoglobin still remain (Figure 1).

Following this study, many protocols have been developed in order to generate erythrocytes from HSC (reviewed by Migliaccio *et al*^[11]). In 2005, Giarratana *et al*^[12] published what could be considered as the reference protocol to generate erythrocytes from HSC. Briefly, after isolation of HSC from diverse origins (peripheral blood, umbilical cord blood, bone marrow and leukaferesis product) through a magnetic assorted cell sorting (MACS) selection based on the CD34⁺ expression, cells were co-cultured with mouse stromal cells (MS5). The cells were cultured in the presence of a cocktail of specific growth factors to allow a correct differentiation toward erythroid commitment: interleukin 3 (IL-3), hydrocortisone (HDS), stem cells factor (SCF) and EPO. After 20 d in culture, pure population of erythrocytes could be isolated from the supernatant. Nonetheless, production of erythrocytes from HSC faced some difficulties that limited the amount of cells which are produced as well as the ability to produce mature red blood cells (RBCs) (as the

hemoglobin isoforms remain at fetal state).

The stem cell-derived erythrocytes have recently been intensively used in the malaria field to try to solve the challenging *in vitro* culture of *Plasmodium vivax* (*P. vivax*)^[13]. Unlike *P. falciparum* that can invade erythrocytes of all ages, *P. vivax* shows a preference for invading immature erythrocytes (named reticulocytes)^[14]. This preference for reticulocyte invasion makes use of peripheral blood as a source of cells to culture parasites *in vitro* nearly impossible as reticulocyte are only 0.5%-1% of erythrocytes in the blood stream and their lifespan prior to maturation is only 24 h. Thus a reticulocyte-enriched source of blood is needed in order to grow *P. vivax in vitro*. Early studies used several methodologies to concentrate reticulocytes from blood by ultracentrifugation^[15], centrifugation on Percoll layer^[8,16] or lysis buffer^[17]. However, more recent studies demonstrated the preference of *P. vivax* for CD71^{high} cells (reticulocytes)^[18-20] revealing the possibility of using stem cell-derived reticulocytes. The first report attempting to establish an *in vitro* culture of *P. vivax* using HSPC-derived reticulocytes showed that parasites could be maintained in culture for more than 50 d using stem cell-derived reticulocytes^[21]. This important study confirmed that stem cells could be used as a source of reticulocytes for *P. vivax in vitro* culture. However conditions still needed to be optimized as reticulocyte production were only 0.5% (after 14 d) and the parasitemia reached very low levels (below 0.0013%). In a more recent study, Noulin *et al*^[22] were able to generate, after 14 d of culture, up to 18% of reticulocytes which were permissive to *P. vivax* invasion. They were also able to successfully cryopreserve reticulocytes in order to create a stock of cells to provide to *P. vivax* at each schizogony cycle. Nevertheless, the amount of reticulocytes generated remained extremely low and the parasite could still not multiply *in vitro*.

Before HSPC-derived reticulocytes can be used for successful *P. vivax in vitro* culture, the problems of low reticulocyte yield and the lack of intra-erythrocyte development of the parasite must be addressed. Very recently, Roobsoong *et al*^[23] proposed optimized *P. vivax* culture conditions in order to better maintain the parasite *in vitro*. As a source of reticulocytes, they differentiated CD34⁺ cells into reticulocytes using the previously described protocol and interestingly purified the reticulocyte population passing the cells through leukocyte reduction filters to get rid of nucleated cells. Alternatively, they also concentrated reticulocytes from peripheral blood (PB) and umbilical cord blood (UCB) on a 19% Nycodenz layer. They also tested different culture media (McCoy's 5A, RPMI or Waymouth) supplemented with different serum concentrations. The authors claimed they could maintain the parasite *in vitro* for 26 mo, though the parasite density dramatically dropped from the first day to an almost undetectable level after the second day. We could conclude from these observations that *P. vivax* did not grow *in vitro* and thus more improvements are needed to reach a viable *in vitro* system.

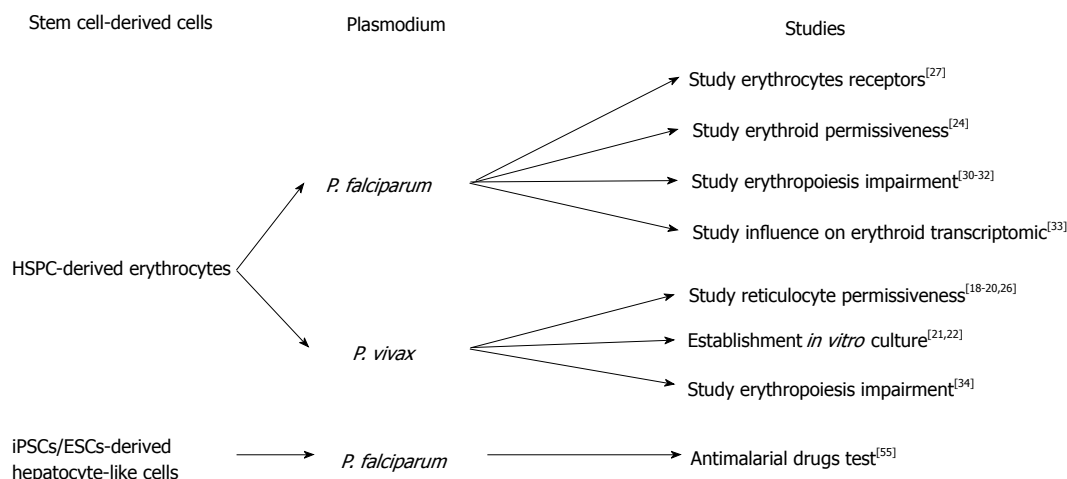


Figure 1 Chart of the different stem cells used for *Plasmodium in vitro* modeling and their applications. Scheme of the different sources of stem cells used for liver and blood cycles of *Plasmodium falciparum in vitro* studies. The main aims of each study are indicated on the right.

The parasite's ability to invade and replicate within reticulocytes generated from HSPC is a precondition for the establishment of *in vitro* culture that relies on stem cells as a source.

Tamez *et al*^[24] were interested in identifying the earliest erythroid stage, which is permissive to *P. falciparum* invasion. They differentiated HSPC according to a previously published protocol^[25]. Briefly, after CD34⁺ isolation, HSPCs were cultured for 8 d in the presence of IL-3, EPO and SCF with medium refreshment at day 3 and 6 without IL-3 and decreased SCF concentrations. A selection was done after 7 d by FACS sorting based on CD71⁺ expression and the cells were cultured with only EPO supplementation for an additional 10 d. They found that the polychromatic erythroblasts were poorly invaded while ortho-erythroblasts could be invaded and allowed for parasite intra-cellular maturation, indicating the permissiveness of erythroid progenitors to *P. falciparum*.

Fernandez-Becerra *et al*^[26] generated reticulocytes starting from HSPC isolated from adult PB, umbilical cord blood UCB and bone marrow (BM). Notably, they used 3T3 cells instead of the more-commonly used MS5 cells as a layer for differentiating erythroid progenitors. They could reach a significant yield of reticulocytes (up to 83.5%) and observed the presence of adult hemoglobin in reticulocytes derived from PB and BM. However, no information was given about the level of parasitemia post-invasion. A year later, Noulin *et al*^[20] investigated different sources of HSPC. Remarkably, after CD34⁺ isolation and before differentiation, they applied an expansion step to increase their HSPC population. They could dramatically increase the HSPC population up to 10 fold for UCB source, 3 fold for BM source and 1 fold for PB source. They also observed better enucleation in PB source (32%) vs BM (20.5%) and UCB (18%). All three sources tested had similar permissiveness and better invasion rates compared to reticulocyte-enriched blood leading to the hypothesis that *P. vivax* prefers immature reticulocytes.

Recently, Egan *et al*^[27] used a reverse genetics approach to investigate the role of RBC receptors that are involved in *P. falciparum* invasion. Using lentiviral shRNA delivery, they performed gene knockdown (kd) of different genes encoding for potential receptors in erythroid cells (starting from PB/HSPC isolated from Granulocyte-colony stimulating factor-stimulated patient or BM). They differentiated those that were genetically modified erythroid progenitors on stromal cell layer to obtain enucleated cells used further for *P. vivax* invasion assays. The authors observed a dramatic invasion decrease in CD55^{kd} as well as in CD44^{kd} RBCs. This work was the first to highlight the possibility of using genetically modified erythrocytes to study *Plasmodium* biology. Nevertheless, since it is impossible to maintain HSPC as stem cells it is essential to repeat the kd procedure every time, which causes some variability between kd experiments.

Significant blood hemolysis was reported during malaria infection^[28] and thus, HSPC are also of particular interest to study erythropoiesis impairment that leads to anemia during malaria episodes^[29]. Several studies tried to investigate the mechanism by which *Plasmodium* infection causes erythropoiesis impairment. In this scope Hemozoin (Hz) attracted particular interest. Hz is produced by the parasite when it metabolizes heme in its food vacuole^[30]. Casals-Pascual *et al*^[31] and Skorokhod *et al*^[32] investigated the influence of Hz on erythroid development. They isolated CD34⁺ cells by MACS followed by a well-established differentiation protocol^[12] and noticed a marked decrease in erythroid production in presence of Hz.

The influence of Hz on erythropoiesis was investigated by Malleret *et al*^[19] using a different protocol. Starting from UCB, they differentiated CD34⁺ cells based on a 3-step process^[20]: 7 d in presence of Fms-like tyrosine kinase 3 (FLT-3) and thrombopoietin (TPO), 7 d with an addition of insulin growth factor-1 (IGF-1), SCF and EPO, and finally 2 to 7 d without SCF. They found that the main reasons for hemolysis are soluble mediators

from Hz-stimulated PBMC rather than erythropoiesis impairment due to Hz itself.

It appears that *P. falciparum* infection significantly influences transcription in erythroid progenitors as shown by Tamez *et al*^[33]. Following the erythroid development protocol they previously developed^[24], they observed an up-regulation of 35 genes in polychromatophilic erythroblasts and 609 regulated genes in ortho-erythroblasts. These results may indicate a negative effect (direct or indirect) of *P. falciparum* on erythropoiesis.

P. vivax infection has a similar effect on erythroid development^[34]. Using a modified protocol previously developed by Giarratana *et al*^[12] (without stromal feeder cells) the authors co-culture erythroid cells with *P. vivax*-infected reticulocytes (intact or lysed), uninfected erythrocytes, in presence of tumor necrosis factor alpha (TNF- α) or interferon gamma (IFN- γ). They observed a decrease of the erythroid multiplication and development in the presence of infected reticulocytes lending support to the idea that that *P. vivax* might have a negative effect on erythropoiesis.

The recent important development of stem cell research contributed to the production of stem cell-derived erythrocytes, and enabled testing the use of human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSC). Indeed, recent findings have demonstrated that those pluripotent cells can be maintained and expanded *in vitro* prior to differentiation into specific lineage^[35,36]. To date, several protocols have been developed to generate mature erythrocytes from hESC or hiPSCs with partial success. Lu *et al*^[37] developed a protocol to produce enucleated red blood cells from ESC. The differentiation was initiated by dispensing hESC as erythroid bodies (EBs) in presence of bone morphogenetic protein 4 (BMP4), vascular endothelial growth factor 165 (VEGF₁₆₅), beta fibroblast growth factor (β -FGF), TPO, FLT-3 and SCF to induce mesodermal commitment. The second step, which is the erythropoiesis leading to the last stages of the blood production, was performed in co-culture with OP9 cells or human mesenchymal stem cells (huMSC) in presence of IL-3, HDS, EPO and SCF. Using this technique, they could get up to 40% of enucleated cells. In 2010 Lapillonne *et al*^[38] reported that by starting from hESC and hiPSCs they could get up to 66% of enucleated erythrocytes. To achieve this amount, they used a two-step protocol where the cells were initially cultivated as EBs in the presence of 5% human plasma with BMP-4, VEGF₁₆₅, TPO, FLT3, SCF, IL-3, EPO and IL-6 for 20 d. After dissociation into single cells with collagenase B, cells were cultured in feeder-free condition with 10% human serum together with growth factor cocktail as previously described^[12] for 25 d.

Although discussed in many papers, to the best of our knowledge, there is no record for the application of ESCs/iPSCs in malaria research thus far. The development of such techniques and the possibility to permanently edit the genome of erythroid cells will make a great contribution for a deeper study of intra-erythrocyte parasite biology.

Stem cell derived-hepatocytes

Studying the liver stage of *Plasmodium* parasites is of great importance for understanding the establishment of infection, and for immunogenic and therapeutic purposes^[39,40]. In addition, *P. vivax* can produce dormant forms known as hypnozoites in the liver^[41]. These hypnozoites are responsible for its ability to maintain long term infections and relapsing episodes that contribute to the difficulties of eradicating *P. vivax*^[42].

An immortalized HepG2 cell line^[43] has been extensively used as starting material to investigate the exo-erythrocytic (E.E) cycle of *P. berghei*^[44], *P. vivax*^[45], *P. falciparum*^[46] and *P. gallinaceum*^[47]. However, even though they were able to infect liver cells with *P. falciparum* sporozoites, it is still difficult to get these parasites to successfully complete the cycle and infect RBCs. This difficulty was overcome by Sattabongkot *et al*^[48] that generated a hepatocyte cell line (HC04) that enabled the full development of both *P. vivax* and *P. falciparum*. Briefly, hepatocytes isolated from a hepatoma patient were cultivated with insulin, epidermal growth factor, thyrotropin releasing factor, HDS, glucagon, nicotinamide, linoleic acid, L-glutamine, pyruvic acid and MEM essential amino acids at 37 °C and 5% CO₂. Interestingly, the levels of enzyme activities and protein secretions were higher than the ones observed in HepG2 cell line. These cells were infected with *P. falciparum* and *P. vivax* sporozoites and when RBCs were added to the culture, blood stage parasites were observed after 7 and 10 d for *P. falciparum* and *P. vivax*, respectively.

One of the drawbacks of using immortalized cell lines is that the metabolism of those cells might differ from the *in vivo* ones. To solve this problem Mazier *et al*^[49] used primary rodent hepatocytes to try and mimic the *in vivo* conditions. They found that *P. vivax* parasites were able to develop and after 10 d, rings could be observed in reticulocytes added in co-culture. Recently, using human and primate primary hepatocytes, Dembélé *et al*^[50] successfully cultured *in vitro* E.E stages of *P. falciparum* as well as *P. cynomolgi*, for which they able to get the hypnozoite forms.

Using primary cells for parasite cultures requires that fresh cells be constantly available. To bypass this obstacle, March *et al*^[51] were able to culture previously frozen primary hepatocytes that remained permissive to *Plasmodium* sporozoites, in a microsystem surrounded by fibroblast stromal cells.

Primary simian hepatocytes were used to evaluate the effect of a drug (KAI407) on *P. cynomolgi* liver stage^[52]. Primary hepatocytes isolated from rhesus macaques were infected with *P. cynomolgi* sporozoites in the presence or absence of the KAI407 compound. The development of E.E stage was well established *in vitro*, but the incubation with KAI470 was shown to inhibit formation of liver schizonts as well as hypnozoites. Similarly, *P. berghei* liver stages could develop within murine primary hepatocytes *in vitro* but their ability to infect RBCs remain unexplored in this study^[53].

Immortalized murine hepatocytes (Hepa1-6) were

used to test malaria vaccine candidates on *P. berghei* E.E stage^[54]. It appears that the TRAP-based vaccine in the presence of CD8⁺ enriched splenocytes inhibits the parasite development in the liver. This method was proposed as an *in vitro* system to screen possible vaccine candidates but its suitability to human *Plasmodium* vaccines needs further investigation.

Surprisingly, stem cell-derived hepatocytes have not been widely used for malaria research. Nonetheless, Ng *et al*^[55] generated hepatocyte-like cells (HLCs) from ESC or iPSCs originating from human foreskin fibroblasts, which were permissive to different *Plasmodium* species including *P. falciparum*, *P. vivax*, *P. berghei* and *P. yoelii*. The hepatocyte differentiation protocol was adapted from the one previously described^[56], in which the ESCs/ iPSCs were cultivated for the first 5 d in presence of activin A (100 ng/L) to induce endodermal commitment. The following 10 d led to hepatoblast formation through hypoxia culture condition and was divided into two steps; the first 5 d in presence of BMP-4 and FGF-2 and the last 5 d in presence of hepatocyte growth factor (HGF). During the last 5 d, the cells were maintained with oncostatin to generate mature hepatocyte-like cells. Their HLCs allowed them to test different antimalarial drugs such as Atovaquon or Primaquine on liver stages. However, iPSC-derived HLCs have low levels of enzymes that metabolize drugs as they remain immature hepatocytes and thus are not optimal for antimalarial drug screen.

Many protocols to generate HLCs from ESCs/iPSCs (reviewed in Schwartz *et al*^[57]) or adult stem cells (reviewed in Zhang *et al*^[58]) are available. However, one should note that different protocols seem to create a variety of HLCs with different characteristics. A general scheme can be drawn with a 4-steps protocol: Mesodermal differentiation (in presence of activin A), hepatic specification, hepatoblast expansion and hepatic maturation. For each of these 4 steps, growth factor concentrations as well as the time of exposure remain variable between different studies.

Optimizing the generation of stem cell-derived hepatocytes, which are more similar to the adult hepatocyte, would have great impact on understanding the biology of *Plasmodium* E.E stages and lead to improved testing of potential antimalarial drugs and vaccine candidates.

ANIMAL MODELS

Murine models

Besides the use of *in vitro* modeling to study *Plasmodium* biology, there are several *in vivo* models that aim to mimic human infections. Several animal models are available for diverse *Plasmodium* species. Among those, the most common one remains the mouse model, which is less costly, more available and much more convenient to maintain than primates or other large models. *P. berghei* and *P. yoelii* (both rodent *Plasmodia*) are commonly used for *in vivo* studies as these species share important similarities with primate and human parasites^[59]. *P. yoelii*

shares common features with *P. vivax* [*i.e.*, *P. yoelii* virulence genes (*yir* genes) homolog of *P. vivax* *vir* genes] that make this parasite ideal for *in vivo* studies^[60]. *P. berghei* seems to be a better model for *P. falciparum* *in vivo* studies, especially concerning blood stage vaccine studies^[61] (Figure 2).

Nevertheless, even in murine *Plasmodium* species that share some features with human *Plasmodium*, the ability to interpret and draw conclusion from phenotypic observations from murine to human species remains questionable.

Recent developments has partially resolved this problem, through the use of humanized mice (reviewed in Kaushansky *et al*^[62]). This system represented an important breakthrough in the field of laboratory modeling^[63] and the application of this model through infections of humanized mice with *P. falciparum* begins to make an impact the malaria field. Infection of humanized mice with *P. falciparum* infected RBCs was done by directly injecting human red blood cells (huRBCs) into the mouse blood stream *via* the intra-peritoneal route^[64] or intravenous route^[65]. The main problem using intra-peritoneal injections is the difference in migration of the injected huRBCs into the blood stream between experiments and the lack of reproducibility from one mouse to another. Intravenous delivery as proposed by Arnold *et al*^[65] allows a more stable and long-lasting presence of huRBCs within mouse host. Notably, they also injected parasitized huRBCs and could reach significant parasitemia (up to 10%) by adding new huRBCs intravenously every 2-3 d.

An alternative method that is now more commonly used is the engraftment of HSPCs into immune-deficient mice^[66]. This method allows for continuous production of human RBC in the mouse blood stream. The main obstacles of this methodology are the short lifespan of those cells within mouse bone marrow and the variability in the engraftment success^[67]. Technically speaking, many protocols have been investigated with different combination of mice and HSC sources. Generally, CD34⁺ cells mainly isolated from umbilical cord blood were isolated *via* MACS selection and injected intrahepatic or intravenously within immune-depressed mice. Interestingly, TPO was shown to increase engraftment of CD34⁺ cells in mouse host^[68].

Recently, Amalados *et al*^[69] were able to generate huRBC permissive to different *P. falciparum* strains by transfecting the HSC with a plasmid that expresses IL-3 and EPO. Using this procedure they were able to produce only low amounts of huRBC (1.5%-2.8%) and parasite density was decreasing constantly and thus will require further optimization. Unfortunately, to the best of our knowledge there is no report on using similar systems of humanized mice to study the *P. vivax* asexual cycle *in vivo*.

To date, the major contribution of using humanized mice was to study liver stage parasites which remain the main target for vaccine development^[70].

To engraft human hepatocytes (huHep) within immune-

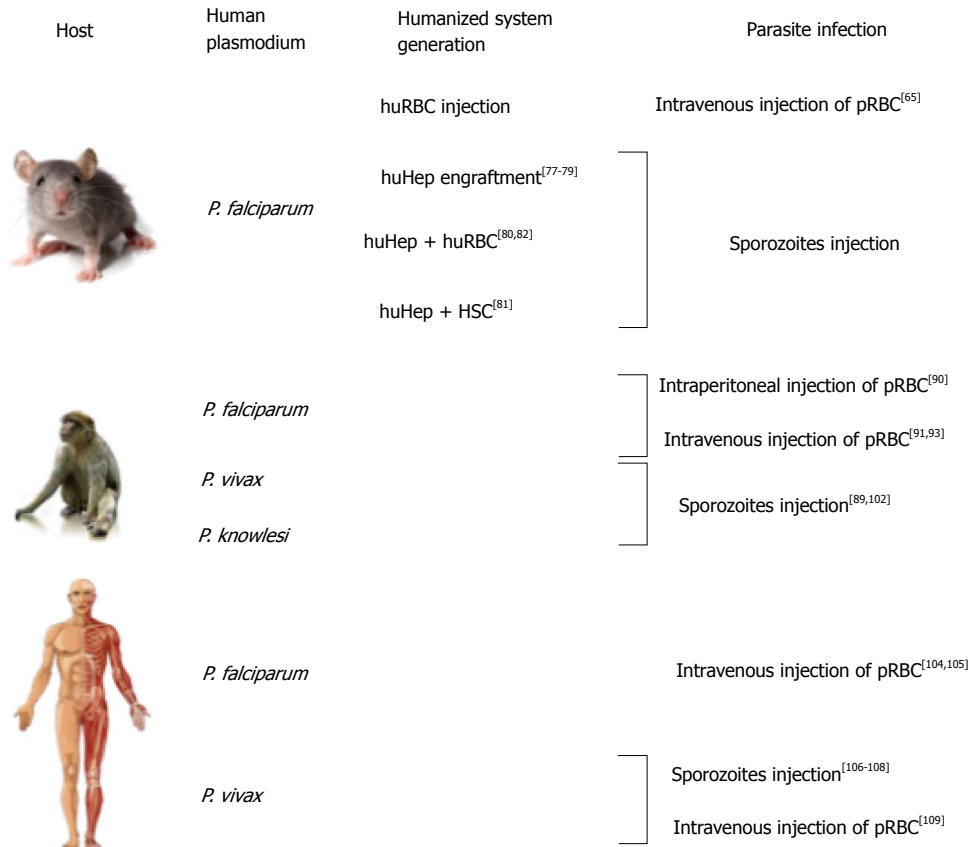


Figure 2 Chart of the different combinations animal model/human *Plasmodia* for *in vivo* studies. Scheme of the different animal models coupled with human *Plasmodium* studies. The different cell types injected within humanized mice are indicated in the column "Humanized system generation" and the *Plasmodium* injection mode under the column "Parasite infection".

depressed mice, it is essential to initially generate damage in order to activate the liver cell repopulation to allow integration of delivered human cells. Several options are available to manipulate mouse hepatocytes: (1) use urokinase plasminogen activator (uPA) toxin^[71]; (2) use fumarylacetoacetate hydrolase knockout mice, dependent of the protection of the 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) drug^[72]; (3) use herpes simplex virus type 1 thymidine kinase transgene dependent of ganciclovir (GGV) activation^[73]; (4) use caspase 8 oligomerization activated with AP20187 drug^[74]; and (5) use diphtheria toxin receptor transgenic mice^[75]. According to the different reports the success rate of colonization of the mouse liver by human hepatocytes range between nearly 50%^[76] to 60%^[75]. A higher ratio of 90% remains so far untenable as murine hepatocytes are needed for metabolic functions.

P. falciparum was shown to be able to invade and develop within hepatocytes of several humanized mice models. The first successful attempt was reported by Morozan *et al*^[77]. Using uPA mice, they were able to engraft human hepatocytes (up to 26%) and after 3 mo, injected them with *P. falciparum* sporozoites. This methodology allowed them to complete the EE development of the parasite to the final schizogony stage. One of the applications of humanized mice is the investigation of potential hepatocyte receptor for *Plasmodium* sporozoites

invasion. Foquet *et al*^[78] investigated the possibility that CD81 receptor and scavenger receptor type B class I (SR-BI) are the entry gates for *P. falciparum* into the liver. They showed that in presence of anti-CD81, the hepatocyte invasion was prevented while anti-SR-B1 did not alter the infection process. The humanized mice used for this study were uPA mice but no indication on the level of human hepatocytes engrafted was indicated. Drug tests can be performed as well in humanized mice as demonstrated by Douglass *et al*^[79]. They monitored the clearance of GFP-luc transgenic *P. falciparum* from the liver after treatment with different antimalarial drugs and could observe a complete clearance of the parasite using Atovaquone (inhibitor of mitochondrial electron transport chain), Serdemetan (p-53 activator) and Obatoclax (BCL-2 family inhibitor). This work shed light on the use of humanized mice to test potential antimalarial drug effect in the human hepatocyte niche.

Recently, humanized mice have been described as a perfect environment to genetically cross *Plasmodium* strains in order to study genetic determinants^[80]. Sporozoites of two different *Plasmodium* strains were injected intravenously into FRG NOD HuHep mice and the EE stage monitored by bioluminescence. The injected sporozoites could mature and invade huRBC injected within the same mice. Unfortunately, the mice were rapidly euthanized and thus no information on the parasite

development in the huRBC could be documented.

Combining human liver and blood stages to get a whole vertebrate cycle within humanized mice remains the ultimate goal of those models. Wijayalath *et al*^[81] reported this full cycle in a humanized mouse. To create the mouse model, they injected CD34⁺ cells that can be the origin of many cell types (*i.e.*, cardiomyocytes, endothelial cells or hepatocytes) in order to be able to recreate a whole human system suitable for the parasite. They could observe an engraftment of 0.023% of huHep, 11% of human Kupffer cells and only 0.2%-1% of huRBC due to poor erythroid differentiation. Sporozoites were injected intravenously, developed within the liver (as shown by immunohistochemistry) and then reached the blood circulation with a very low parasite density of 2-5 parasite/L (parasite density 0.0001%). The asexual stages were then cultivated *in vitro* to obtain gametocytes that could develop into oocytes and sporozoites within mosquitoes. The possibility of getting the whole *P. falciparum* cycle in humanized mice would be a great achievement. Nevertheless, in this study, the low levels of engraftments of human cells, the low parasite density as well as the obligation to generate gametocytes *in vitro* indicate that this system needs further optimization for studying the complete cycle of *Plasmodium* parasites. More recently, Soulard *et al*^[82] achieved the complete *P. falciparum* cycle in humanized mice, from liver stages to sexual forms in the blood. They got up to 80% of huHep and above 80% of huRBC that can be maintained for 5 wk in mice with daily injections. *P. falciparum* sporozoites could migrate into huHep in the mice liver and schizonts were observed 7 d post-infection. The parasite asexual stages could be detected in the blood from 8 d post-infection and sexual stages after 21 d. The parasitemia reached up to 1.52%. In addition, they could infect hepatocytes with *P. ovale* sporozoites and observe the formation of several hypnozoites, but there was no indication for *P. ovale* asexual stages in the blood.

These mice open new frontiers for studying human *Plasmodium in vivo*. Nevertheless, the variations between mice and experiments should be taken into account when designing experiments and analyzing the results. It would be extremely beneficial to expand the use of these mice and apply it to get a mouse model for *P. vivax* (using reticulocyte-enriched huRBC).

While the use of humanized mice obviously offers a wide range of new possibilities to study the biology of human *Plasmodium* spp. *in vivo*, the need to work with immune-depressed mice makes them unsuitable for vaccine development.

Primate models

Beside the use of mice as animal model, primates appear to be a very suitable model to study malaria as they are evolutionary close to humans^[83] and they are natural hosts of human *Plasmodium* spp.^[84,85]. Studies on host-parasite interactions benefit from of this *in vivo* system that allows collecting samples and data regularly.

A complete overview of the use of primates for malaria modeling has already been reviewed by Beignon *et al*^[86].

Nowadays, the uses of non-human primates (NHP) are preferred for *in vivo* research despite ethical reasons that restrict experimenting on primates. Therefore, NHP allows larger sample size and more reagents are available for these models^[87].

To increase parasite density and maintain long-term *Plasmodium* infections within the host, primates need to be splenectomized^[88]. Parasite infection is done either by injecting *Plasmodium* sporozoites^[89] or by direct injection of parasitized RBC (pRBC)^[90,91].

The use of these primate models for *P. falciparum* studies has been restricted mainly to *Aotus* monkeys^[92] that could be infected with several *P. falciparum* strains. The first report of *Aotus* infection with *P. falciparum* was described by Geiman *et al*^[90]. They injected intraperitoneally pRBC from a *P. falciparum* infected woman into a splenectomized *Aotus* monkey. They were able to detect asexual forms of the parasite in the primate blood 54 d post-injection. These primate models were used to test potential blood stage antigens for vaccine development, *i.e.*, MSP-1^[93] or PfEBA-175^[91]. Briefly, *Aotus* primates pre-treated with potential vaccine-candidates were challenged by the injection of pRBC and the parasite density was monitored in order to analyze the protection provided by the initial challenge of the potential vaccines.

Many *P. vivax* isolates have been adapted to several NHP models, among those: The Chesson strain, Salvador I strain and others, which allowed getting an important source of study material. In 1966, Young *et al*^[94] were able to infect *Aotus* primate with pRBC isolated from a *P. vivax* infected patient. Interestingly, they also infected two human volunteers as well as one primate through infected mosquito bites and after 11 d they could only identify *P. vivax* infection in the human volunteers while parasitemia in the monkeys could be observed only after 41 d followed by his death 5 d later.

Primate models have been intensively used to study *P. vivax* liver stages^[87]. The development of an *in vivo* system to study the ability of *P. vivax* to generate dormant forms (hypnozoites) in the liver, which cause relapses of the infective forms^[41] would aid in understanding the dynamics of this process. Collins *et al*^[89] tested different primate species infected with the *P. vivax* Salvador I strain and identified *Saimiri boliviensis* as the most suitable primate species to study *P. vivax* liver stages.

As an alternative to human *Plasmodium* studies in monkeys, researchers recently focused on close simian *Plasmodium* spp in NHP: *P. knowlesi* as a model for *P. falciparum* and *P. vivax*^[95,96] and *P. cynomolgi* for *P. vivax*^[97]. Indeed, these simian *Plasmodium* spp share important features with their human orthologues and can be used to better understand parasite biology or test potential vaccines^[98]. The primate infection remains identical to the process used for *P. falciparum* and *P. vivax*. Krotoski *et al*^[99] were the first to identify the *P. cynomolgi* hypnozoite stage after inoculation of

sporozoites into rhesus monkeys. Akinyi *et al*^[100] were able to create a *P. cynomolgi* transgenic line expressing a red fluorescent protein, which was used to track the parasite *in vivo*.

P. knowlesi can also infect humans can be used as model for both *P. falciparum* and *P. vivax* infections and also to study its own infection traits in human^[101]. Irradiated *P. knowlesi* sporozoites injected into rhesus monkeys achieved a relative protection against further *P. knowlesi* infections, demonstrating the use of *P. knowlesi* as a model for vaccine development^[102].

P. knowlesi can also be applied as a model to study severe malaria usually caused by *P. falciparum*. Barnwell *et al*^[103] could observe a link between the expression of schizont-infected cell agglutination and the severity of the infection in rhesus monkeys, linking between pathogenicity and antigenic variation caused by variant surface antigens in *P. knowlesi* and *P. falciparum*.

One of the major drawbacks of using primate as a model to investigate *Plasmodium in vivo* remains their availability and the significant cost of the colony maintenance that limits the development of this research line.

Human model

Perhaps the most relevant model to study malaria, in such cases that allows experimenting, is the human host itself. Naturally, potential candidate vaccine candidates have to be tested in humans during clinical trial. For example, to test the RTS/S vaccine, healthy volunteers that were prime-boosted immunized with candidate vaccines were infected with *P. falciparum* sporozoites to test the efficacy of these vaccine candidates^[104,105]. We can also cite the control human malaria infection program that allows inoculating parasites in human volunteers in order to test potential vaccines or anti-malarial drugs^[106,107].

Interestingly, the lack of an *in vitro* model for *P. vivax*^[13] pushed researchers to infect human volunteers with *P. vivax* sporozoites in order to develop a model for *P. vivax* drug screening and vaccine development. Herrera *et al*^[108] let *P. vivax*-infected Anopheles mosquitoes feed on different groups of volunteers, each exposed to increasing number of mosquito bites. They observed that malaria symptoms appeared after 9 d and a total clearance of the parasites was observed 48 h post treatment at the latest. No record of *P. vivax* relapsing after the end of the study was reported and thus, the system is claimed to be safe to test antimalarial drugs *in vivo*.

More recently, McCarthy *et al*^[109] infected human volunteers *via* intra-venous injection of pRBC isolated from a *P. vivax* positive woman. The first symptoms appeared 11 d post-inoculation and disappeared 24 h post-antimalarial drug treatment. The advantage of using pRBC instead of sporozoites is to avoid the formation of hypnozoites and thus re-infection. Their

goal was to establish a *P. vivax in vivo* system similar to the work described earlier by Herrera *et al*^[108] and only the inoculation method (sporozoites vs pRBC) and the number of volunteers (18 vs 2) was different. Although biologically, humans are the most relevant models, there are important ethical issues that prevent wide use of human volunteers in *in vivo* experiments out of phase II clinical trials.

CONCLUDING REMARKS

Establishing good experimental models for malaria research has great importance in understanding fundamental aspects of the parasites' biology, the course of infection and disease establishment and progression. It is an important tool for laboratories located in non-endemic areas that have more facilities to perform state of the art research to help fighting malaria.

The development of stem cell research has opened many new options to study parasite interactions with human host. Combining these novel *in vitro* systems with animal models offered a wide range of new avenues to study aspect of the parasite biology, which were not possible before.

Each technique has its advantages and weakness depending on the parasite species and the stage in the cell cycle being investigated.

Thus far, despite their great potential, the use of stem cells for malaria *in vitro* studies is limited. The establishment of good *in vitro* culture of *P. vivax* in reticulocytes originated from HSC has not been successful even though there is a great interest in such a model. On the other hand, the use of HSC to study erythroid impairment during malaria episodes was shown to be a great tool, which is expected to have a significant contribution to the field in coming years. Very surprisingly, although understanding the biology of *Plasmodium* liver stages is of major importance for drug and vaccine development, the use of hepatocyte-derived stem cells is poorly developed and there is a great need for a better cell line that differentiates into mature hepatocytes.

Animal model have been intensively developed to gain an understanding that will be able to be rapidly translated to the clinic. Monkeys appear to be the most suitable models, especially for *P. vivax*, but the cost of colony maintenance limits the use of this model. Nevertheless, *P. vivax* primate model are used successfully and are currently the best option for research since an *in vitro* culture of this parasite remains challenging. The development of *in vitro* stem cell techniques would offer an important tool to study *P. vivax* biology, especially for the intra-erythrocytic cycle. Recent protocol improvements give great hope that with additional optimization these systems will be available in coming years.

The use of humanized mice to study *Plasmodium* biology through an *in vivo* system offers new opportunities, however, the short-term life span of engraftment and the low levels of chimeric systems eventually obtained have to

be taken into account in the process of data analyses.

The use of simian *Plasmodium* species that could infect humans to overcome the difficulties in maintaining cultures seems like an attractive option, however, the use of the human *Plasmodium* spp. will yield the most relevant observations that could be directly translated to human malaria.

Additional tools such as mathematical and bio-informatics modeling could also become valuable as recently shown by MacDonald *et al.*^[110] that used computational methods to investigate potential antimalarial drugs.

The combination of stem cell research and animal modeling such as humanized mice could be the key to move a step forward in the study of *Plasmodium* biology. Optimization of those techniques and generation of new animal/human stem cell combinations could bring malaria modeling to the next level.

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Role of adipose-derived stromal cells in pedicle skin flap survival in experimental animal models

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Abstract

The use of skin flaps in reconstructive surgery is the first-line surgical treatment for the reconstruction of skin defects and is essentially considered the starting point of plastic surgery. Despite their excellent usability, their application includes general surgical risks or possible complications, the primary and most common is necrosis of the flap. To improve flap survival, researchers have used different methods, including the use of adipose-derived stem cells, with significant positive results. In our research we will report the use of adipose-derived stem cells in pedicle skin flap survival based on current literature on various experimental models in animals.

Key words: Pedicle skin flap; Adipose stromal cells; Flap survival; Stem cell; Skin defect; Reconstructive surgery

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Core tip: The use of skin flaps in reconstructive surgery is the first-line surgical treatment for the reconstruction of skin defects and is essentially considered the starting point of plastic surgery. Our work, summarizing the current literature, presents the role of adipose-derived stromal cells in pedicle skin flap survival in experimental animal models.

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INTRODUCTION

Flaps are used in plastic surgery for wound coverage

when insufficient blood supply impedes the viability of skin grafts. Examples of such applications include large wounds over a flexion crease or wounds with exposed bone, tendon, or other vital structures. Flaps are also preferred in plastic surgery over free grafts because they have a better aesthetic and functional result^[1]. A first distinction of cutaneous flaps was established in the 1970s. Skin flaps were classified depending on the blood irrigation into the axial pattern flaps, which have an anatomically recognized arteriovenous system running along their long axis, and random pattern flaps, which lack any significant bias in their vascular patterns^[2].

Since then, there has been a rapid development of reconstructive surgery, which has kept pace with the goal of understanding, improving, and developing methods to avoid partial or total flap necrosis, the main complication of the use of skin flaps. Although the cause of skin flap necrosis has not been fully resolved yet, the lack of adequate nutrient blood supply certainly plays a significant role in the pathophysiology of necrosis. To reverse this phenomenon and strengthen vascular reserves, various therapeutic approaches have been pursued. For example, the administration of exogenous agents such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) has been shown to enhance revascularization and improve survival of ischemic flaps^[3-5]. However, the beneficial effect of such exogenous factors is reduced due to their short half-life^[6] and the limited number of existing endothelial cells. Hence, the abovementioned factors are not enough to control the complex cascade of wound healing.

In recent years the rapid development of cell biology and genetics has helped to highlight the ability of somatic stem cells, especially bone marrow-derived stem cells (BSCs) and adipose-derived stem cells (ADSCs), to promote neovascularization^[7-12]. Various studies conducted to compare the forms of stem cells derived from bone marrow, umbilical cord, or adipose tissue showed no significant differences in terms of morphology, immunogenicity, and pluripotent differentiation^[13]. The proangiogenic effect of ADSCs and BSCs has been well established, however the two groups seem to have different promoting angiogenesis mechanisms^[14]. This fact, combined with the minimally invasive techniques in extraction, isolation, and culture from ADSCs^[15-17], places them in the first line of research for various therapeutic purposes in medical science^[18].

This review presents the therapeutic benefits of ADSCs in pedicle skin flap survival based on current literature on various experimental models in animals.

EFFECT OF ADSCS ON VIABILITY OF RANDOM PEDICLE SKIN FLAPS

The first time adipose stem cells were used as an antinecrotic treatment in random pedicle flaps was by Lu *et al.*^[19] in 2008. Intracutaneous injection of (DiI)-

labeled (*i.e.*, chemical used for labeling cell membranes and hydrophobic structures) adipose-derived stem cells in ICR mice (*i.e.*, mice originating from a Swiss mice strain from Institute for Cancer Research in Philadelphia) led to a statistically significant increase in survival of the flaps with considerable improvement in capillary density. Furthermore, the immunohistochemical test showed that on some occasions there was *in vivo* differentiation of ADSCs in endothelial cells. Uysal *et al.*^[20] examined the behavior and properties of adipose-derived stem cells in an ischemia-reperfusion model in ICR mice. They established that ADSCs could prevent ischemia-reperfusion injury, mainly by regulating growth factors, especially VEGF, bFGF, and transforming growth factor-beta (TGF- β). Gao *et al.*^[21] showed that topical use of ADSCs could improve viability of ischemic random pedicle skin flap in streptozotocin-induced diabetic mice *via* expression of hypoxia-inducible factor-1 α . Sheng *et al.*^[22] implicated the beneficial effect of BSCs vs stromal vascular factor (SVF), which contains a group of heterogeneous cells in the adipose tissue, including ADSCs. No statistically significant difference in promoting vascularization and survival of pedicle skin flaps in Wistar rats could be observed.

In 2013, Karathanasis *et al.*^[23] examined whether genetically modified autologous ADSCs increase graft survival. They conducted an experimental study in which autologous green fluorescent protein (GFP)-producing ADSCs were injected intracutaneously into random-pattern skin flaps in Wistar rats. The results indicated that transplantation of modified GFP-ADSCs improves the survival of the flaps. GFP-ADSCs were detected in the endothelium of blood vessels co-expressing the endothelial marker von Willebrand factor, suggesting that they promoted blood vessel regeneration *in vivo*^[23]. The same year, Yue *et al.*^[24], using a hypoxic preconditioning experimental flap model, showed that preoperative transplantation of ADSCs, combined with hypoxic preconditioning, effectively improves the survival of ischemic skin flaps in Lewis rats by enhancing neovascularization associated with the production and activation of hypoxia-inducible factor 1 alpha (HIF-1 α), together with an increase in VEGF. Comparing the effectiveness of different administration routes of ADSCs in improving the viability of random-pattern skin flaps, Lee *et al.*^[25] indicated that the collagen sponge method delivers ADSCs most effectively within the flap, increasing flap vascularity. Nevertheless, the intravascular administration of ADSCs also positively affects the skin-flap survival, as shown in experiments established by Suartz *et al.*^[26] in Wistar rats.

Recently, Park *et al.*^[27] investigated the effects of low-level light therapy (LLLT) on transplanted human adipose-derived mesenchymal stromal cells in the skin flaps of mice. The results indicated that LLLT is an effective biostimulator of ADSCs in vascular regeneration, which enhances the survival of ADSCs and stimulates the secretion of growth factors in skin flaps. Therefore, although the use of ADSCs led to improved viability

Table 1 The most relevant studies on the effect of adipose-derived stem cells on viability of pedicle skin flaps in experimental animal models

Ref.	Year	Contribution
Lu <i>et al</i> ^[19]	2008	Intracutaneous injection of (DiI)-labeled ADSCs improves capillary density
Uysal <i>et al</i> ^[20]	2009	ADSCs prevent ischemia-reperfusion injury by regulating growth factors, especially VEGF, bFGF, TGF- β
Gao <i>et al</i> ^[21]	2011	Human-ADSCs improve viability of ischemic random pedicle skin flap in mice <i>via</i> expression of hypoxia-inducible factor-1 α
Sheng <i>et al</i> ^[22]	2011	BSCs vs SVF promotes vascularization
Karathanasis <i>et al</i> ^[23]	2013	Transplantation of modified GFP-ADSCs promotes blood vessel regeneration <i>in vivo</i>
Yue <i>et al</i> ^[24]	2013	Transplantation of ADSCs, combined with hypoxic preconditioning, enhances neovascularization associated with the production and activation of HIF-1 α , together with an increase in VEGF
Lee <i>et al</i> ^[25]	2014	ADSCs delivered <i>via</i> sponge method increase flap vascularity
Suartz <i>et al</i> ^[26]	2014	Administration of ADSCs affects positively in skin-flap survival
Derby <i>et al</i> ^[28]	2014	Genetic modified GFP-ADSC improves overlying skin composition and appearance after fat graft transplantation
Park <i>et al</i> ^[27]	2015	LLLT on transplanted human-ADSCs in the skin flaps of mice stimulates the secretion of growth factors in skin flaps
Reichenberger <i>et al</i> ^[29]	2012	Topical application of ADSCs embedded in a fibrin matrix, increases ischemic tissue survival, blood flow and expression of pro-angioactive genes in an animal epigastric skin flap model
Reichenberger <i>et al</i> ^[30]	2012	ADSCs in an extended inferior epigastric artery skin flap enhance blood supply and tissue regeneration
Feng <i>et al</i> ^[31]	2014	Heterologous transplantation of human ADSCs in axial pedicle skin flaps improves viability of axial skin flap in mice
Xu <i>et al</i> ^[32]	2015	Transplantation of ADSCs promotes capillary formation
Tomita <i>et al</i> ^[33]	2013	Utilization of ADSCs in Lewis rats improved the sensory capability of skin flaps <i>via</i> the production of neurotrophic factors and nerve growth factors
Uysal <i>et al</i> ^[36]	2010	ADSCs and BSCs increased the vascular density, and the VEGF
Li <i>et al</i> ^[37]	2010	ADSCs increase the vascular density and the survival percentage of the flaps producing high cytokine levels such as VEGF-A

ADSCs: Adipose-derived stem cells; HIF-1 α : Hypoxia-inducible factor 1 alpha; SVF: Stromal vascular factor; VEGF-A: Vascular endothelial growth factor A; VEGF: Vascular endothelial growth factor; bFGF: Basic fibroblast growth factor; TGF- β : Transforming growth factor-beta; BSCs: Bone marrow-derived stem cells; GFP: Green fluorescent protein; LLLT: Low-level light therapy.

of skin flaps, their combination with LLLT significantly enhanced their action.

Derby *et al*^[28] used the well-documented epithelial stem cell marker p63 to identify *in vivo* transdifferentiation of genetic modified GFP-ADSC in epithelial cells, and therefore show, their contribution to the improvement of overlying skin composition and appearance after fat graft transplantation.

EFFECT OF ADSCS ON VIABILITY OF AXIAL PEDICLE FLAPS

To the best of our knowledge, the first attempt to examine the effect of ADSCs in axial pedicle skin flap survival took place in 2012^[29]. Reichenberger *et al*^[29] indicated that the topical application of ADSCs embedded in a fibrin matrix increases ischemic tissue survival, blood flow, and expression of pro-angioactive genes in an animal epigastric skin flap model. In the same year it was also shown that the administration of ADSCs in an extended inferior epigastric artery skin flap-which was used as a flap ischemia reperfusion injury (IRI) model-may protect axial skin flaps from IRI by enhancing blood supply and tissue regeneration^[30]. The heterologous transplantation of ADSCs in axial pedicle skin flaps was examined by Feng *et al*^[31], in which an increase in the viability of human adipose-derived stem cells was observed after local intra-arterial injection in the superficial epigastric arteria of axial skin flaps in mice. A further study was conducted by Xu *et al*^[32] in which stem cells were shown to contribute positively to the survival of axial flaps. Xu

and his team established a rabbit ear venous-congested skin flap model, where they transplanted ADSCs. After histological and immunofluorescence evaluation, it was indicated that ADSCs not only increase the survival of venous-congested skin flaps but also promote capillary formation.

Tomita *et al*^[33] investigated the phenomenon of flap reinnervation through the utilization of ADSCs. They indicated that the use of the aforementioned cells improved the sensory capability of skin flaps in Lewis rats *via* the production of neurotrophic factors and nerve growth factors^[33].

EFFECT OF ADSCS ON VIABILITY OF PREFABRICATED PEDICLE FLAPS

The concept of flap prefabrication is relatively new to the field of reconstructive surgery and was first introduced by Yao^[34] in the 1980s. In the procedure of flap prefabrication, a vascular pedicle is introduced in a donor area that lacks any axial vascularization, improving the blood supply and enhancing the viability of the surrounding tissues. Although the above flaps can be used for wound coverage in almost any part of the body, their use in head and neck regions has prevailed, especially after extensive burns in which the available reconstructive options are scarce^[35].

Despite the undeniable utility of prefabricated flaps in plastic surgery, the risk of total or partial necrosis after flap transplantation remains a problem for further investigation. Among the concepts employed to resolve this potential complication is the application of ADSCs.

There are two studies in the literature in which ADSCs have been used in prefabricated flaps as an anti-necrosis therapy. Uysal *et al*^[36] used the femoral artery, vein, and fascia of Wistar rats as a vascular crane for a prefabrication model in which they introduced ADSCs and BSC. Their experiments showed that both of the aforementioned cells increased the vascular density, and the VEGF indicated that mesenchymal stem cells could be useful in any prefabrication procedure in which neovascularization is necessary. Li *et al*^[37] applied a prefabricated abdominal island flap model in rats, also using the right femoral artery, in which ADSCs were injected. The post-operative control demonstrated that ADSCs increased the vascular density and the survival percentage of the flaps producing high cytokine levels such as vascular endothelial growth factor A. Table 1 summarizes the most relevant studies on the effect of ADSCs on viability of pedicle skin flaps in experimental animal models.

CONCLUSION

The current literature shows that in all cases where ADSCs were applied to investigate their effect on pedicle skin flap survival, they led to improved viability of the flaps. This was established through the increase of skin flap vascularity *via* the production of growth factors and/or ADSCs' direct transformation into epithelial cells with neoangiogenesis. Although the number of experimental studies on the application of stem cells as an anti-necrosis therapy is limited, an increasing number of researchers have been focusing on this field. This tendency, combined with the already successful clinical application of adipose stem cells in other fields of medical science, might show that their future use in the field of reconstructive surgery - where skin flaps are widely used-is no longer utopian.

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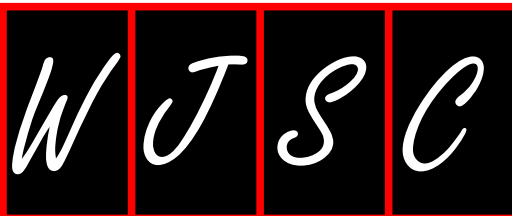
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Basic Study

Updates in the pathophysiological mechanisms of Parkinson's disease: Emerging role of bone marrow mesenchymal stem cells

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Author contributions: Ahmed HH designed and coordinated the research as well as wrote the paper; Salem AM analyzed the data; Atta HM performed the isolation and preparation steps of bone marrow mesenchymal stem cells from rats; Eskandar EF participated in the designation of the research; Farrag AH performed the immunohistochemical examination and histopathological investigations; Ghazy MA performed the molecular investigations; Salem NA and Aglan HA participated in the induction of Parkinson's disease in rats and treatment as well as performed the biochemical measurements.

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Abstract

AIM: To explore the approaches exerted by mesenchymal stem cells (MSCs) to improve Parkinson's disease (PD) pathophysiology.

METHODS: MSCs were harvested from bone marrow

of femoral bones of male rats, grown and propagated in culture. Twenty four ovariectomized animals were classified into 3 groups: Group (1) was control, Groups (2) and (3) were subcutaneously administered with rotenone for 14 d after one month of ovariectomy for induction of PD. Then, Group (2) was left untreated, while Group (3) was treated with single intravenous dose of bone marrow derived MSCs (BM-MSCs). *SRY* gene was assessed by PCR in brain tissue of the female rats. Serum transforming growth factor beta-1 (TGF- β 1), monocyte chemoattractant protein-1 (MCP-1) and brain derived neurotrophic factor (BDNF) levels were assayed by ELISA. Brain dopamine DA level was assayed fluorometrically, while brain tyrosine hydroxylase (TH) and nestin gene expression were detected by semi-quantitative real time PCR. Brain survivin expression was determined by immunohistochemical procedure. Histopathological investigation of brain tissues was also done.

RESULTS: BM-MSCs were able to home at the injured brains and elicited significant decrease in serum TGF- β 1 (489.7 ± 13.0 vs 691.2 ± 8.0 , $P < 0.05$) and MCP-1 (89.6 ± 2.0 vs 112.1 ± 1.9 , $P < 0.05$) levels associated with significant increase in serum BDNF (3663 ± 17.8 vs 2905 ± 72.9 , $P < 0.05$) and brain DA (874 ± 15.0 vs 599 ± 9.8 , $P < 0.05$) levels as well as brain TH (1.18 ± 0.004 vs 0.54 ± 0.009 , $P < 0.05$) and nestin (1.29 ± 0.005 vs 0.67 ± 0.006 , $P < 0.05$) genes expression levels. In addition to, producing insignificant increase in the number of positive cells for survivin (293.2 ± 15.9 vs 271.5 ± 15.9 , $P > 0.05$) expression. Finally, the brain sections showed intact histological structure of the striatum as a result of treatment with BM-MSCs.

CONCLUSION: The current study sheds light on the therapeutic potential of BM-MSCs against PD pathophysiology *via* multi-mechanistic actions.

Key words: Parkinson's disease; Pathophysiology; Bone marrow derived mesenchymal stem cells; Rotenone; Anti-inflammatory action; Ovariectomy; Anti-apoptotic effect; Neurogenic potential

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Core tip: The current study was planned to clarify the mode of action of mesenchymal stem cells (MSCs) in targeting multiple systems implicated in the pathophysiology of Parkinson's disease (PD) in the rat model. For this purpose, the MSCs were isolated from bone marrow (BM) of rat femur bone and PD was induced in ovariectomized rats by rotenone administration for 14 d. Our results provided clear evidences for the therapeutic role of BM-derived MSCs against PD pathophysiology through their immunomodulatory properties, anti-inflammatory and anti-apoptotic effects as well as neurotrophic and neurogenic potentials.

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INTRODUCTION

Parkinson's disease (PD) is one of the most common neurodegenerative diseases, associated with extrapyramidal motor dysfunction^[1] due to the progressive and specific loss of dopaminergic neurons in the substantia nigra pars compacta and declining levels of dopamine (DA) in the striatum^[2]. It affects approximately seven million people globally^[3]. The commonness of PD raises with age, as 1% of people over 60 years of age, 3.4% of those over 70, and 4% of those over 80 were affected by the disease^[1]. Epidemiological studies and pathological investigations exhibit a mean period of onset of 70 in sporadic PD, which represents about 95% of patients^[4,5]; but familial forms of the disease linked to transformation in a limited number of genes account for 4% and these patients suffer from early-onset disease before the age of 50^[6].

Growing body of evidences have demonstrated that environmental factors play a critical role in the etiology of PD^[7]. For example, the environmental toxin 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was identified as one the causative agents of Parkinsonism^[8]. Also, herbicides or pesticides usage increase the risk of PD^[9,10]. As, the pesticide rotenone and the herbicide paraquat reproduce the PD phenotype in animals^[11]. Additionally, it has been suggested that exposure to organic solvents, carbon monoxide and carbon disulfide^[12] play roles in the etiology of PD. Epidemiological studies have proposed a potential link between pesticide exposure and increased risk of PD. For example, agrarian laborers, particularly individuals who work with pesticides, are at increased risk for suffering from PD^[13].

At present, there is no therapy clinically accessible to postpone neurodegeneration, thusly modulation of the disease course is an imperative unmet clinical need. Along these lines, understanding of the pathophysiology and etiology of the disease at cellular and molecular levels to find new targets against which neuroprotective/disease-modifying therapy may be developed is the pivotal issue in the field of PD research^[7].

Mesenchymal stem cells (MSCs) are a heterogeneous subset of stromal stem cells that have the capacity of self-renewal and differentiation into mesodermal lineage cells and other embryonic lineages, including adipocytes, osteocytes, chondrocytes, hepatocytes, neurons, muscle cells, epithelial cells, etc.^[14]. Additionally, these cells have several advantages, such as easy availability as well as few ethical concerns and low immunogenicity. An expanding number of data has demonstrated that MSCs not only depend on their differentiation capacity to repair damaged tissue, but also rely on their ability to modify local environment, activate endogenous progenitor

cells, and secrete several factors^[15]. The aforementioned properties make MSCs perfect candidate cell type for tissue engineering, regenerative medicine and autoimmune disease treatment^[14].

The focus of our interest was to clarify the mode of action of bone marrow derived MSCs (BM-MSCs) in targeting multiple systems implicated in the pathophysiology of PD in the rat model.

MATERIALS AND METHODS

Preparation of BM-MSCs

BM was harvested by flushing the tibiae and femurs of 6-wk-old male Sprague Dawley rats with Dulbecco's modified Eagle's medium (DMEM; GIBCO/BRL, Grand Island, New York, United States, Cat. #42430-082) supplemented with 10% fetal bovine serum (FBS; GIBCO/BRL, Cat. #16000-044). Nucleated cells were isolated with a density gradient [Ficoll/Paque (Pharmacia)] and resuspended in complete culture medium supplemented with 1% penicillin-streptomycin (GIBCO/BRL, Cat. #10378-016). Cells were incubated at 37 °C in 5% humidified CO₂ for 12-14 d as primary culture or upon formation of large colonies. When large colonies developed (80%-90% confluence), cultures were washed twice with phosphate buffer saline (PBS; Gibco/BRL, Cat. #10010056) and the cells were trypsinized with 0.025% trypsin and 0.01% ethylenediaminetetraacetic acid (EDTA) (Gibco/BRL, Cat. #R-001-100) for 5 min at 37 °C. After centrifugation, cells were resuspended with serum-supplemented medium and incubated in 50 mL falcon tube. The resulting cultures were referred to as first-passage cultures. MSCs in cultures were characterized by their adhesiveness and fusiform shape^[16].

Experimental set up

Twenty four adult female Sprague-Dawley rats weighing 130-150 g were obtained from the Animal House Colony of the National Research Centre, Giza, Egypt and acclimated in a specific area where temperature (25 °C ± 1 °C) and humidity (55%). Rats were controlled constantly with a 12 h light/dark cycles at National Research Centre Animal Facility Breeding Colony. Rats were individually housed with *ad libitum* access to standard laboratory diet consisted of casein 10%, salt mixture 4%, vitamin mixture 1%, corn oil 10%, cellulose 5% and completed to 100 g with corn starch and tap water. Rats were cared for according to the guidelines for animal experiments which were approved by the Ethical Committee of Medical Research at National Research Centre, Giza, Egypt.

After the acclimatization period (2 wk), the female rats were ovariectomized surgically in Hormones Department, Medical Research Division at the National Research Centre. Then, after one month from ovariectomy the animals were classified into 3 different groups (8 rats/group). The first group (Ovariectomized control group) was untreated ovariectomized control group. While, the second and third groups were subcutaneously injected

with rotenone (Sigma, United States, Cat. #R8875) in a dose of 12 mg/kg b. wt.^[17] daily for 14 d for induction of PD. Thereafter, the second group (PD untreated group) was left untreated for 4 mo while, the third group (PD + BM-MSCs group) was infused intravenously with a single dose (3×10^6 cells/rat) of BM-MSCs^[18]. For MSCs infusion, the PD induced rats were deeply anaesthetized *via* diethyl ether and MSCs were suspended in 100 µL PBS before transplantation and then slowly injected into the tail vein in 5 min with a 27G needle. The needle was kept in the tail vein for another 5 min to avoid regurgitation and then withdrawn.

At the end of the experimental period (4 mo), all animals were fasted for 12 h and the blood samples were collected from retro-orbital venous plexus under diethyl ether anaesthesia. The blood samples were left to clot and the sera were separated by cooling centrifugation (4 °C) at $1800 \times g$ for 10 min and then stored immediately at -20 °C in clean plastic Eppendorf until analyzed. Moreover, the whole brain of each rat was rapidly and carefully dissected. Then, each brain was sagittally divided into two portions. The first portion was immediately frozen in liquid nitrogen and stored at -80 °C prior to extraction for molecular study and DA level determination. While, the second portion was fixed in formalin buffer (10%) for histological investigation and immunohistochemical study.

Detection of male-derived MSCs in the brain of females

The genomic DNA was isolated from the brain tissues of female rats which were treated with BM-MSCs using phenol/chloroform extraction and ethanol precipitation method according to Sambrook *et al.*^[19] with minor modifications. The presence or absence of the sex determination region on the Y chromosome male (SRY) gene in recipient female rats was assessed by PCR. Primer sequences for SRY gene (forward 5'-CATCGAAGGGTTAAA-GTGCCA-3', reverse 5'-ATAGTGTGTAGTTGTTGTCC-3', Invitrogen) were obtained from published sequences^[20] and amplified to a product of 104 bp. The PCR conditions were as follows: Incubation at 94 °C for 4 min; 35 cycles of incubation at 94 °C for 50 s, 60 °C for 30 s, and 72 °C for 1 min; with a final incubation at 72 °C for 10 min. PCR products were separated using 2% agarose gel electrophoresis and stained with ethidium bromide.

Biochemical analyses

Serum transforming growth factor beta-1 (TGF-β1) level was assayed by enzyme linked immunosorbent assay (ELISA) using kit purchased from DRG Diagnostics Co., Germany (Cat. #EIA-1864), according to the method described by Kropf *et al.*^[21]. While, serum monocyte chemoattractant protein-1 (MCP-1) level was determined by ELISA method using kit purchased from Bender MedSystems GmbH, Europe (Cat. #BMS631INST), according to the method described by Baggiolini *et al.*^[22]. Moreover, serum brain derived neurotrophic factor (BDNF) level was evaluated by ELISA method using kit purchased

from Millipore Corporation, United States (Cat. #CYT306), according to the method described by Laske *et al.*^[23]. Finally, the quantitative determination of brain DA level was carried out according to the method described by Ciarlone^[24] using a fluorometric method.

Detection of tyrosine hydroxylase and nestin genes expression level

Total RNA was isolated from brain tissues of female rats by the standard TRIzol[®] reagent extraction method (Invitrogen, Cat. #15596-026). Then, the complete Poly(A)⁺ RNA was reverse transcribed into cDNA in a total volume of 20 μ L using RevertAid[™] First Strand cDNA Synthesis Kit (MBI Fermentas, Germany, Cat. #K1631). An amount of total RNA (5 μ g) was used with a reaction mixture, termed as master mix. The MM was consisted of 50 mmol/L MgCl₂, 5 \times reverse transcription (RT) buffer (50 mmol/L KCl; 10 mmol/L Tris-HCl; pH 8.3; 10 mmol/L of each dNTP, 50 μ mol/L oligo-deoxyribonucleotide primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M-MuLV reverse transcriptase. The RT reaction was carried out at 25 $^{\circ}$ C for 10 min, followed by 1 h at 42 $^{\circ}$ C, and the reaction was stopped by heating for 5 min at 99 $^{\circ}$ C. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through semi-quantitative real time PCR (sqRT-PCR). An iQ5-BIO-RAD Cyler (Cepheid, United States) was used to determine the rat cDNA copy number. PCR reactions were set up in 25 μ L reaction mixtures containing 12.5 μ L 1 \times SYBR[®] Premix Ex Taq[™] (TaKaRa, Biotech. Co. Ltd., Germany, Cat. #RR820A), 0.5 μ L 0.2 μ mol/L forward primer, 0.5 μ L 0.2 μ mol/L reverse primer (Invitrogen), 6.5 μ L distilled water, and 5 μ L of cDNA template. Primer sequences were F: 5'-ACTGTGGAATTCGGGCTATG-3', R: 5'-GACCTCAGGCTCCTCTGACA-3' for tyrosine hydroxylase (TH)^[25]; F: 5'-TGGAGCGGGAGTTAG-AGGCT-3', R: 5'-ACCTCTAAGCGACACTCCCGA-3' for nestin^[26] and F: 5'-CTGTCTGGCGGCACCACCAT-3', R: 5'-GCAACTAAGTCATAGTCCGC-3' for β -actin^[27]. The reaction program was allocated to 3 steps. First step was at 95.0 $^{\circ}$ C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (1) denaturation at 95.0 $^{\circ}$ C for 15 s; (2) annealing at 58.0 $^{\circ}$ C for 30 s, 55.0 $^{\circ}$ C for 5 s and 60 $^{\circ}$ C for 30 s for TH, *nestin* and β -actin genes respectively; and (3) extension at 72.0 $^{\circ}$ C for 30 s. The third step consisted of 71 cycles started at 60.0 $^{\circ}$ C and then increased about 0.5 $^{\circ}$ C every 10 s up to 95.0 $^{\circ}$ C for melting curve analysis which was performed at the end of each sqRT-PCR to check the quality of the used primers. Each experiment included a distilled water control.

Immunohistochemical examination of brain survivin expression

Samples were taken from brain of rats of the different groups and fixed in 10% formalin buffer for 24 h. Washing was done in tap water then ascending grade of ethyl alcohol (30%, 50%, 70%, 90% and absolute) was used for dehydration. Specimens were cleared in xylene and

embedded in paraffin (melting point 58 $^{\circ}$ C-60 $^{\circ}$ C) for 24 h. Sections were cut into 4 μ thick by sledge microtome then fixed on positive slides in a 65 $^{\circ}$ C oven for 1 h. Slides were placed in a coplin jar filled with 200 mL of triology working solution (Cell Marque, CA-United States, Cat. #920P-04) which combines the three pretreatment steps: Deparaffinization, rehydration and antigen unmasking. Then, the jar is securely positioned in the autoclave which was adjusted so that temperature reached 120 $^{\circ}$ C and maintained stable for 15 min after which pressure is released. Thereafter, the coplin jar is removed to allow slides to cool for 30 min. Sections were then washed and immersed in Tris-buffer saline to adjust the pH and these were repeated between each step of the immunohistochemical procedure. Quenching endogenous peroxidase activity was performed by immersing slides in 3% hydrogen peroxide for 10 min. Broad spectrum LAB-SA detection system (Invitrogen, Cat. #85-8943) was used to visualize any antigen-antibody reaction in the tissue. Background staining was blocked by putting 3 drops of 10% goat non immune serum blocker on each slide and incubating them in a humidity chamber for 10 min. Without washing, excess serum was drained and the working solution (1:100) of survivin mouse monoclonal (Thermo Scientific, United States, Cat. #RB-9245-P1) was prepared. Three drops of the working solution were applied and slides were incubated in the humidity chamber overnight at 4 $^{\circ}$ C. Henceforward, biotinylated secondary antibody from ultravision detection system anti-polyvalent HRP/3,3'-diaminobenzidine (DAB) (Thermo Scientific, Cat. #TP-015-HD) was applied on each slide for 20 min followed by 20 min incubation with the streptavidin HRP enzyme conjugate (Thermo Scientific, Cat. #TP-015-HD). Then, DAB chromogen (Thermo Scientific, Cat. #TP-015-HD) was prepared and 3 drops were applied on each slide for 2 min. DAB was rinsed, after which counterstaining with Mayer hematoxylin and cover slipping were performed as the final steps before slides were examined under the light microscope (Olympus Cx21 with attached digital camera)^[28]. Image analysis was performed using the image J, 1.41a NIH, United States analyzer.

Histopathological investigation of brain tissue of rats

Samples were taken from brain of rats in different groups and fixed in 10% formalin buffer for 24 h. Washing was done in tap water then ascending grade of ethyl alcohol (30%, 50%, 70%, 90% and absolute) was used for dehydration. Specimens were cleared in xylene and embedded in paraffin (melting point 58 $^{\circ}$ C-60 $^{\circ}$ C) for 24 h. Paraffin wax tissue blocks were prepared for sectioning at 4 μ by sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained by hematoxylin and eosin (H and E) stain^[29] for histopathological examination through the electric light microscope.

Statistical analysis

In the present study, all results were expressed as mean \pm SE of the mean. Data were analyzed by one

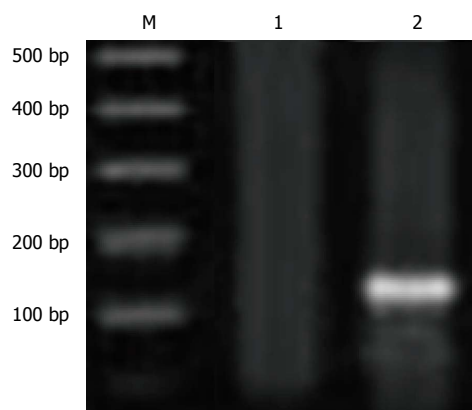


Figure 1 An agarose gel electrophoresis of DNA fragments showed *SRY* gene in recipient female rats for bone marrow derived mesenchymal stem cells in Parkinson's disease model. Lane (M) represents DNA ladder; Lane (1) represents ovariectomized control sample; Lane (2) represents sample from PD group treated with BM-MSCs. PD: Parkinson's disease; BM-MSCs: Bone marrow derived mesenchymal stem cells.

way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 14 followed by least significant difference (LSD) to compare significance between groups. Difference was considered significant when P value was < 0.05 .

Animal care and use statement

The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (25 °C, 12 h/12 h light/dark, 55% humidity, *ad libitum* access to food and water) for 2 wk prior to experimentation. The animals were deeply anaesthetized *via* diethyl ether for intravenous infusion of MSCs. Also, blood samples were collected from retro-orbital venous plexus under diethyl ether anaesthesia.

RESULTS

BM-MSCs homing

To confirm that the intravenously transplanted MSCs derived from male bone marrow migrate and home to the female injured brain, DNA was isolated from the brain tissues of female rats and the presence or absence of the responsible region for sex determination on Y chromosome (*SRY* gene) was assessed by PCR. The agarose gel demonstrated that *SRY* gene was present in the brain tissues obtained from the group of rats treated with BM-MSCs. While, *SRY* gene was absent in the brain tissues obtained from the ovariectomized control rats (Figure 1).

Effect of treatment with BM-MSCs on inflammatory markers

Since, TGF- β 1 has a pivotal role in the control of the transition between pro-inflammatory and anti-inflammatory response^[30] and MCP-1 has a vital role in the migration of inflammatory cells across the blood-brain barrier as well

Table 1 Effect of treatment with bone marrow derived mesenchymal stem cells on serum transforming growth factor beta-1 and monocyte chemoattractant protein-1 levels in Parkinson's disease model

	TGF- β 1 (pg/mL)	MCP-1 (pg/mL)
Ovariectomized control	481.5 \pm 7.5	88.1 \pm 0.9
PD untreated	691.2 \pm 8.0 ^a	112.1 \pm 1.9 ^a
PD + BM-MSCs	489.7 \pm 13.0 ^c	89.6 \pm 2.0 ^c

Data are represented as mean \pm SE of 8 rats/group. ^aSignificant change at $P < 0.05$ in comparison with the ovariectomized control group; ^cSignificant change at $P < 0.05$ in comparison with the untreated PD group. PD: Parkinson's disease; BM-MSCs: Bone marrow derived mesenchymal stem cells; TGF- β 1: Transforming growth factor beta-1; MCP-1: Monocyte chemoattractant protein-1.

as forms chemotactic gradients within the CNS to control the local inflammatory response^[31]. Serum TGF- β 1 and MCP-1 levels were determined by ELISA to evaluate the anti-inflammatory and immunomodulatory effects of the injected BM-MSCs in PD model.

Our data revealed that rotenone administration causes significant ($P < 0.05$) elevation in serum TGF- β 1 (43.6%) and MCP-1 (27.2%) levels vs the ovariectomized control group (Table 1). While, treatment with BM-MSCs elicits a significant ($P < 0.05$) reduction in both serum TGF- β 1 and MCP-1 levels by 29.2% and 20.1% respectively relative to the group of rats left untreated.

Effect of treatment with BM-MSCs on neurotrophic and neurogenic markers

Brain derived neurotrophic factor plays an important role in supporting the survival of existing neurons and encouraging the growth as well as differentiation of new neurons and synapses^[32]. Thusly, serum BDNF level was estimated by ELISA to evaluate the neurotrophic capacity of the injected BM-MSCs in PD model. In view of the data of the current work, rotenone administration experiences significant ($P < 0.05$) decline in serum BDNF level by 21.5% (Table 2) as compared to the ovariectomized control group. In contrast, treatment with BM-MSCs elevates serum BDNF level significantly ($P < 0.05$) by 26.1% (Table 2) relative to the group of rats left untreated.

Brain DA level was determined by a fluorometric method, while brain TH and nestin genes expression level was detected by sqRT-PCR to evaluate the neurogenic potential of the injected BM-MSCs in PD model. It is well known that DA is a neurotransmitter released by nerve cells to play crucial role in motor control, motivation, arousal, cognition and reward^[33]. Furthermore, TH enzyme catalyzes the conversion of L-tyrosine to L-3,4-dihydroxy-phenylalanine^[34]. While, nestin is one of the markers of neural precursors^[35]. The data of our work revealed that rotenone administration leads to significant ($P < 0.05$) depletion of brain DA level (32.1%) and significant ($P < 0.05$) down-regulation in the expression level of brain TH and nestin genes by 54.6% and 48.5% respectively (Table 2) as compared to the ovariectomized control group.

Table 2 Effect of treatment with bone marrow derived mesenchymal stem cells on serum brain derived neurotrophic factor and brain dopamine levels as well as brain tyrosine hydroxylase and nestin genes expression level in Parkinson's disease model

	BDNF (pg/mL)	DA (μ g/g tissue)	Relative expression of TH gene (TH/ β -actin)	Relative expression of nestin gene (nestin/ β -actin)
Ovariectomized control	3700 \pm 26.4	882 \pm 20.3	1.19 \pm 0.004	1.30 \pm 0.004
PD untreated	2905 \pm 72.9 ^a	599 \pm 9.8 ^a	0.54 \pm 0.009 ^a	0.67 \pm 0.006 ^a
PD + BM-MSCs	3663 \pm 17.8 ^c	874 \pm 15.0 ^c	1.18 \pm 0.004 ^c	1.29 \pm 0.005 ^c

Data are represented as mean \pm SE of 8 rats/group. ^aSignificant change at $P < 0.05$ in comparison with the ovariectomized control group; ^cSignificant change at $P < 0.05$ in comparison with the untreated PD group. PD: Parkinson's disease; BM-MSCs: Bone marrow derived mesenchymal stem cells; BDNF: Brain derived neurotrophic factor; DA: Dopamine; TH: Tyrosine hydroxylase.

Table 3 Effect of treatment with bone marrow derived mesenchymal stem cells on brain survivin expression in Parkinson's disease model

	Survivin (cell number)
Ovariectomized control	288 \pm 16.5
PD untreated	271.5 \pm 13.9
PD + BM-MSCs	293.2 \pm 15.9

Data are represented as mean \pm SE of 8 rats/group. PD: Parkinson's disease; BM-MSCs: Bone marrow derived mesenchymal stem cells.

However, treatment with BM-MSCs produces significant ($P < 0.05$) elevation in brain DA level by 45.9% and significant ($P < 0.05$) up-regulation in brain TH and nestin genes expression level by 122.2% and 92.5% respectively (Table 2) vs the group of rats left untreated.

Effect of treatment with BM-MSCs on anti-apoptotic marker

The anti-apoptotic action of the single intravenous dose of BM-MSCs in PD model was evaluated through the detection of brain survivin expression using immunohistochemical technique. As, survivin belongs to a family of endogenous cellular inhibitors of caspases that directly repress apoptotic cell death through interactions with pro-apoptotic caspases^[36]. In view of the current data, rotenone administration causes insignificant ($P > 0.05$) decrease in the number of positive cells for survivin expression by 5.7% (Table 3 and Figure 2B) relative to the ovariectomized control group. While, treatment with BM-MSCs produces insignificant ($P > 0.05$) increase in the number of positive cells for survivin expression by 8.0% (Table 3 and Figure 2C) in comparison with the group of rats left untreated.

Effect of treatment with BM-MSCs on brain structure

The brain section photomicrograph of ovariectomized control rat shows congestion in the blood vessels in striatum area (Figure 3A). While, brain section photomicrographs of untreated rotenone administered rat show congestion in the blood vessels and capillaries (Figure 3B) in the striatum as well as hyalinization and plaques formation in the matrix of the striatum indicating the occurrence of neurodegeneration (Figure 3C). Finally, the brain section photomicrograph of rotenone administered rat treated with BM-MSCs shows

intact histological structure of the striatum (Figure 3D).

DISCUSSION

MSCs have been considered as an effective tool for regenerative cell therapy. These cells could be isolated from both healthy and patient tissues and expanded *in vitro* on a therapeutic scale without posing significant ethical or procedural problems^[37]. Furthermore, it has been proposed that stem cells may replace lost cells by differentiating into functional neural tissue; provide source of trophic support for the diseased nervous system or alter the immune system to prevent further neurodegeneration^[38]. Therefore, the current study was planned to elucidate the mechanisms by which BM-MSCs could attenuate PD pathophysiology in the experimental model.

In consistent with Yoon *et al.*^[39] who found that intravenously transplanted BM-MSCs could migrate and home into the brain, the data presented in this work demonstrated that the intravenously transplanted MSCs were able to migrate to the site of injury (brain). The homing property afforded by MSCs was likely attributable to their broader expression of homing molecules^[40]. Furthermore, it has been reported that, chemokines released from tissue or endothelial cells may contribute to the activation of adhesion ligands, transendothelial migration, chemotaxis, and/or subsequent retention in surrounding tissue^[41].

In view of the data of the current work, rotenone administration for 14 d in ovariectomized rats elevated the level of serum TGF- β 1 and MCP-1 significantly. This finding is greatly supported by those of Rota *et al.*^[42] and Reale *et al.*^[43] who stated that both TGF- β 1 and MCP-1 levels are increased in several chronic neurodegenerative pathologies such as PD. It has been reported that the inflammatory response due to Parkinsonism is characterized by activation of microglia in the brain. The proposed explanation in regards to the reason of degeneration in dopaminergic neurons is that PD is caused by activation of microglial cells as a result of increased levels of cytokines^[44]. Activated microglia release a wide array of pro-inflammatory and cytotoxic factors as well as eicosanoids and nitric oxide^[45], which work in concert to develop neurodegeneration^[46]. Moreover, Gao *et al.*^[47] reported that the dopaminergic neurodegeneration enhanced by rotenone might be attributed primarily to

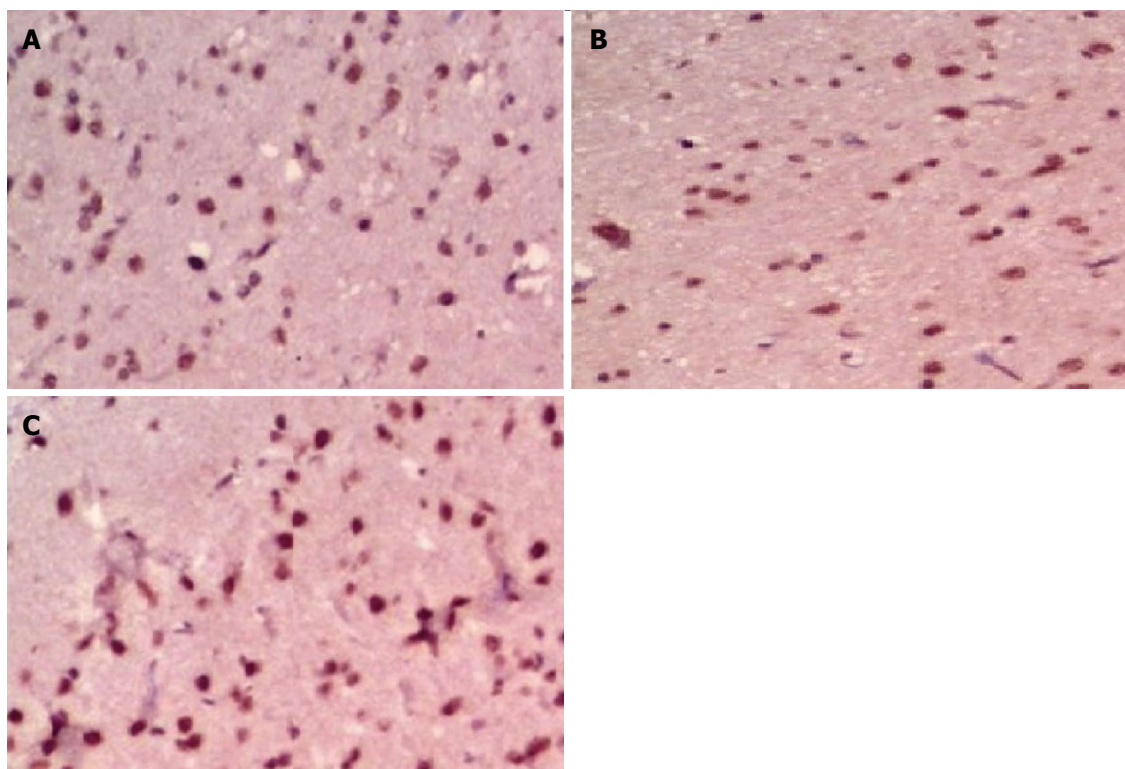


Figure 2 Immunohistochemical examination of survivin expression in Parkinson's disease model groups. A: Ovariectomized control; B: PD untreated; C: PD + BM-MSCs. PD: Parkinson's disease; BM-MSCs: Bone marrow derived mesenchymal stem cells.

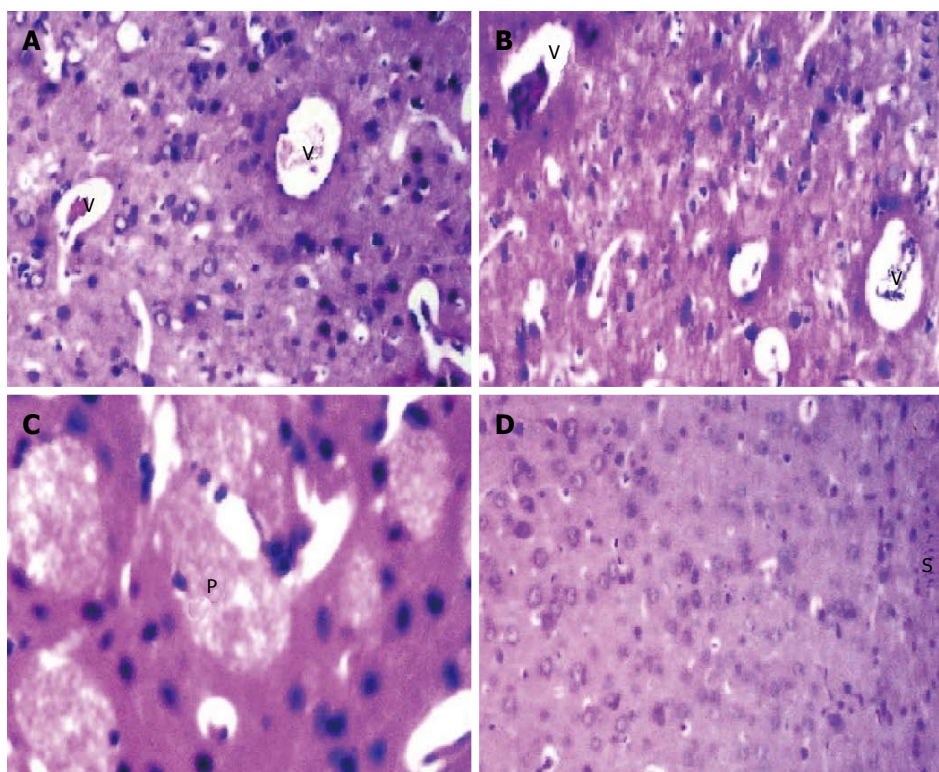


Figure 3 Photomicrograph of brain section of: A: Ovariectomized control group shows congestion in blood vessels of striatum (v) (H and E \times 80); B: untreated Parkinson's disease (PD) group shows congestion in blood vessels and capillaries of striatum (v) (H and E \times 80); C: Untreated PD: Parkinson's disease group shows hyalinization with plaques formation in the matrix of striatum (H and E \times 160); and D: PD group treated with bone marrow derived mesenchymal stem cells shows intact histological structure of the striatum (H and E \times 80).

the activation of microglia and consequently their release of superoxide free radicals that play an important role in

the inflammation mediated oxidative damage to neurons. This effect might be ascribed to the known susceptibility of dopaminergic neurons to oxidative stress as a result of reduced antioxidant capacity, high content of iron and DA, and possible defect in mitochondrial function^[48]. The release of cytokines from the brain into the peripheral blood supply through the blood brain barrier^[49] could explain the observed increase in serum TGF- β 1 and MCP-1 levels.

The results of the current study manifested that treatment with BM-MSCs lessen the level of serum TGF- β 1 and MCP-1 significantly. This finding is in great accordance with our previous work on adipose tissue derived MSC^[50] that proved its anti-inflammatory and immunomodulatory activities which are implicated in mitigating neuroinflammation characterizing PD. Accordingly, the observed role of BM-MSCs in depleting serum TGF- β 1 and MCP-1 levels could be allied to the ability of BM-MSCs to modulate microglia/macrophage activation including inflammatory responses as documented by Németh *et al.*^[51] and Choi *et al.*^[52].

Growing body of evidence indicates that there is a link between pro-inflammatory cytokines and neurotrophic factors in the CNS^[53]. It has been postulated that there is a balance between cytokine and neurotrophin in the brain and disruption of this balance cause injurious changes in the CNS^[54]. Moreover, Borchelt^[55] observed that astrocytes stimulated by mediators released from microglia down-regulate neurotrophic factors expression and release additional inflammatory mediators that in turn activate microglia. Parallel to these evidences, our results indicated that rotenone administration elicited significant decrease in serum BDNF level. This finding could be allied to the diminished level of brain BDNF due to inflammation. As, Klein *et al.*^[56] reported that BDNF level in the blood correlates with alteration in the level of BDNF in the brain.

In view of the current data, treatment with BM-MSCs experienced significant increase in serum BDNF level. This preferable effect could be related to the ability of MSCs to secrete BDNF as observed by Lattanzi *et al.*^[57] and Han *et al.*^[58]. Blandini *et al.*^[59] documented that MSCs have the ability to differentiate into glial cells that release diverse neurotrophic factors to provide protection against neurotoxin after their grafting into Parkinsonian rat brains. Additionally, there is an evidence that MSCs may modulate the expression of neurotrophic factors according to the environment in which they exist^[60,61].

The data presented in this work revealed that rotenone administration led to significant down-regulation in brain *TH* gene expression level in concomitant with significant decline in brain DA level. This observation could be ascribed to the dopaminergic degeneration^[62] due to elevated sensitivity of dopaminergic neurons to oxidative damage^[47] as well as inhibition of complex I activity and decrement of the mitochondrial membrane potential as a result of rotenone administration^[47,63].

Our previous findings indicated the neurotrophic and neuroprotective potentials of adipose tissue derived MSC

against neurodegenerative insult of PD^[50]. Similarly, the data of the present work demonstrated that treatment with BM-MSCs elicited significant increase in brain DA level as well as brain *TH* gene expression level. This finding comes in line with the study of Shetty *et al.*^[64] who demonstrated that BM-MSCs can be transdifferentiated efficiently into functional dopaminergic neurons capable of secreting DA and alleviating behavioral deficiencies. Moreover, the results of Bouchez *et al.*^[25] study showed that grafting of BM-MSCs caused an increase in the immunostaining of *TH* in striatum associated with elevation in the number of *TH*⁺ neurons in the substantia nigra pars compacta. Also, Blondheim *et al.*^[65] and Offen *et al.*^[66] stated that the transplantation of BM-MSCs into the animal model induced with 6-hydroxydopamine resulting in an increase in the level of *TH* in the striatal region thus improving motor behavior in a mouse model of PD. Since, *TH* is the rate-limiting enzyme in DA synthesis, the increase in the level of *TH* would increase the production of DA. Additionally, the observed increase in brain DA content and *TH* expression level as a result of treatment with BM-MSCs could be explained by the ability of MSCs to secrete a wide array of cytokines and growth factors, including BDNF^[57] which exert neurotrophic and neuroprotective effects on DA neurons^[67]. Furthermore, Trzaska *et al.*^[68] reported that BDNF has a crucial role in the functional maturation of MSC-derived DA progenitors.

In line with previous studies reported by Höglinger *et al.*^[69] and Abdipranoto *et al.*^[70], the current study manifested that rotenone administration caused significant down-regulation in brain nestin gene expression level. This finding could be imputed to the depletion in DA level due to degeneration of dopaminergic neurons as documented by Crews *et al.*^[71]. In contrast, treatment with BM-MSCs induced significant up-regulation in nestin gene expression level. Bouchez *et al.*^[25] found that rat MSCs express neuronal proteins such as nestin at the RNA and protein levels. Moreover, the study of Ye *et al.*^[72] indicated the presence of nestin positive cells in brain tissue of PD rat after transplantation of undifferentiated BM-MSCs. The suggested mechanism by which BM-MSCs treat PD rat model could be related to that transplanted BM-MSCs might become nestin-positive stem cells that differentiate into astrocytes or other non-dopaminergic neurons and participate in the reconstruction of dopaminergic neurons circuits^[72].

The data of this work revealed that rotenone administration produced slight decrease in the number of positive cells for survivin expression. This finding harmonizes with that of Zhang *et al.*^[73] who reported that degenerating neurons lacked survivin expression. Jiang *et al.*^[74] results showed that survivin is critically required for the survival of developing CNS neurons. Moreover, Zhang *et al.*^[75] suggested that there is a connection between the expression of survivin and adult neurogenesis. Thus, the observed decrement in survivin expression might be attributed to the decreased neurogenesis due to DA depletion^[71]. Another possible mechanism by which rotenone could decrease survivin expression might be related to its effect on p53

which was shown to be over expressed by rotenone^[76]. Under normal conditions, p53 protein levels are low and regulated by I κ B kinase (IKK) and prominently by mouse double minute 2 (Mdm2), an ubiquitin ligase responsible for p53 degradation. Cellular stress reduces the interaction between p53 and Mdm2 leading to accumulation of the former^[77]. Wu *et al.*^[76] reported that the degeneration of dopaminergic neurons by rotenone was accompanied by an increase in p53 protein level which in turn induces p21 expression. Then, the increased level of p21 suppresses the expression of cyclin dependent kinases leading to accumulation of hypophosphorylated retinoblastoma that interact with E2F (a transcriptional activator) to repress survivin expression^[78].

In the light of our results, treatment with BM-MSCs caused insignificant increase in the number of positive cells for survivin expression. This increment is in agreement with Okazaki *et al.*^[79] and it could be imputed to the ability of MSCs to enhance neurogenesis and inhibit apoptosis through their secreted BDNF as documented by Ye *et al.*^[72]. Moreover, Kim *et al.*^[80] reported that grafted MSCs attenuate dopaminergic neuronal loss through their anti-apoptotic effects. Also, the increase in survivin expression by MSCs treatment might be related to their inhibitory action on P53 through the inactivation of ERK1/2^[81].

In view of the histopathological investigations of brain tissues section of the current work, rotenone administration resulted in congestion in the blood vessels and capillaries of striatum. Also, there were hyalinization and plaques formation in the matrix of striatum indicating the occurrence of neurodegeneration. Sai *et al.*^[82] demonstrated that rotenone causes dopaminergic neurons degeneration *in vivo* and substantia nigra pars compacta and striatum are the main targets of rotenone in the rat brain. These findings could be allied to the inhibition of neuronal mitochondrial complex I activity^[47] and consequently oxidative damage^[83] as a result of rotenone administration.

Brain tissue sections examination indicated that single infusion with BM-MSCs resulted in intact histological structure of the striatum. This finding coincides with Dezawa *et al.*^[84] who reported that nerve system recovery after BM-MSCs transplantation could be related to their secretion of neurotrophic factors that restore the function of nervous system, promotion of local angiogenesis and vascular reconstruction and neuronal regeneration through promotion of autologous neuronal regeneration and differentiation of transplanted cells into neural cells.

In conclusion, the current study provided experimental evidences for the ability of BM-MSCs to mitigate PD pathophysiology through multi-mechanistic approaches (immunomodulatory, anti-inflammatory and anti-apoptotic effects as well as neurotrophic and neurogenic potentials). These promising results pave the way for the clinical trial application of MSCs in the treatment of neurodegenerative diseases particularly PD.

COMMENTS

Background

Parkinson's disease (PD) is one of the neurodegenerative diseases,

accompanied by extrapyramidal motor dysfunction due to the progressive and selective loss of dopaminergic neurons in the substantia nigra pars compacta and declining levels of dopamine in the striatum. So, it is very important to stop or halt neurodegeneration. However, to date, there is no therapy clinically available that delays the neurodegenerative process itself, therefore modification of the disease course is an important unmet clinical need. Transplantation of mesenchymal stem cells (MSCs) for treating neurodegenerative disorders has received growing attention recently because these cells are readily available, easily expanded in culture, and when transplanted survive for relatively long periods of time.

Research frontiers

MSCs are a heterogeneous subset of stromal stem cells that have the ability of self-renewal and multipotency. In the area of neurodegenerative disorders treatment, the current research hotspot is how to modify the disease course by specifically target the pathophysiologic cascade, hoping to delay the onset of the disease and slow its progression.

Innovations and breakthroughs

Modern research has focused on discovering effective disease-modifying therapies, which specifically target the pathophysiologic cascade, hoping to delay the onset of the disease and slow its progression. The study provided a non invasive approach for mitigating PD pathophysiology via bone marrow derived MSCs (BM-MSCs) transplantation which has immunomodulatory, anti-inflammatory and anti-apoptotic effects as well as neurotrophic and neurogenic potentials.

Applications

The study results shed light on the therapeutic potential of BM-MSCs against PD pathophysiology via multi-mechanistic actions.

Terminology

PD is the second most common neurodegenerative disease, accompanied by extrapyramidal motor dysfunction which resulting from the progressive and selective loss of dopaminergic neurons in the substantia nigra pars compacta and declining levels of dopamine in the striatum. MSCs are a heterogeneous subset of stromal stem cells that have the ability of self-renewal and multipotency, which could differentiate into cells of the mesodermal lineages and other embryonic lineages, including adipocytes, osteocytes, chondrocytes, hepatocytes, neurons, muscle cells, epithelial cells, etc.

Peer-review

This article is well written, clearly demonstrating the therapeutic effect of BM-MSCs for the treatment of PD. Authors also presented the molecular basis for the amelioration of PD pathology by showing decrements and increments in inflammatory mediators and neurotrophic factors in the serum, respectively. The overall data presented in this manuscript are sound.

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Human induced pluripotent stem cells for monogenic disease modelling and therapy

Paola Spitalieri, Valentina Rosa Talarico, Michela Murdocca, Giuseppe Novelli, Federica Sangiuolo

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Abstract

Recent and advanced protocols are now available to

derive human induced pluripotent stem cells (hiPSCs) from patients affected by genetic diseases. No curative treatments are available for many of these diseases; thus, hiPSCs represent a major impact on patient health. hiPSCs represent a valid model for the *in vitro* study of monogenic diseases, together with a better comprehension of the pathogenic mechanisms of the pathology, for both cell and gene therapy protocol applications. Moreover, these pluripotent cells represent a good opportunity to test innovative pharmacological treatments focused on evaluating the efficacy and toxicity of novel drugs. Today, innovative gene therapy protocols, especially gene editing-based, are being developed, allowing the use of these cells not only as *in vitro* disease models but also as an unlimited source of cells useful for tissue regeneration and regenerative medicine, eluding ethical and immune rejection problems. In this review, we will provide an up-to-date of modelling monogenic disease by using hiPSCs and the ultimate applications of these *in vitro* models for cell therapy. We consider and summarize some peculiar aspects such as the type of parental cells used for reprogramming, the methods currently used to induce the transcription of the reprogramming factors, and the type of iPSC-derived differentiated cells, relating them to the genetic basis of diseases and to their inheritance model.

Key words: Human induced pluripotent stem cells; Gene therapy; Monogenic diseases; Gene editing; Foetal cells; Reprogramming techniques; Differentiation

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Core tip: With the development of human induced pluripotent stem cells (hiPSCs) deriving from patients, we can begin to understand the molecular mechanisms underlying monogenic diseases and consequently identify new drugs for their treatment. hiPSCs can differentiate into many disease-relevant cell types, providing in this way to innovative applications in the field of cell replacement therapy, disease modelling, drug testing and

drug discovery.

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INTRODUCTION

For many years the research and experimentation on stem cells have been made, taking advantage of their extraordinary ability to divide and self-renew into undifferentiated cells. Pluripotent stem cells are able to differentiate into all cell types. These characteristics offer the possibility of different applications from the use of stem cells for model disease, for cell therapy and tissue regeneration and pharmacological and toxicological tests.

Researchers have always worked with both embryonic and adult stem cells for the study of disease and for gene therapy. Despite the unique characteristic of embryonic stem cells (ESCs), there are controversies moral, ethical and legal regarding their use.

Adult stem cells have limited differentiation potentiality, so this aspect reduces the options for their use.

Thus, induced pluripotent stem cells (iPSCs), derived from somatic cells, have equal characteristics of ESCs. It is possible reprogramming cells from patients with human diseases that reproduce a model of disease *in vitro* and summarize the pathological phenotypes and the etiopathology of the diseases. So, their use allows the development of innovative therapies, drug screening and toxicological testing^[1-3].

For some genetic diseases no therapeutic treatment is available and the animal model does not always fully possess the variability of the disease. In addition, the understanding of the pathogenetic mechanism at the base of the disease is slow.

The ultimate goal of reprogramming is the transplantation of progenitor cell, genetically corrected *in vitro* before transplantation, derived from a patient-specific human induced pluripotent stem cells (hiPSCs). These cells will not trigger an immune response, avoid tumour formation and recover the target-damaged tissue.

In 2007, iPSCs were obtained from human fibroblasts by manipulation and expression of genes involved in dedifferentiation and in the maintenance of "stemness"^[4,5]. Reprogramming somatic cells using the defined OCT4, SOX2, KLF4, and c-MYC (OSKM) factors led Yamanaka S and Gurdon JB to win the Nobel Prize in Physiology or Medicine in 2012. Thomson's group follow-up research produced iPSCs using NANOG and LIN28 instead of KLF4 and c-MYC^[5] and later, many other researchers developed alternative methods of reprogramming^[6].

The most commonly used method is the use of viral transduction of defined factors to somatic cells.

Lentiviral-based systems, for example, are the most efficient and reproducible, driving the integration of the reprogramming factors. Unfortunately, viral-based disease models still bear the risks of oncogene reactivation, insertional mutagenesis, immunogenicity, reactivation of reprogramming genes or their uncontrollable silencing, making them unacceptable for human clinical applications. In terms of the aspect of safety of reprogramming, various alternative approaches of gene delivery have been developed. Instead of integrating vectors^[1], plasmids^[7], Cre/loxP system^[8], piggy Bac vectors^[9], and minicircle vectors have been investigated^[10], in order to partially prevent transgene integrations and in the same time to simplify the methods to obtain cell reprogramming^[6].

Current studies have successfully reported the generation of transgene-free iPSCs using different approach, such as: Protein transduction^[11], non-integrating viral vectors such as: The Sendai virus^[12], episomal vectors^[13], transfection of modified mRNA transcripts^[14], and chemicals^[15]. Nevertheless, when using protein as inducing factor for reprogramming the efficiency is lower (approximately 0.001).

A modified mRNA-based strategy is currently being explored to produce transgene-free iPSCs^[16,17]. Other methods dealing with small molecules have also been reported to enhance the performance of iPSCs derivation^[18-25]. Similarly human telomerase reverse transcriptase (hTERT), P53 siRNA and Simian Vacuolating Virus 40 large T (SV40LT) Antigen successfully stimulate the reprogramming kinetics^[26,27]. Some others, like Estrogen related receptor β (Esrrb), Utf1, Lin28, and developmental pluripotency-associated 2 (Dppa2) generate iPSCs without of OSKM factors with single-cell level identification of reprogramming events^[28].

The typical yields of iPSCs production by the methods aforesaid range from 0.01%-5%, depending on the target cell and reprogramming system. Rais *et al.*^[29] reported the reprogramming efficiency of methyl-binding protein 3 deletion that reached up to nearly 100% within a few days, supporting that iPSCs reprogramming represents a deterministic process.

One factor strongly influencing the efficiency of reprogramming is the type of cell used as target. This choice can depend on the amount of DNA methylation, gene expression and stability of the pluripotent phenotype, as well as the epigenetic memory of the cell type. In addition, the gene delivery methods, and culture conditions as well as the transcription factors combination might also be the reason of the differences between the various iPS cell populations created. At least, several uncontrollable stochastic events can influence the success of the reprogramming^[30].

For all these reasons, researchers have studied the "best candidate parental population" to create iPSCs for *in vitro* investigations and eventual clinical trials^[31]. Fibroblasts are the main source of iPSCs, although other sources for iPSCs have been reported, like hepatocytes and mature B cells^[32]. To better understand

developments in personalized medicine, we will focus our review on the production and application of human iPSCs derived from foetal tissues, highlighting their higher responsiveness during the reprogramming.

Stem cells have been identified in several foetal tissues and from amniotic fluid, umbilical cord blood, and placenta at term^[33-35]. These cells have mesenchymal origin and they are capable of self-renewal and differentiation into multiple tissue types^[36-39]. According to their tissue derivation and to the gestational age, the heterogeneity of foetal stem cell populations is emblematic, in agreement to their phenotypic characteristic, properties and cell marker expression.

HUMAN FOETAL TISSUES

Human trophoblast stem cells

Trophoblast cells form the foetal part of the placenta. The placenta is an organ indispensable for the growth and survival of the developing embryo. The placenta is constituted by different trophoblast cell types aimed for embryo implantation, for vascular connection to the maternal circulation and nutrition of the fetus and immunological adaptation. Trophoblast cells derive from the trophoectoderm, which gives rise to both attachment and implantation of the embryo. The trophoectoderm is composed of floating and anchored villi and their specialized cell types, the syncytium and the cytotrophoblast. For the first 3 wk of pregnancy, it represents a continuously renewing epithelium^[40]. During gestation, the trophoblast changes morphologically and functionally. In particular, the cytotrophoblast, a self-renewing population located in the proliferation zone, divides continuously and fuses to form syncytiotrophoblasts, in which some authors report the presence of possible stem cells important for its renewal^[41,42]. Spitalieri *et al.*^[39] has isolated and characterized a subpopulation of multipotent cells, named human cytotrophoblastic-derived multipotent cells (hCTMCs) obtained from human chorionic villus sampling (hCVSs) with characteristics that are "intermediate" between mesenchymal and pluripotent stem cells. These cells express stem cell markers, such as ALP, SSEA4, OCT-4, CD117, NANOG, and SOX2. Also, these cells are capable of generate *in vitro* cells belonging to ectoderm, mesoderm and endoderm layers, but, if inoculated into Nod/SCID mice, they are unable to form teratomas. If injected into mouse blastocysts, hCTMCs are integrated and could be tracked into various tissues of the adult chimeric mice. These cells may be also a promising target for gene editing approaches, such as small fragment homologous recombination, as we report for an *in vitro* genetic modification of *SMN* gene in a fetus affected by spinal muscular atrophy (SMA). They can be genetically edited with high performance, allowing an innovative therapeutic approach to cure genetic defects^[39,43,44].

Human amniotic fluid stem cells

During amniocentesis, at the sixteenth week of gestation, small amount of fluid carry hAFSCs. Stem cells are

present also in amniotic fluid at term (from routine caesarean deliveries). Kaviani *et al.*^[45] collected up to 20000 cells from 2 mL of amniotic fluid, 80% of which are having the ability to grow. Membrane receptor c-kit (CD117), marker of stemness, is expressed on ESCs^[46], primordial germ cells and many others somatic stem cells, including a sort multipotent subpopulation of human amniotic fluid stem cells (hAFSCs) (round 1%)^[47]. Moreover, hAFSCs show a high telomerase activity with a self-renewal capacity, and display normal G1 and G2 cell cycle checkpoints. On top, at late passages they maintain a normal karyotype. Like ESCs, hAFSCs are capable of differentiating into all three germ layers, their efficiency depends on the gestational age^[33,48-50]. But, hAFSCs are unable to form teratoma, when inoculated into immunodeficient mice *in vivo*. It is possible, also, to use hAFSCs in a technique involving retrovirally tagged cells, because they have a high clonal capacity.

Amnion epithelial cells

From a single term amnion membrane it is possible isolate a multipotent epithelial cell population and obtain approximately 120 million viable epithelial cells^[51-53]. hAECs possess ability of multipotent differentiation^[54], low immunogenicity^[55] and anti-inflammatory functions^[56].

Therefore, as reported in the literature, both hCVSs and hAFSCs are heterogeneous populations composed by several stem/progenitor lineages^[57]. In response to external stimuli, they modulate gene and protein expression for their high plasticity, as already published^[58]. Moreover foetal stem cells have an higher proliferative capacity if compared to adult cells. Again, congenital malformations or genetic diseases in newborns could be treated thanks to the capacity to separate pluripotent autogenic progenitor cells during pregnancy^[59]. Only recently have these cells, traditionally used for prenatal diagnosis, been explored for their stemness and for reprogramming efficiency^[3,60].

HiPSCs vs hESC

When compared with embryonic cells, iPSCs differentiate less efficiently into specialized cell lines, due to their "molecular identity"^[61]. Moreover, some iPSCs have a greater capacity to silence some genes demonstrated to be required for foetal development and differentiation^[62]. These differences constitute an active area of research that still requires a direct comparison of the pluripotency of hiPSCs vs hESCs. An explanatory situation is represented by the *FMR1* gene, involved in Fragile X syndrome (#300624), a genetic condition characterized by learning disabilities and cognitive impairment. The protein product is necessary for normal brain development. In Fragile X syndrome the *FMR1* gene acquires a silencing mutation. While this gene functions normally in human embryonic cells and becomes silenced as the cells differentiate, in hiPSCs remains inactive^[63].

Several studies suggest that cells undergoing reprogramming go through an intermediary state *via* resetting of the epigenetic landscape, whereby *c-MYC* and *KLF4*

are initially required to prime the cells that are then driven towards pluripotency by *OCT4* and *SOX2*. These observations open interesting scenarios for further investigations focused to discover methods to directly create progenitor therapeutic cell types from somatic cells^[6], bypassing the pluripotency step.

Thus, iPSCs that conserve genomic stability and free from any integrated agents represent an important aim for therapeutic uses. Recent studies suggest the use of a high-resolution method, such as the Affymetrix Cytoscan HD array (Affymetrix, Santa Clara, CA, United States), for monitoring genomic alterations throughout iPSC preparation to preserve clinical applications^[64].

Given the high level of manipulation and the lack of knowledge about their role *in vivo*, the use of hiPSCs in human trials is complex. Whether hiPSCs will prove an useful substitute for hESCs has yet to be determined, hESCs are still considered the gold standard for embryonic cell lines^[65].

At the same time, hiPSCs represent an ideal autologous cellular model for the study and the treatment of diseases, reaching the goal of personalized medicine. The field of personalized medicine is based on the idea that life is variable and that individuals behave differently from each other under disease conditions. By taking into account individual clinical, genetic and environmental information, personalized medicine optimizes medical care and outcomes for customized disease prevention, detection and treatment.

hiPSCs coincide perfectly with the concept of personalized medicine for disease modelling and further clinical application. In fact, hiPSCs bypass the limitation of immune rejection, being patient-specific cells, united to a "rejuvenation" of telomere length during reprogramming^[66], epigenetic memory and functional properties, offering enormous clinical potential.

The tumorigenic risk of hiPSCs arising from the use of integrating vectors for their derivation supports the use of integrating vectors that can be subsequently removed from the genome. Sommer *et al.*^[67], for example, reports the use of the human STEMCCA excisable polycistronic lentiviral vector^[3]. The delivery vectors are designed so that the ectopic genes are flanked by *loxP* sites, thus enabling their removal by transient Cre protein expression^[68,69]. This approach generates hiPSCs free of transgenic sequences that can improve and increase the safety of derivation methods.

HIPSCS-BASED MONOGENIC DISEASE MODELS

Due to ethical and technical challenges, human embryonic stem cells (hESCs) can represent unsuitable candidates for disease modelling. Therefore, hiPSCs, closely resembling the key features of hESCs such as self-renewal and pluripotent potentials, can be extensively exploited to study various inherited disorders.

Dimos *et al.*^[70] (2008) and Park *et al.*^[71] (2008) reported

for the first time disease-specific iPSC lines in 2008, mimicking human disease. This new strategy consists of screening patients for genetic mutations, isolating cell lines, returning them to iPSCs, and finally differentiating the iPSCs into one or more cell types phenotypically developing the disease.

The possibility of dedifferentiating patient-specific cells back to stem cells and again to differentiate them into cells representative of the disease organ or tissue, allows one to faithfully replicate the key aspects of the disease in a "petri dish" and to quantify disease phenotype in different tissues (Figure 1). Each of these points is difficult to obtain; however, many disorders have been successfully recapitulated *in vitro* contributing to research in the field of disease modelling. Most of these studies have focused on monogenic disorders that exhibit strong phenotypes *in vitro*. Moreover, obtaining pluripotent cells from patients with developmental or degenerative disorders also allows for new opportunities for drug discovery^[72]. When differentiated *in vitro* into relevant somatic lineages, hiPSCs are used both for assessing personalized pharmacological therapy and for *in vivo* cell therapy.

Therefore, iPSCs-disease models mimic human pathological development rather than trials utilizing conventional rodent and cell lines^[73]. Human cell culture assists the research using animal models of disease. Murine models of human inherited and acquired diseases are helpful systems but human pathophysiology cannot always be faithfully reproduced. When murine and human physiology are different, disease-specific pluripotent cells able of differentiation into the several cells affected establish disease pathophysiology, allowing *in vitro* investigation in a human tool monitored and proving a large number of genetically-modifiable cells in a specific manner for each genetic defects.

The main advantages of iPSCs-based model systems are: (1) iPSCs can be obtained from several sources (adult somatic cells, embryonic/foetal cells, adult stem cells and cancer cells); (2) iPSCs naturally maintain the genetic background; (3) iPSCs have the ability to differentiate into any desired cell types *in vitro*; (4) iPSCs can self-renew and maintain their undifferentiated state and pluripotent capacity; and (5) iPSCs resume early human embryo development during differentiation *in vitro*.

For all these reasons, iPSCs represent an available model system for studying the pathogenetic mechanisms of various diseases, particularly in those cases where animal models do not exactly reproduce human phenotype or when disease-target cells types are not available for research.

This review provides an overview of the current state of modelling monogenic disease by using hiPSCs and the ultimate applications of these *in vitro* models for cell therapy. We consider and summarize some peculiar aspects such as the type of parental cell used for reprogramming, the methods currently used to induce expression of the reprogramming factors, and the type of iPSC-derived differentiated cells, relating them to the

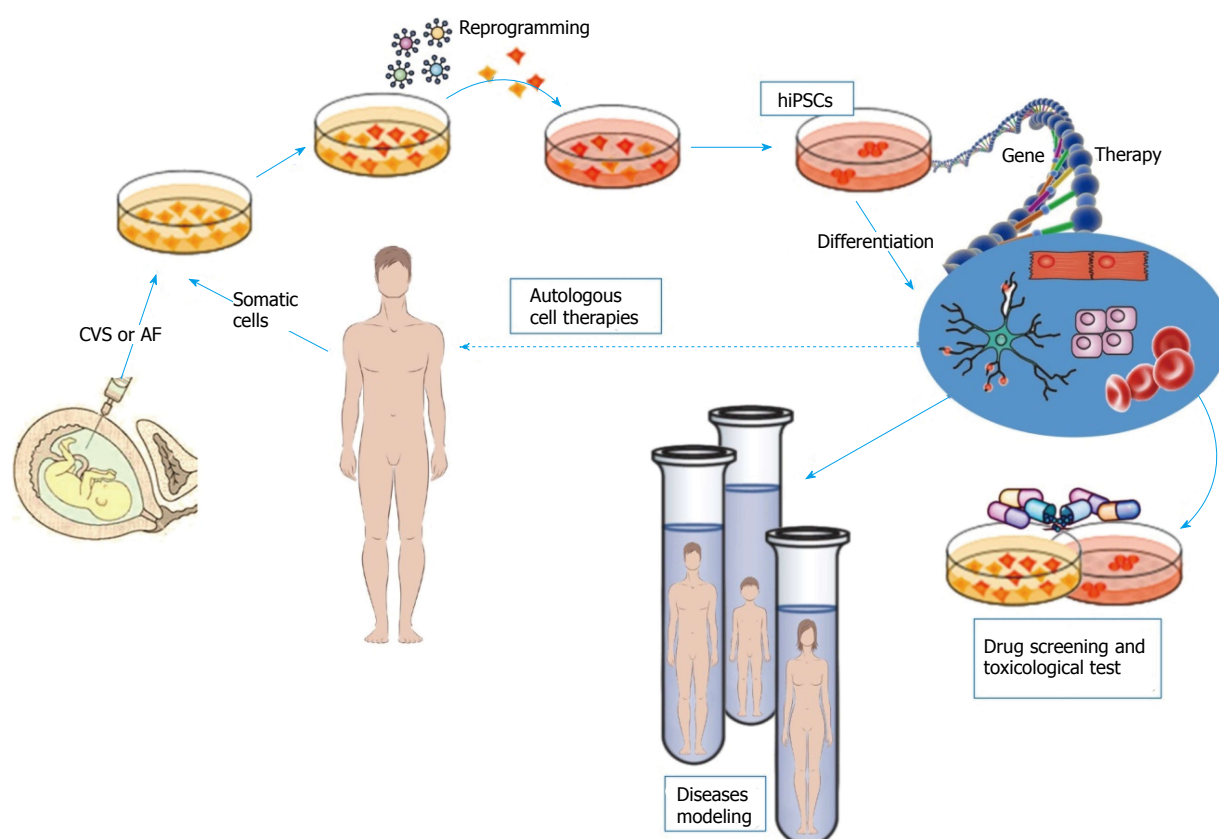


Figure 1 Representation of the workflow for the derivation of patient-specific induced stem cells from foetal or somatic cells and successively their use for the development of personalized therapy protocols. CVS: Chorionic villus sampling; AF: Amniotic fluid; hiPSCs: Human induced pluripotent stem cells.

genetic basis of diseases and to their inheritance model (Tables 1-3).

hiPSCs for modelling autosomal dominant disorders

Huntington's disease (HD; OMIM #143100) is an autosomal dominant neurodegenerative disorder that shows up later in life. It is caused by an expansion of the "CAG" triplets repeated in exon 1 of the *HTT* gene. The protein encoded by *HTT* gene is expressed in many tissues and organs, especially in the brain and testis^[74-77]. The normal function of *HTT* is still not fully identified as it differs from other known proteins.

HD is known as a neurological disease; however, peripheral HD-associated pathologies, such as cardiac defect and skeletal muscle malfunction, have also been described. The generation of HD-iPSC lines can facilitate the match of the affected phenotypes in "dish" with clinical discoveries in sick individual of their families, highlighting the genetic basis and molecular mechanism leading to the development of the HD disease^[78]. For this purpose, the HD-iPSC consortium recently promoted and defined an *in vitro* model of HD based on the creation of iPSCs and established by multiple lines, clones and repeat lengths, exhibiting that the clear association between the extension of the CAG repeats and the clinical pathology severity observed in HD patients could also be reproduced *in vitro* by HD-iPSC differentiated into neurons^[79].

Juopperi *et al.*^[80] generated an *in vitro* system deriving patient-specific iPSCs to study HD pathogenesis. Thanks to this model, they were able to describe a specific vacuolation phenotype in iPSCs-derived astrocytes. The same characteristics were previously observed in primary lymphocytes derived from HD patients^[80]. This study opens up new potential investigations using human iPSCs for model HD and for therapeutic drug screening. iPSC lines obtained from two homozygous individuals bringing 42/44 and 39/42 CAG repeats and from one heterozygote having 17/45 CAG repeats show a lysosomal activity increased when cultured *in vitro* and when differentiated in neurons^[81]. The sizes of the CAG repeats persisted constant during the period of culture. On the other hand, another HD-iPSC line carrying 72 CAG repeats had no phenotype when cultured *in vitro* both in undifferentiated state and in neural precursors. However, the HD-iPSCs display manifestation of HD disorder under condition of oxidative stress^[82], or proteasome inhibitors or if injected into neonatal brains for 33 wk^[83]. Increased caspase activation in HD-iPSCs is also observed in iPSCs-derived neurons^[84]. Thus, HD-iPSCs recapitulate the disease phenotype and represent an available tool to study HD and to develop novel therapeutics.

Marfan syndrome (MFS; OMIM #154700) is an autosomal dominant hereditary disorder of connective tissue strongly involving skeletal, ocular, and cardiovascular systems^[85,86]. The mutated gene responsible for MFS is

Table 1 Models of monogenic dominant diseases

Diseases	Genetic defects	Target cells for reprogramming	Delivery methods	Disease relevant cells	Ref.
Huntington's disease (OMIM #143100)	Expanded CAG repeat in <i>HTT</i> gene	HF	Lentiviral vectors (O, S, K, M, NANOG and LIN28)	Neurons	[79]
		HF	Retroviral vectors (O, S, K, M)	Astrocytes	[80]
		HF	Lentiviral vectors (O, S, K, M)	Neurons	[81]
		HF	Retroviral vectors (O, S, K, M)	Neurons	[82]
		HF	Retroviral vectors (O, S, K, M)	GABAergic neurons	[83]
		HF	Retroviral vectors (O, S, K, M)	Striatal neurons	[84]
Marfan syndrome (OMIM #154700)	<i>FBN1</i> mutations	HF	Retroviral vectors (O, S, K, M)	Osteogenic cells	[87]
Myotonic dystrophy type 1 (OMIM #160900)	Expanded CTG repeat in <i>DMPK</i> gene	HF	Retroviral vectors (O, S, K, M)	NS	[92]
		HF	Retroviral vectors (O, S, K, M)	NSC	[94]
Achondroplasia (OMIM #100800)	<i>FGFR3</i> mutations	HF	Episomal plasmid vectors (O, S, K, M, LIN28 and p53 shRNA)	Chondrocytes	[97]
Familial hypercholesterolemia (OMIM #143890)	<i>LDLR</i> mutations	HF	Retroviral vectors (O, S, K, M)	HLC	[99]
		HF	Lentiviral vectors (O, S, NANOG, LIN28)	Hepatocytes	[100]
Timothy syndrome (OMIM #601005)	<i>CACNA1C</i> mutations	HF	Retroviral vectors (O, S, K, M)	HLC	[101]
		HF	Retroviral vectors (O, S, K, M)	Cardiac myocytes	[102]
		HF	Retroviral vectors (O, S, K, M)	Cortical neuronal precursor cells and neurons	[103]
		HF	Retroviral vectors (O, S, K, M)	Cortical neuronal precursor cells and neurons	[104]

NS: Neurosphere; NSC: Neural stem cell; HLC: Hepatocyte-like cells; HF: Human fibroblasts; O: OCT4; S: SOX2; K: KLF4; M: C-MYC.

located on chromosome 15 and encodes for fibrillin-1 (*FBN1*).

Originally, the fibrous connective tissue disorders of MFS were attributed to structural weakness of the fibrillin-rich extracellular matrix. Increased bioavailability of TGF β is associated with the pathological signs, indicating the resemblance to MFS-related disorders. In fact, mesenchymal cells, derived from both MFS iPSCs and ESCs differentiate spontaneously into chondrogenic cells contrary to wild type iPSC/ESC-derived mesenchymal cells that need exogenous TGF β chondrocytes, demonstrating an alteration of TGF β signaling in MFS cells^[87]. This model is in agreement with the skeletal manifestations of MFS and increases the knowledge of molecular mechanisms underlying the pathogenesis of abnormal skeletogenesis in human diseases caused by mutations in *FBN1*.

The use of the iPSC technology permits the reprogramming of MFS adult fibroblasts containing different *FBN1* mutations, allowing the clearance of the mechanisms underlying the pathological variability, and shows the benefit of personalized therapeutic interventions.

Myotonic dystrophy type 1 (DM1; or Steinert's disease, OMIM #160900) is the most common muscular dystrophy in adults^[88]. DM segregates as an autosomal dominant pathology and is caused by the expansion of a CTG repeat located within the 3'-untranslated region of the dystrophin myotonia protein kinase (*DMPK*) gene on chromosome 19q13.3^[89]. In the classic form, the major features include myotonia, muscle weakness and wasting, cardiomyopathy with conduction defects, insulin-resistance, frontal balding, cataracts and disease-specific serological abnormalities^[90]. hiPSCs offer the possibility to study unstable repeat expansions by generating a model

disease and disease-impaired cells in culture. These aspects are helpful in order to investigate unstable repeat pathologies^[91].

In particular, DM1 patient-derived iPSCs could be an ideal model to study triplet-repeat instability. Du *et al.*^[92] first generated iPSCs from DM1 patient fibroblasts and detected CTG.CAG triplet repeats in each iPSC clone. Homogeneous lengths of CTG.CAG triplet repeats in each iPSC clone allows for the study of the mechanisms of repeat expansion, and offers knowledge of a general mechanism of triplet-repeat expansion in iPSCs^[92].

Recently, Xia *et al.*^[93,94] reported neural stem cells (NSCs) derived from iPSCs of DM1 patients, a helpful device for the study of DM1-NSCs neuropathogenesis. Both DM1 iPSCs and iPSC-derived NSCs show the presence of nuclear RNA foci, representing a molecular hallmark of disease, allowing them to be used as cellular models to understand the dynamic changes of RNA foci during the cell cycles^[93,94].

Achondroplasia (ACH; OMIM #100800) is the most common skeletal dysplasia, with disproportionate short-limb dwarfism. The mutated gene encodes for fibroblast growth factor receptor 3 (*FGFR3*)^[95,96]. The study of skeletal dysplasia, as well as many other diseases, exploits the development of iPSCs technology.

Yamashita *et al.*^[97] demonstrated that the chondrogenically differentiated ACH-hiPSCs adequately recapitulate the primary abnormalities found in *FGFR3*-related disease patients. These cells manifested lower proliferation and higher apoptosis when differentiated into chondrocytes^[97]. Thus, hiPSCs technology is instrumental in investigating the effects of several therapeutic molecules, including statins, on ACH iPSCs-derived chondrocytes^[98] (Table 1).

Table 2 Models of monogenic recessive diseases

Diseases	Genetic defects	Target cells for reprogramming	Delivery methods	Disease relevant cells	Ref.
Spinal muscular atrophy (OMIM #253300)	<i>SMN1</i> mutations	HF	Lentiviral vectors (O, S, NANOG, LIN28)	Neurons/astrocytes/motor neurons	[109]
		HF	Retroviral vectors (O, S, K, M)	Motor neurons	[110]
		HF	Lentiviral vectors (O, S, NANOG, LIN28)	Motor neurons	[112]
		HF	Retroviral vectors (O, S, K, M)	GABAergic neurons	[113]
β -thalassaemia (OMIM #613985)	Point mutations or deletions in the β -globin (<i>HBB</i>) gene	HF/AF/CVS	Retroviral vectors (O, S, K, M)	Hematopoietic cells	[116]
		AF	Lentiviral vectors (O, S, K, M)	Hematopoietic cells	[118]
		HF/MSCs	Lentiviral vectors (O, S, K, M)	Erythroid cells	[119]
		HF	PiggyBac transposon	Hematopoietic cells	[120]
Cystic fibrosis (OMIM #219700)	<i>CFTR</i> mutations	HF	Lentiviral vectors (O, S, K, M)	Mature airway epithelial cells	[126]
		HF	Modified RNAs (iPSC)	Mature airway epithelial cells	[127]
		HF	Retroviral vectors (O, S, K, M)	Mature airway epithelial cells	[128]
		HF	Retroviral vectors (O, S, K, M)	CLCs	[130]
Sickle cell disease (OMIM #603903)	<i>HBB</i> mutations	HF	Retroviral vectors (O, S, K, M)	Erythrocytes	[135]
		HF	Lentiviral vectors (O, S, K, M)	None	[136]
Hutchinson-gilford progeria syndrome (OMIM #176670)	<i>LMNA</i> mutations	HF	Retroviral vectors (O, S, K, M)	Neural progenitors, endothelial cells, fibroblasts, VSMCs, and MSCs	[137]
		HF	Retroviral vectors (O, S, K, M)	Vascular SMCs	[138]
		HF	Retroviral vectors (O, S, K, M)	MSCs and osteogenic cells	[139]
Niemann-pick disease type C1 (OMIM #257220)	<i>NPC1</i> mutations	HF	Retroviral vectors (O, S, K, M)	Neurons	[140]
		HF	Lentiviral vectors (O, S, K, M)	Neurons	[141]
		HF	SeV vectors (O, S, K, M)	HLCs and neural progenitors	[142]

CLCs: Cholangiocytes; VSMCs: Vascular smooth muscular cells; MSCs: Mesenchymal stem cells; SMCs: Smooth muscle cells; MSCs: Mesenchymal stem cells; HLCs: Hepatocyte-like cells; SeV: Sendai virus; AF: Amniotic fluid cells; CVS: Chorionic villus; HF: Human fibroblasts; O: OCT4; S: SOX2; K: KLF4; M: C-MYC.

hiPSCs for modelling autosomal recessive disorders

SMA (OMIM #253300) is an autosomal recessive neuro-degenerative disorder. SMA is caused by mutation or deletion of the survival motor neuron-1 (*SMN1*) gene^[105,106]. The clinical phenotype is typically characterized by the degeneration of α -motor neurons in the spinal cord, leading to muscle weakness, atrophy and premature death^[107,108].

All SMA patients have also a highly homologous gene copy (*SMN2*) in different copy number. *SMN2* is not able to produce sufficient levels of SMN protein, due to its defective splicing pattern. However *SMN2* copy number is inversely correlated with the severity of the SMA phenotype.

One study describes human iPSCs derived from skin fibroblasts to model SMA^[109]. The main characteristic is the degeneration of motor neurons caused by a loss of SMN1 protein in all cells of the body. The use of SMA-iPSCs-derived motor neurons may help to elucidate the role of *SMN1* in disease initiation and progression, but also to screen new drug useful in future pharmacological therapies for SMA.

Later studies reported the establishment of five iPSC lines from type 1 SMA fibroblasts. iPSCs-derived neurons with a decreased ability to generate motor neurons and an altered neurite outgrowth. Exogenously induced expression of *SMN* in these iPSC lines determined a normal motor neuron differentiation and rescued the aberrant neurite outgrowth, confirming the role of the *SMN* defect in the disease^[110]. Successively, several reports have been published using these cells to test novel compounds for efficacy prior to administration to patients, increasing the possibility of success in the

treatment of this serious disorder^[111-113].

β -thalassemia (β -Thal; OMIM #613985) is an inherited autosomal recessive blood disorder, caused by either point mutations or deletions of nucleotides in the β -globin gene, provoking a reduced/abnormal or absent synthesis of β -globin chains that make up hemoglobin. Affected patients have severe anemia and an shortened life span^[114].

The generation of patient-specific iPSCs and the subsequent editing of the disease-causing mutations provide an ideal therapeutic solution to β -thalassemia and other haemoglobinopathies^[115]. Disease-specific autologous iPSCs have been generated from somatic cells and differentiated into haematopoietic cells, both *in vitro* and *in vivo* in SCID mice^[116,117]. Fan *et al.*^[118] used cultured β -thalassemia-amniotic fluid cells as target cells for an efficient reprogramming by using a single polycistronic lentiviral vector. iPSCs producing insufficient amounts of β -globin can be induced to increase β -globin product by infecting them with a viral vector carrying an exogenous copy of the β -globin gene and successively to differentiate into "restored" erythrocytes^[119]. Recently, other approaches have been used for targeting the *HBB* gene in β -thalassemia-derived iPSCs, demonstrating how TALENs was able to mediate a higher homologous recombination efficiency than that obtained by CRISPR/Cas9^[120]. The development of innovative gene editing protocols opens new promising prospects for the use of iPSCs as a target of gene therapy for monogenic diseases.

Cystic fibrosis (CF; OMIM #219700) is an autosomal recessive disorder. The primary defect is the regulation of

Table 3 Models of monogenic X-linked recessive diseases

Diseases	Genetic defects	Target cells for reprogramming	Delivery methods	Disease relevant cells	Ref.
Fragile X syndrome (OMIM #300624)	<i>FMR1</i> silencing	HF	Retroviral vectors (O, S, K, M)	Neurons	[146]
		HF	Retroviral vectors (O, S, K, M)	Forebrain Neurons	[147]
		HF	Sendai virus	NPC	[148]
Duchenne muscular dystrophy (OMIM #310200)	Dystrophin gene mutations	HF	Retroviral vectors (O, S, K, M)	Myogenic cells	[154]
		HF	Retroviral vectors (O, S, K, M)	CMs	[155]
		HF	Retroviral vectors (O, S, K, M)	Neurons	[156]
Wiskott-aldrich syndrome (OMIM #301000)	<i>WASP</i> mutations	HF	Retroviral vectors/sendai virus vectors (O, S, K, M)	Megakaryocytes	[157]
Rett syndrome (OMIM #312750)	<i>MeCP2/CDKL5</i> mutations	HF	Retroviral vectors (O, S, K, M)	Neurons	[158]
		HF	Retroviral vectors (O, S, K, M)	NPCs/mature neurons	[159]
		HF	Retroviral vectors (O, S, K, M)	NPCs/mature neurons	[160]
Hemophilia A (OMIM #306700)	Deficiency of factor VIII	Urine cells	Episomal vectors (O, S, K, SV40LT)	Hepatocytes	[161]

NPC: Neural progenitor cells; CMs: Cardiomyocytes; HF: Human fibroblasts; O: OCT4; S: SOX2; K: KLF4; M: C-MYC.

epithelial chloride transport by a chloride channel protein, encoded by the CF transmembrane conductance regulator (*CFTR*) gene^[121]. CF is a multisystem disorder characterized by loss of function in the *CFTR* in organs with secretory function^[122,123]. Recurrent pulmonary infections are responsible for 80%-90% of the deaths in CF patients^[124].

Fibroblasts from patients with CF can be reprogrammed to iPSCs and differentiated into lung airway epithelium^[125]. From the point of view of translational medicine, patient-specific iPSC-derived airway epithelial cells open the way to personalize therapeutic interventions for the treatment of serious lung diseases. Different groups report the generation of iPSCs from CF patients and their differentiation into pulmonary cells, creating a platform for dissecting human lung disease^[126-129]. Unfortunately, the development of iPSCs-based models of human lung disease is hampered by the inability to differentiate hiPSCs into lung progenitors and subsequently into mature pulmonary epithelial cell types.

Moreover mutations in the *CFTR* gene are also responsible for CF-associated pathologies, such as cholangiopathy, resulting in reduced intraluminal chloride secretion, increased bile viscosity and focal biliary cirrhosis. Sampaziotis *et al.*^[130] generated hiPSCs from skin fibroblasts of a CF patient and differentiated them into cholangiocytes (CLCs), showing that CF-hiPSC-derived CLCs (CF-CLCs) represent a good model CF biliary disease *in vitro*. In fact, their use for treatment with the experimental CF drug VX809 has demonstrated the *in vitro* rescue of the disease phenotype (phase 2a clinical trials)^[131,132]. The use of gene targeting specific nucleases to correct *CFTR* gene sequences has been reported^[129,133,134].

Crane *et al.*^[124] designed zinc-finger nucleases to target endogenous *CFTR* for editing the inherited genetic mutation in patient-derived iPSCs *via* homology-directed repair (HDR). When induced to differentiate *in vitro*, modified hiPSCs demonstrated a restored expression of the *CFTR* gene, recovering the expression of the mature *CFTR* glycoprotein and of the chloride channel functions^[124] (Table 2).

hiPSCs for modelling X-linked disorders

Fragile X syndrome (FXS, OMIM #300624) is an inherited disorders due to CGG triplet expansion located within the 5' untranslated region of the Fragile X mental retardation gene (*FMR1*). The expansion causes the epigenetic silencing and the consequent loss of the Fragile X mental retardation protein (FMRP), a cytoplasmic mRNA transport factor^[143-145]. Species-specific differences in molecular and neurodevelopmental aspects of FXS require a human FXS model and hiPSCs enable disease modelling.

It has been reported that FXS hESCs and hiPSCs differ in the epigenetic state of the *FMR1* gene. In fact, the *FMR1* gene is unmethylated and expressed in hESCs, presenting full-mutation repeats, converting to methylated and silenced in the differentiated state^[64]. In contrast, FXS hiPSCs do not return to the naive epigenetic state because the *FMR1* gene remains methylated and silenced during reprogramming^[64,146]. Although FX-hiPSCs do not reproduce the methylation state of the *FMR1* gene, they represent a useful model for studying the role of *FMR1* in neural cells. The distinction between FX-hES and FX-hiPSCs at the *FMR1* locus suggests a more general epigenetic phenomenon in human pluripotent stem cells, which highlights the need for more studies to clarify the similarity and differences between ESCs and iPSCs.

Different hiPSCs cell lines have been generated from multiple patients with FXS and successively induced to differentiate into post-mitotic neurons and glia^[146]. In these cells, an aberrant neuronal differentiation of FXS hiPSCs is observed, directly associated to the epigenetic modification of the *FMR1* gene and to a loss of FMR protein expression, evidencing a key role for the FMR protein early in human neurodevelopment prior to synaptogenesis. iPSCs-derived neurons represent disease-associated cellular phenotypes, very useful for discovering novel therapies for FXS and other diseases sharing common pathophysiology.

Another paper reports the reprogramming of hiPSC lines from FXS fibroblasts. FXS forebrain neurons have been differentiated from these iPSCs, displaying both

defective neurite initiation and extension^[147]. iPSCs constitute a platform to examine potential neuronal deficits caused by FXS and develop assays for drug discovery^[148].

Because the main consequence of the lack of FMRP in FXS is the synaptic defect, another group has reported a cellular model focused on neuronal cells that well represent the disease and express a transcriptional and proteomic pattern similar to that present in the neurons of the brain^[148]. This aspect is important for identification of the target that modulates FMRP expression because its study in other cellular models could be erroneous. Current therapy for FXS is only at the behavioral level.

Duchenne muscular dystrophy (DMD; OMIM #310200) is the most prevalent congenital pediatric muscular dystrophy. It is an X-linked genetic degenerative myopathy and multisystem disease characterized by disease-specific serological abnormalities, dilated cardiomyopathy, cataracts, insulin-resistance, cardiac conduction defect, myotonia and muscular dystrophy, which can lead to the loss of motor function in puberty^[149].

The disease is a myopathy that affects in approximately 1 in 5000 male births and is caused by mutations within the dystrophin gene (locus Xp21.2)^[150,151]. The disease is characterized by a reduction in dystrophin, a protein assembles with the dystrophin glycoprotein complex (DGC), associating the cytoskeleton to the extracellular matrix in skeletal and cardiac muscles^[152]. Consequences of DGC inefficiency are severe muscle wasting, contraction-induced damage, necrosis and inflammation^[153].

Cell transplantation and hiPSCs offers an encouraging way for cell-based therapy, in fact myogenic cells derived from hiPSCs are an unlimited source for cell-based therapy of DMD. Goudenege *et al.*^[154] have established the usefulness of genetically corrected human multipotent cells for muscle repair. Transplantation studies in hiPSCs highlight the advantages to correct the patient own cells, avoiding the immune response against the donor myoblast or mesoangioblast. Lin *et al.*^[155] have utilized DMD iPSCs to replicate and analyze the major phenotypes of dilated cardiomyopathy (CMs) found in DMD-affected individuals, and thus to reveal the disease mechanism. Their study has identified a pathway determining increased apoptosis in DMD-CMs that can be regulated by drug therapy. Thus, these cells might represent an *in vitro* system for preclinical testing of future therapy^[155] (Table 3).

GENE THERAPY APPROACHES IN PATIENT-SPECIFIC HIPSCS FOR THE TREATMENT OF MONOGENIC DISEASES

The opportunity to derive patient-specific iPSCs in combination with the current development of gene modification protocols surely represents a good opportunity for cell therapy of several inherited genetic diseases.

Different gene editing methods have been demonstrated to modify defective genes in hiPSCs. Their choice depends on the gene correction approach and on the mutation type.

In the last few years, substantial progress has

been made by using BACs (bacterial artificial chromosomes)^[162-164], viral vectors^[165-167], and other relatively new methods, such as zinc finger nucleases (ZFNs)^[168-171], transcription activator-like effector nucleases (TALENs)^[172,173] and especially the clusters of regularly interspaced palindromic repeats (CRISPR) /Cas-derived RNA-guided endonucleases^[174,175].

BAC-based targeting vectors can obtain a high efficiency of homologous recombination in iPSCs of different genetic backgrounds, but this approach has the difficulty of confirming the homologous recombination event. Adenoviral and retroviral vector-mediated gene targeting appear to be effective considering the efficiency of transduction and the homologous recombination. The preparation of these viral vectors needs expertise and an enough biosafety facility. Moreover, their use is confined to insertional mutagenesis, although, self-inactivating (SIN) lentiviral vectors have now become almost safe clinical strategies^[167,176-178].

The correction of disease mutations by nucleases in iPSCs has been described for different diseases. These nucleases cleave chromosomal DNA, generating DNA double strand breaks (DSBs), whose repair *via* endogenous mechanisms, such as homologous recombination (HR) or non-homologous end-joining (NHEJ), leads to targeted mutagenesis and chromosomal rearrangements^[179-181]. Hence, before delivery to patients, gene-corrected-iPSCs are differentiated into the appropriate somatic cells, to evaluate the expression of the corrected gene and to avoid teratoma formation in patients. In particular, different research groups have created nucleases for genome engineering in hiPSCs by linking the cleavage domain of the *FokI* restriction enzyme to a designed zinc finger protein (ZFP). The ZFN, which works as a dimer, is mediated by its linked ZFP domain. In particular, the ZFNs are designed to bind to a genomic sequence of sufficient length (18-36 bp)^[134,137,170,173,182,183]. The ZFN method is rapid and suitable but has a poor targeting density and lack of targetable sites for genome editing in small DNA sequences.

A recent and interesting approach for engineering DNA binding specificities is based upon TALEs from *Xanthomonas* plant pathogens. TALEs are a different way to the creation of site-specific nucleases^[184-187]. TALEs are transcription factors that specifically bind and regulate plant genes during pathogenesis^[188,189]. The DNA binding domain of TALEs is composed of multiple 34 amino acid units (TALE repeats) that are organized in tandem. Their sequence is almost equivalent, but presents two highly variable amino acids that set up the base recognition specificity for each unit. Each single domain allows the specificity of binding to one of the four possible nucleotides in the TALE recognition sequence, so any desired genomic sequence can be recognized as a DNA-binding domain. An example of TALENs application to correct a defective gene in iPSCs is the correction of the Niemann-Pick type C (*NPC1*) mutations in iPSCs-derived hepatic and neuronal cells of patients affected by NPC disease, a lipid storage disorder causing severe neurodegeneration and liver dysfunction. This approach allows for the rescue of the

phenotype, including the dysfunctional autophagic flux directly linked to loss of NPC1 protein function^[190]. It is important to keep in mind that the DNA methylation and histone acetylation in inactive chromatin could influence the efficiency of genome editing *via* TALENs.

The latest and the greatest mechanism for gene editing is represented by the bacterial cluster and regularly interspaced short palindromic repeats/CRISPR-associate nuclease 9 (CRISPR-Cas9). CRISPR is yet another example for how scientists have learned from nature's inventions helping them to discover new gene functions with high sensitivity and precision. This technology was created from type II CRISPR-Cas systems, by which bacteria degrade targeted nucleic acids. CRISPRs are components of the genomes of most bacteriophage-resistant *Bacteria* and *Archaea*. Cas9, a CRISPR-associated endonuclease, can be confined to specific DNA loci to induce double-strand breaks beneath the guidance of the trans-activating CRISPR RNA (tracrRNA): CRISPR RNA (crRNA) duplex. The dual tracrRNA:crRNA was additionally evolved as a single guide RNA (sgRNA) for genome engineering and consists of the 5' end 20-nucleotide sequence disposing the DNA target site conforming to Watson-Crick base pairing and 3' end double-stranded structure binding Cas9. The sgRNA could lead CRISPR-Cas 9 to any target DNA sequence with a protospacer-adjacent motif (PAM) by modifying the guide RNA sequences^[174,175]. In a genome editing system, the right choice of delivery system is crucial for the targeted cells.

Different delivery protocols have been adopted to vehicle *in vitro* plasmid DNA encoding Cas9-gRNA complexes through cell membranes in cell culture including electroporation, nucleofection and lipofectamine-mediated transfection^[177,191,192].

An important characteristic of the CRISPR/gRNA is its easy design and preparation. In addition, the system recognizes approximately 23 bp of the target site, which is a relatively shorter sequence than that recognized by TALENs. Recent papers have also corroborated that the RNA-mediated CRISPR/CAS9 system has a high tolerance to a few base pair mismatches towards the 5' half of the target site and a high potential for off-target risk in human cell lines.

A lot of studies have successfully modified genes in monogenic disorders using patient-specific iPSCs.

An interesting use of CRISPR-Cas9 is the correction of the *HBB* gene in human iPSCs generated from patients carrying a homozygous missense point mutation in the *HBB* gene. Using a specific guide RNA and Cas9, Huang *et al.*^[193] corrected, without off-targeting mutations, one allele of the *HBB* gene by homologous recombination with a donor DNA template containing the wild-type *HBB* DNA. Erythrocytes were then generated by differentiating hiPSCs in which normal expression of the β -globin protein (due to CRISPR-Cas9 modified HBB allele) was detected^[193].

Recently, another group has demonstrated the restoration of the dystrophin protein in DMD patient-

derived iPSCs by TALEN or CRISPR-Cas9. They used three correction methods (exon skipping, frameshifting and exon knock-in) to restore protein levels. After investigating the genome integrity, they differentiated the corrected iPSCs towards skeletal muscle cells and detected the expression of the full-length dystrophin protein^[194].

Haemophilia A is an X-linked genetic disorder caused by mutations in the *F8* gene encoding the blood coagulation factor VIII. The CRISPR-Cas9 protocol has been used by Park *et al.*^[195] to repair two large inverted regions back to the normal orientation in Haemophilia A patient-derived iPSCs. They demonstrated the expression of the *F8* gene *in vitro* in endothelial cells after their differentiation. Targeted deep sequencing and whole genome sequencing analyses showed no off-target effects, as they chose a target sequence that differs from any other site in the human genome by three nucleotides^[195].

The CRISPR protocol has been recently adopted for correcting the $\Delta F508$ mutation in CFTR- mutated hiPSCs. An excisable selection system is used to improve the efficiency of the correction. The possibility of any genomic footprint at the target site allowed Firth *et al.*^[196] to edit a point mutation in the genome, while leaving no genomic scar in patient cells. Modified hiPSCs were then differentiated to mature airway epithelial cells in which the functional recovery of the *CFTR* channel protein was demonstrated. Half of the CRISPR-corrected epithelial cells stably responded to stimulation by the whole-cell patch clamp method^[196].

The number of disease-specific hiPSC lines is increasing and the technology of gene editing using engineered nucleases holds considerable promise for progressing science and enhancing human health with the potential to create a variety of novel therapeutics for a range of diseases, many of which are untreatable today. Through the use of guiding RNA and nuclease activity, genes can be located and modified in a less expensive way. These technologies allow researchers to make changes to human DNA (human germline editing) in the nuclei of cells, in eggs, in sperm or in human embryos. In this way replacing or eliminating disease-causing genes could reverse disease symptoms.

However, these technologies also induce several social considerations and have not yet carried out an ethical experimental protocol useful for current medicinal practice. As a result, the *National Academy of Sciences* and the *National Academy of Medicine* are promoting an important initiative to lead decision making about research activities involving human gene editing. Many researchers report problems linked to mutations in other genes or off-target effects of the Cas9 nuclease whose consequence is largely unknown. These problems are most likely caused by the high level of functionality of the Cas9 protein, posing a dilemma in research goals requiring both powerful methods and efficacy. Several methods may minimize these problems, selecting for example unique target sequences and changing the

structure of the crRNA or sgRNA, strengthening nuclease expression levels, making use of truncated sgRNAs or recombinant proteins instead of plasmids.

Nonetheless, more detailed and comprehensive studies will be needed to determine the relative merits of these technologies in different experimental procedures to open the next era of gene therapy.

CONCLUSION

Human iPSCs are central in modelling and studying monogenic diseases. They represent an affordable and applicable tool to investigate the pathogenesis and the progression of human disease and, at the same time, can be used for the *in vitro* screening of new therapeutic compounds.

The advantages of induced pluripotent stem cells are represented by the generation of patient-derived cells and the maintenance of a versatile differentiation potential, opening new perspectives for the development of cell-based therapy and personalized medicine.

The possibility of obtaining a wide variety of disease relevant cells would allow investigators to design an efficient test system that permits large-scale screening of drugs for the targeted treatment of specific human diseases *in vitro*.

Moreover, this technology offers the opportunity to develop cell therapy protocols for several serious diseases requiring the restoration of cells or organs damaged in the pathological process. The possibility of using powerful methods for genome engineering, such as ZFNs, TALENs, and the CRISPR/Cas9 system, allows the correction of mutated genes *in vitro* and thus safe transplantation back to the patients to assess their therapeutic efficacy.

When concerning iPSC-derived models, it is necessary to examine the inherited genetic and epigenetic variations among patients in the phenotypic analysis. Regardless their potential, iPSCs-based clinical trials cannot be used except if all obstacles discussed in this review are overcome. For example, the residual pluripotent stem cells form teratomas after cells transplantation. Any aberrations regarding tumour formation or malfunctions of epigenetic memory, as well as the genomic instability, the delivery and expression of the reprogramming factors, and the growth of cells in culture, should also be closely monitored.

However, promising results have already been obtained in pre-clinical studies in different disease models, and also in the first clinical study currently on-going in Japan^[197,198]. In summary, only 8 years after their first report, iPSC cell technologies provide a promising prospect for clinical uses.

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Migration of bone marrow progenitor cells in the adult brain of rats and rabbits

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Abstract

Neurogenesis takes place in the adult mammalian brain in three areas: Subgranular zone of the dentate gyrus (DG); subventricular zone of the lateral ventricle; olfactory bulb. Different molecular markers can be used to characterize

the cells involved in adult neurogenesis. It has been recently suggested that a population of bone marrow (BM) progenitor cells may migrate to the brain and differentiate into neuronal lineage. To explore this hypothesis, we injected recombinant SV40-derived vectors into the BM and followed the potential migration of the transduced cells. Long-term BM-directed gene transfer using recombinant SV40-derived vectors leads to expression of the genes delivered to the BM firstly in circulating cells, then after several months in mature neurons and microglial cells, and thus without central nervous system (CNS) lesion. Most of transgene-expressing cells expressed NeuN, a marker of mature neurons. Thus, BM-derived cells may function as progenitors of CNS cells in adult animals. The mechanism by which the cells from the BM come to be neurons remains to be determined. Although the observed gradual increase in transgene-expressing neurons over 16 mo suggests that the pathway involved differentiation of BM-resident cells into neurons, cell fusion as the principal route cannot be totally ruled out. Additional studies using similar viral vectors showed that BM-derived progenitor cells migrating in the CNS express markers of neuronal precursors or immature neurons. Transgene-positive cells were found in the subgranular zone of the DG of the hippocampus 16 mo after intramarrow injection of the vector. In addition to cells expressing markers of mature neurons, transgene-positive cells were also positive for nestin and doublecortin, molecules expressed by developing neuronal cells. These cells were actively proliferating, as shown by short term BrdU incorporation studies. Inducing seizures by using kainic acid increased the number of BM progenitor cells transduced by SV40 vectors migrating to the hippocampus, and these cells were seen at earlier time points in the DG. We show that the cell membrane chemokine receptor, CCR5, and its ligands, enhance CNS inflammation and seizure activity in a model of neuronal excitotoxicity. SV40-based gene delivery of RNAi targeting CCR5 to the BM results in downregulating CCR5 in circulating cells, suggesting that CCR5 plays an important role in regulating traffic of BM-derived cells into the CNS, both in the basal state and in response to injury. Furthermore, reduction in CCR5 expression in

circulating cells provides profound neuroprotection from excitotoxic neuronal injury, reduces neuroinflammation, and increases neuronal regeneration following this type of insult. These results suggest that BM-derived, transgene-expressing, cells can migrate to the brain and that they become neurons, at least in part, by differentiating into neuron precursors and subsequently developing into mature neurons.

Key words: Stem cells; Bone marrow; Hippocampus; Cell therapy; SV40; Brain; Nestin; Doublecortin; Neurons; Development; Epilepsy; Seizures

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Core tip: It was previously thought that the development of new neurons did not take place in the adult brain of higher vertebrates. There has been substantial progress in understanding neurogenesis in the adult brain during the last decade, showing that neural progenitor cells can induce neurogenesis, mainly in three areas: Subventricular zone, subgranular zone of the hippocampal dentate gyrus, and olfactory bulb. More recently, it has been shown that bone marrow progenitor cells can participate in neurogenesis in the adult brain. In this review, we discuss the mechanisms of the migration, differentiation, and maturation of bone marrow progenitor cells in the adult brain. We also consider the increase of adult neurogenesis following experimental seizures, provided that neuroinflammation is decreased by reducing the expression of chemokines, and consequently the related migration of inflammatory cells into the brain parenchyma.

Dennie D, Louboutin JP, Strayer DS. Migration of bone marrow progenitor cells in the adult brain of rats and rabbits. *World J Stem Cells* 2016; 8(4): 136-157 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v8/i4/136.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v8.i4.136>

ROLE OF BONE MARROW PROGENITOR CELLS IN ADULT NEUROGENESIS

Neurogenesis in the adult brain is a relatively new concept. There are three regions of the adult brain in Mammals where neurogenesis can take place in the adult mammal: Subgranular zone (SGZ) of the dentate gyrus (DG); subventricular zone (SVZ) of the lateral ventricle; and olfactory bulb (OB). The cells participating in neuronal development in adults have been characterized using molecular markers. It has been more recently suggested that a population of bone marrow (BM) progenitor cells could also contribute to adult neurogenesis. One way to verify if this hypothesis is correct would be to stain BM stem cells *in situ* and to track them in the body. In this review, we report that injecting the BM of rats and rabbits with SV40 vectors

results in the transduction of BM precursor cells that are migrating, among other organs, to the brain where they differentiate in neurons and microglial cells. It has also been previously shown that neuroinflammation can hamper the process of neuroregeneration following insult in the DG. We show here that reducing the levels of certain chemokine receptors in circulating cells by gene transfer of siRNA against these receptors in a context of a rat model of neurotoxicity leads to a decrease in inflammation and an increase of BM-derived cells migrating to the brain.

GENE DELIVERY TO BONE MARROW PROGENITOR CELLS

Ex vivo gene delivery is the most utilized procedure for transducing hematopoietic stem cell (HSC). However, in order to replace the *ex vivo* approach of transduction and reimplantation HSC, direct delivery of viral vectors into the BM has been proposed^[1-3]. This procedure has been suggested because *ex vivo* gene transduction and reimplantation may modify the homing properties and can change the functions of progenitor cells and HSC^[4-6]. Furthermore, HSC transduced by *ex vivo* gene delivery procedures may become exposed to infectious agents^[4]. We tried here to assess the efficiency of intramarrow injection in the femoral cavity of rats using rSV40 vectors. Levels of transgene expression were evaluated in peripheral blood population during several months^[7]. Transgene expression was observed during several months in multiple BM and peripheral blood lineages by using this method^[7]. Long term expression of transgene in platelets and the correction of haemophilia phenotype for at 5 mo were observed in other studies^[8]. Sustained gene expression was also found present in neuronal cell after *in vivo* gene transfer^[9]. The direct injection of viral gene delivery in the bone marrow can take full advantage of the stem cells that are present within the bone marrow including non hematopoietic cells^[8,10]. The targeting of HSC within their niche may be advantageous in the treatment of Fanconi anemia (FA) by ensuring that they maintain their function and by enabling the correction of the remaining stem cells^[6]. This approach also eliminates challenges, such as the requirement for preconditioning^[8,10], thus making it very promising in the treatment of FA^[6] and haemophilia^[8]. Moreover, gene transfer based on direct intramarrow injection should prevent difficulties seen during *ex vivo* approach such as stimulation by cytokines, putative loss of engraftment, and keeping HSC properties overtime^[8,10]. Gene transfer to the BM improves the efficiency of HSC viral vector transduction and strengthens the supportive microenvironment by opposition to intravenous inoculation that delivers vector to blood^[8,10]. Consequently, this procedure leads to better preservation of stem cell viability and capacity^[10]. We used the intrafemoral gene delivery approach to BM progenitor cells to study their fate in the body, and more particularly in the brain. We will first review the cells

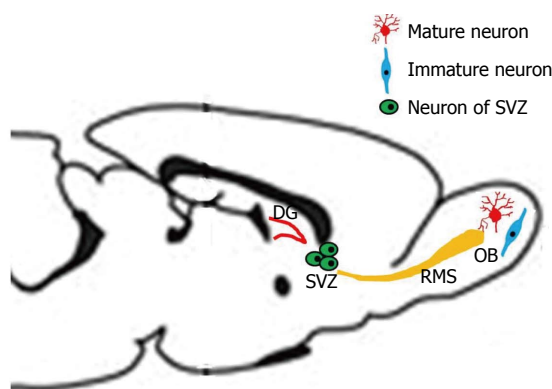


Figure 1 Different areas of neurogenesis in the adult rodent. The three areas of neurogenesis in the adult are the dentate gyrus of the hippocampus, the subventricular zone, and the olfactory bulb. Some progenitor cells are migrating from the SVZ to the OB, along a rostral migratory stream. DG: Dentate gyrus; SVZ: Subventricular zone; OB: Olfactory bulb; RMS: Rostral migratory stream.

involved in adult neurogenesis, their molecular markers, the different areas involved in adult neurogenesis, and the factors influencing neuronal development. Then, by using gene delivery into the femoral cavity, we will show the migration and differentiation of transduced BM progenitors to the brain. Finally, we will demonstrate that reducing neuroinflammation in an animal model of experimental seizure leads to an increase of BM progenitor cells migrating towards the brain.

NEUROGENESIS IN THE ADULT BRAIN

The traditional concept was that the development and growth of new neurons from neuronal stem precursor cells does not take place in the adult brain of higher vertebrates. In 1962, Altman^[11] demonstrated neurogenesis in the brain of an adult rat. There was no further report until Goldman and Nottebohm^[12] reported evidence of neurogenesis in canaries. This was further substantiated in 1992 when studies reveal similar evidence of adult neurogenesis^[13]. There has been substantial progress in understanding neurogenesis in the adult brain during the last decade. However, molecular events leading to the increase number, migration, and differentiation of progenitor cells in the brain need to be better characterized. The participation of BM progenitor cells to adult neurogenesis has also been suggested more recently.

Adult neurogenesis occurring in mammalian brain is now a well accepted idea, essentially in three regions: SGZ of the DG, SVZ of the lateral ventricle, and olfactory bulb^[14]. Dividing cells see their number reduced after birth, except in the SGZ of the DG and the SVZ (Figure 1)^[15].

Neuroblasts and progenitor cells from the SVZ also migrate through the rostral migratory stream (RMS) to the OB maturing into new neurons (Figure 1). The same process has been documented in primates, including humans^[16-18]. Neurons, produced in the DG and the SVZ are primarily granular neurons^[17] and to a lesser extent

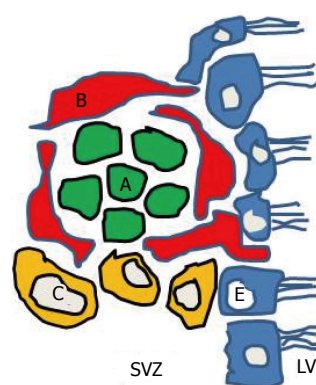


Figure 2 Four different types of progenitor cells in the subventricular zone. Type-A cells are migrating towards the olfactory bulb along the rostral migratory stream. Type B cells are believed to be the neural precursor cells and will give rise to both type C and type A cells. Type C cells are rapidly dividing immature precursor cells arranged in clusters along the migrating chains, and they are found only in the SVZ. E cells belong to ependymocytes lineage. LV: Lateral ventricle; SVZ: Subventricular zone.

periglomerular neuronal cells for the OB^[19].

The SVZ of the lateral ventricle is mainly located in the lateral wall of the lateral ventricle which is facing the striatum^[20]. It contains various neural progenitor cells along its wall. Four different types of cells have been identified in the SVZ of the lateral ventricle of mice^[20,21]. They are described as types A, B, C and E cells (Figure 2). These cells differ from each other based on the ultrastructure, morphology and molecular markers expressed by the cells. Type A cells, the most common ones, are darker than B cells in electron microscopy. Their cell bodies are elongated and contain up to two processes; they have abundant lax chromatin with two to four nucleoli; the cytoplasm is dark and contained free ribosomes. The rough endoplasmic reticulum has only a few short cisternae, the Golgi apparatus is small, and many microtubules are arranged along the long axis of the cell^[20]. These type A cells were described as being connected to each other by junctional complexes^[20]. Type A cells are neuroblasts expressing polysialated form of the neural cell adhesion molecule (PSA-NCAM). Type A cells are migratory in nature and course tangentially to the walls of the lateral ventricle. Type B cells are slow dividing astrocytes that enclose the migrating neuroblasts. These cells have different characteristics compared to type A cells^[14]. Their nuclei is irregular and the cytoplasm is lighter stained. They are characterized by their abundant intermediate filaments and the dense bodies within their cytoplasm. A further subdivision of type B cells into type B1 and a type B2 has been reported^[20]. Type B1 cells are larger, and contain more cytoplasm. It is believed that type B cells are the neural precursor cells and that they give rise to both type C and type A cells^[22]. Type C cells are found only in the SVZ and they are rapidly dividing immature precursor cells arranged in clusters along the migrating chains. Type C cells are larger, more spherical, contain larger golgi apparatus than type B cells; however their size is similar to type B1 but they have fewer processes^[20].

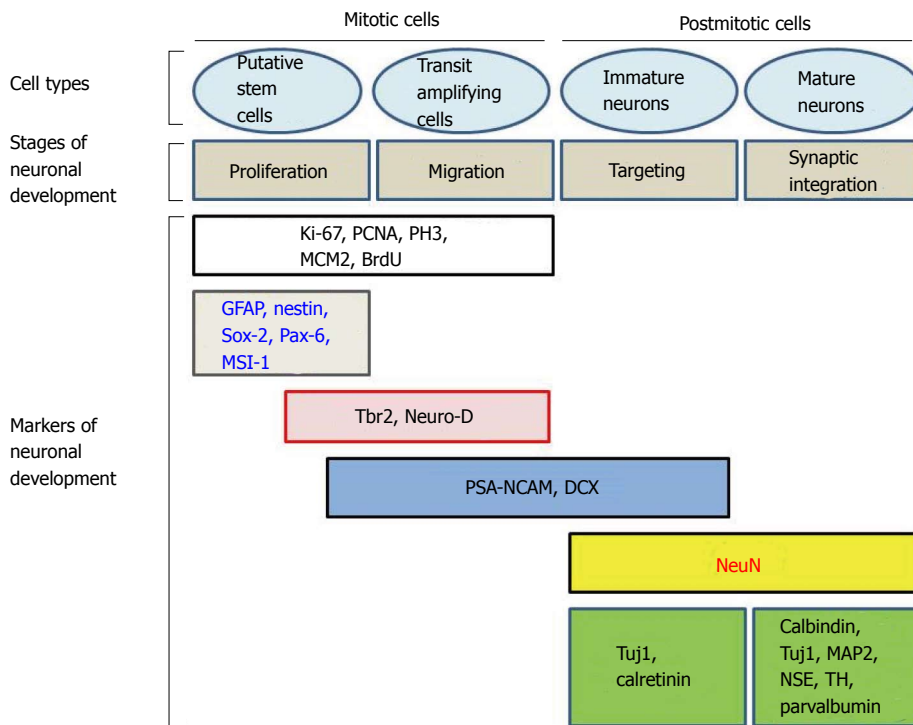


Figure 3 Different markers of neuronal development in mitotic and postmitotic cells (modified from Ref. [21,35]). PSA-NCAM: Polysialated form of the neural cell adhesion molecule; DCX: Doublecortin; GFAP: Glial fibrillary acidic protein; PCNA: Proliferating cell nuclear antigen; PH3: Phosphorylated form of histone 3; MCM2: Minichromosome maintenance protein 2; MSI-1: Musashi-1; MAP2: Microtubule-associated protein 2; NSE: Neuron specific enolase; TH: Tyrosine hydroxylase.

Type E cells belong to ependymocytes lineage (Figure 2).

Cells in the SVZ that are originating from the lateral wall of the lateral ventricle migrate along the RMS pathway to be incorporated into the OB^[14,23]. The rostral migratory stream pathway is constituted mainly of type A and type B cells^[20]. There has been no evidence so far of type C cells within the RMS and studies suggest that this region is devoid of these cells. Type A cells are arranged in chains surrounded by type B cells within the RMS, similarly as within the SVZ. These cells reach the olfactory bulb and become interneurons. In addition, microglial cells and endothelial cells are also present in the RMS^[24]. The division and migration of the neuroblasts within and to the olfactory bulb is independent of the process that takes place within the olfactory bulb^[25].

SGZ of the DG is situated between the hilus and the granular cell layer of the DG in the hippocampus. This is one of the main areas where neurogenesis takes place^[17,26] with an estimated 9000 new cells generated each day^[27]. Not all of the neurons generated in the hippocampus will survive and become incorporated into the neuronal circuit of the brain. Approximately 50% of the neurons born in the SGZ will die after birth without being incorporated in the neuronal circuit of the brain. Within the hippocampus, three types of cells are identified: (1) Type 1, radial cells, which give rise to type 2 cells; (2) Type 2, non-radial intermediate, neural progenitor cells; and (3) Neuroblasts, derived from type 2 cells^[28]. Type 1 cells are found to be progenitor cells similar to type B cells found in SVZ and are slow dividing cells, whereas type 2 cells are rapidly dividing ones^[29].

MOLECULAR MARKERS OF NEUROGENESIS

Adult neurogenesis involves a succession of events occurring in a specific order^[30-32]. For example, hippocampal neurogenesis is thought to begin with stem cells located in the SGZ of the DG. There, these cells proliferate, differentiate and give rise to new neurons. Neurons at various stages of neurogenesis express different markers. Consequently, the fate, and differentiation, of cells during neurogenesis can be followed^[33,34] (Figure 3 and Table 1).

Different markers help to determine the cells in the stage of neurogenesis and their role during particular stages. Some of the most commonly used markers include glial fibrillary acidic protein (GFAP), nestin, Neuro D, Hu, Tuj1, doublecortin (DCX), PSA-NCAM, neuron specific enolase (NSE) and neuronal specific nuclear protein (NeuN). Markers are useful to distinguish cellular proliferation, early adult neurogenesis, later steps of adult neurogenesis, and mature stage.

Different markers of proliferation can be used. The most utilized markers are proliferating cell nuclear antigen, and Ki-67, respectfully characterized by a long half-life, and a short half-life of about one hour. Ki-67 is rarely used in this application. Other markers include minichromosome maintenance protein 2 and phosphorylated form of histone 3^[35]. Bromodeoxyuridine (BrdU) incorporates into DNA during S phase and is also used as a marker of proliferating cells. To conclude that BrdU-positive cells are of neuronal lineage, and to characterize the cell lineages involved, BrdU-labeling needs to be combined with markers of neurons at different stages of neurogenesis^[33,35].

Table 1 Markers of cells involved in neurogenesis with corresponding stages of neurogenesis

Marker	Cells	Stage of neurogenesis
Nestin	Neuronal stem cells, radial glia cells, transit amplifying cells	Proliferation, differentiation
GFAP	Neuronal stem cell, mature astrocytes	Proliferation, differentiation
PSA-NCAM	Migrating neuroblast, immature neuron	Differentiation, migration, targeting
Tuj1	Migrating neuroblast, immature neuron, mature neuron	Differentiation, migration, targeting
Doublecortin	Migrating neuroblast, immature neuron	Differentiation, migration, targeting
NeuN	Mature neuron	Targeting, synaptic integration

GFAP: Glial fibrillary acidic protein; PSA-NCAM: Polysialated form of the neural cell adhesion molecule.

Markers of early stages of adult neurogenesis include GFAP, which is an intermediate filament protein expressed by mature astrocytes within the adult brain^[33,36,37]. Its expression has been described in type B progenitor cells present in the SVZ and type 1 cells described in the SGZ^[21,36-38]. Nestin is a structural analog to intermediate filament protein, found to be positive in type B neural progenitor cells and transit amplifying type C cells in the SVZ^[21], in type 1 (radial glial cells) and 2 cells in the SGZ^[29,39]. Nestin is thought to be a marker of precursor cells. Consequently, nestin synthesis decreases, as differentiation of nervous tissue progresses. Neurofilament and glial (e.g., GFAP) proteins begin to be expressed during the differentiation of neurons and astrocytes respectively when nestin expression starts to be reduced. Nestin expression declines during the postnatal period. However, rare cells expressing nestin are present in the adult DG and the SVZ^[40]. Expression of nestin is abruptly terminated. Other early markers of adult neurogenesis are Sox-2, a SRY-related HMG-box gene 2, paired box gene 6 (*Pax-6*), and musashi-1 (*MSI-1*), a RNA-binding protein preferentially expressed in the CNS. However, neuronal specificity of some of these markers is not absolute^[35].

The markers of later stages of adult neurogenesis include DCX and PSA-NCAM, both markers of immature neurons. DCX is a protein positive in cells within the RMS and is present in migrating progenitor of neurons^[41,42]. DCX induces polymerization of microtubules. DCX is a marker expressed by neuroblasts during their migration and immature neurons of the granular layer of the DG. It is also present in newly generated cells located at the border of the granular layer of the DG and in the SGZ. DCX is expressed in newly generated olfactory, hippocampal, and striatal neurons, but not in the cortex. DCX is a marker of late mitotic neuronal precursors and early postmitotic neurons. There is no overlap between the expression of DCX and nestin^[33,34]. As such, DCX is a good marker for adult neurogenesis. Expression of the (PSA-NCAM) is observed at the same stage. Thus, both mitotic neuronal precursors and early postmitotic new neurons are positive for PSA-NCAM and DCX^[35]. The basic helix-loop-helix protein NeuroD and T-box brain protein 2 (*Tbr2*) are also expressed during later steps of adult neurogenesis, and show some overlap with the expression of Pax-6. Neuro D is a protein inducing microtubule polymerization. NeuroD is expressed in proliferating neurons and in migrating neuroblasts^[33].

Both *Tbr2* and *NeuroD* are coexpressed with DCX and PSA-NCAM. Moreover, *Tbr2* is downregulated when cells are committed to neuronal lineage^[35].

Finally, NeuN is a soluble protein that is found in the nucleus and cytoplasm of postmitotic neurons. NeuN is expressed by mature neurons^[33]. In the hippocampus (HC), postmitotic cells are immunopositive for NeuN. NeuN is a marker of both newly generated postmitotic neurons and "normal" postmitotic neurons. There is a correlation between the decreased expression of DCX and the beginning of NeuN expression^[33]. Additional markers can be used to characterize mature neurons, and some of them can be specific of certain areas of the brain. Hu is a RNA binding protein from the *elav* family expressed from the early stages of neurogenesis to the end^[43]. Tyrosine hydroxylase is the enzyme that catalyzes the formation of L-DOPA, precursor of dopamine. Several calcium-binding proteins can be expressed in mature neurons: Calbindin-D28k is particularly abundant in the cerebellum; calretinin is a 29 k protein with 58% homologies to calbindin-D28k; parvalbumin is a small calcium-binding albumin protein involved in calcium signaling. Microtubule-associated protein 2 induces assembly of microtubules, an essential stage in neuritogenesis. NSE is often used as a marker of mature neurons. Neuron-specific class III β -tubulin (*Tuj1*) contributes to axonal transport and provides stability to microtubules in axons and somas. However, the list of the markers for mature neurons is not exhaustive (Figure 3).

Markers expressed in the SVZ and the subgranular zone of the DG during adult neurogenesis are presented in Tables 2 and 3 respectively.

MIGRATION OF BONE MARROW PROGENITORS TO THE BRAIN AND THEIR DIFFERENTIATION INTO CELLS OF DIFFERENT CNS LINEAGES

rSV40-transduced bone marrow progenitor cells migrate to the brain

Gene delivery to the brain has focused mainly on transducing neurons directly. However, an alternative approach may be to consider those areas of the brain where neurogenesis continues well into adult life: The DG of the HC, and the SVZ. New neurons are generated throughout life in the DG. These new neurons are involved

Table 2 Markers expressed during neurogenesis originating in the subventricular zone

Neuronal lineage	Type B cells	Type C cells	Type A cells	Neuronal cells
Cell identity	SVZ Astrocytes Putative stem cells	Transit amplifying progenitor cell	Migrating neuroblast	Mature and immature neuron
Identifying marker	GFAP Nestin	Nestin	PSA-NCAM Tuj1 Hu	DCX NeuN

SVZ: Subventricular zone; GFAP: Glial fibrillary acidic protein; PSA-NCAM: Polysialated form of the neural cell adhesion molecule; DCX: Doublecortin; NeuN: Neuronal specific nuclear protein.

in the repair of brain insults. Targeting endogenous brain cell progenitors *in situ* might be attempted in order to genetically engineer them. For example, proliferation of such engineered cells after injury or during disorder of the brain would lead to functional brain cells expressing a transgene of potential interest. However, this approach is limited by the low number of endogenous progenitors in the adult brain. Furthermore, the life span of endogenous stem cells might be not as long compared to pluripotent stem cells with different properties as well.

Migration of BM stem cells to the brain and their differentiation into different types of brain cells has been reported in rodents^[44,45] as well as in humans^[46]. When they are injected intravascularly or intraperitoneally in rodents, adult BM progenitor cells are able to migrate to the adult CNS where they differentiate into neuronal^[45,47], or non-neuronal cells^[48]. In patients with transplantation of BM, the autopsy of brains demonstrated that human HSC can trans-differentiate into neurons, astrocytes, and microglia following long term marrow engraftment. These results were observed without fusion between cells. They suggest that human HSC coming from BM-transplantation could be used as a potential therapeutic source not only for long term regenerative neurogenesis^[46], but also for gene delivery in the brain.

The potential of direct transduction of HSC in the BM has been raised. However, *in situ in vivo* gene delivery to HSC/progenitors by direct injection of viral vectors in the BM has been rarely described, despite the putative interest of this method. The fate of cells positive for the transgene in the body has never been studied in this context. Our group previously reported that the inoculation of a Tag-deleted recombinant SV40 vector carrying a marker gene (FLAG epitope appended to HIV-1 Nef as a carrier protein) in the femoral BM of rats caused positive results^[7]. Expression of the transgene in the blood lasted throughout the 16 mo of the study. Twenty-five percent of femoral marrow cells and between 4%-12% (average, 5%) of blood nucleated cells of all lineages were positive for the transgene FLAG throughout the whole study. However, it remained to be determined if HSC could migrate to the brain in this experimental paradigm. The

Table 3 Markers expressed during neurogenesis in the subgranular zone of the dentate gyrus

	Mitotic cells		Post mitotic cells	
Stages of neuronal development	Putative stem cell	Transit amplifying progenitors	Immature neurons	Mature neurons
Markers for neuronal development	GFAP Nestin	Nestin Doublecortin	Doublecortin Tuj1	Tuj1 NeuN NSE

GFAP: Glial fibrillary acidic protein; NeuN: Neuronal specific nuclear protein; NSE: Neuron specific enolase.

aim of the study was to determine the localization and the type of transgene-positive cells in the DG and in the SVZ. DG is composed of the hilus area surrounded by the granule cell layer (GCL), formed by an inner (upper) and outer (lower) blades. We therefore investigated by immunohistochemistry^[49,50] if some BM progenitor cells, transduced *in vivo* by intramarrow injection of a rSV40 vector, could migrate to the adult CNS and differentiate into different brain cell lineages^[51,52].

Transgene expression was seen in cells with shape of neurons in the DG 16 mo after intramarrow injection of the vector. Transgene expression was not seen in the DG of control animals whose BM was either inoculated with a control vector [SB(BUGT)], or saline. Transgene-positive cells were mostly observed in the DG and SVZ (Figure 4A). Transgene-positive cells were not seen before 1 mo and few of them were detected at 4 mo. Numerous transgene-positive cells were observed in the DG and SVZ 16 mo after intramarrow injection of the vector. Some of the transgene-expressing cells were also positive for NeuN, a marker of mature neurons, not only in the GCL but also in the hilus. Transgene-positive cells expressing NeuN had the shape of neurons of the DG. There were also some transgene-expressing cells that were not NeuN positive in the same areas, and some of these cells had the morphology of microglial cells and were immunopositive for CD11b-C3bi, and CD68, both markers of microglial cells. Very rare transgene-positive were astrocytes. Sixteen months after intramarrow injection of the vector, approximately 5% of DG cells were positive for the transgene. Forty-eight point six percent, 49.7% and 1.6% of these transgene-positive cells were respectively expressing markers of neurons, microglia, and astrocytes, as assessed by double immunocytochemistry for the transgene and lineage markers. We also determined transgene expression in the SVZ. The pattern of FLAG expression in the SVZ was similar as in the DG. One and 4 mo after intramarrow injection of the vector, no or few cells were expressing FLAG; numerous transgene-positive cells were observed in the SVZ 16 mo after intramarrow injection of SV(Nef-FLAG). About 50% of transgene-positive cells were of neuronal lineage because stained by neurotrace (NT), a neuronal marker. This percentage is close to the one observed in the DG. Thus, transgene-positive cells can be observed in areas of the CNS other

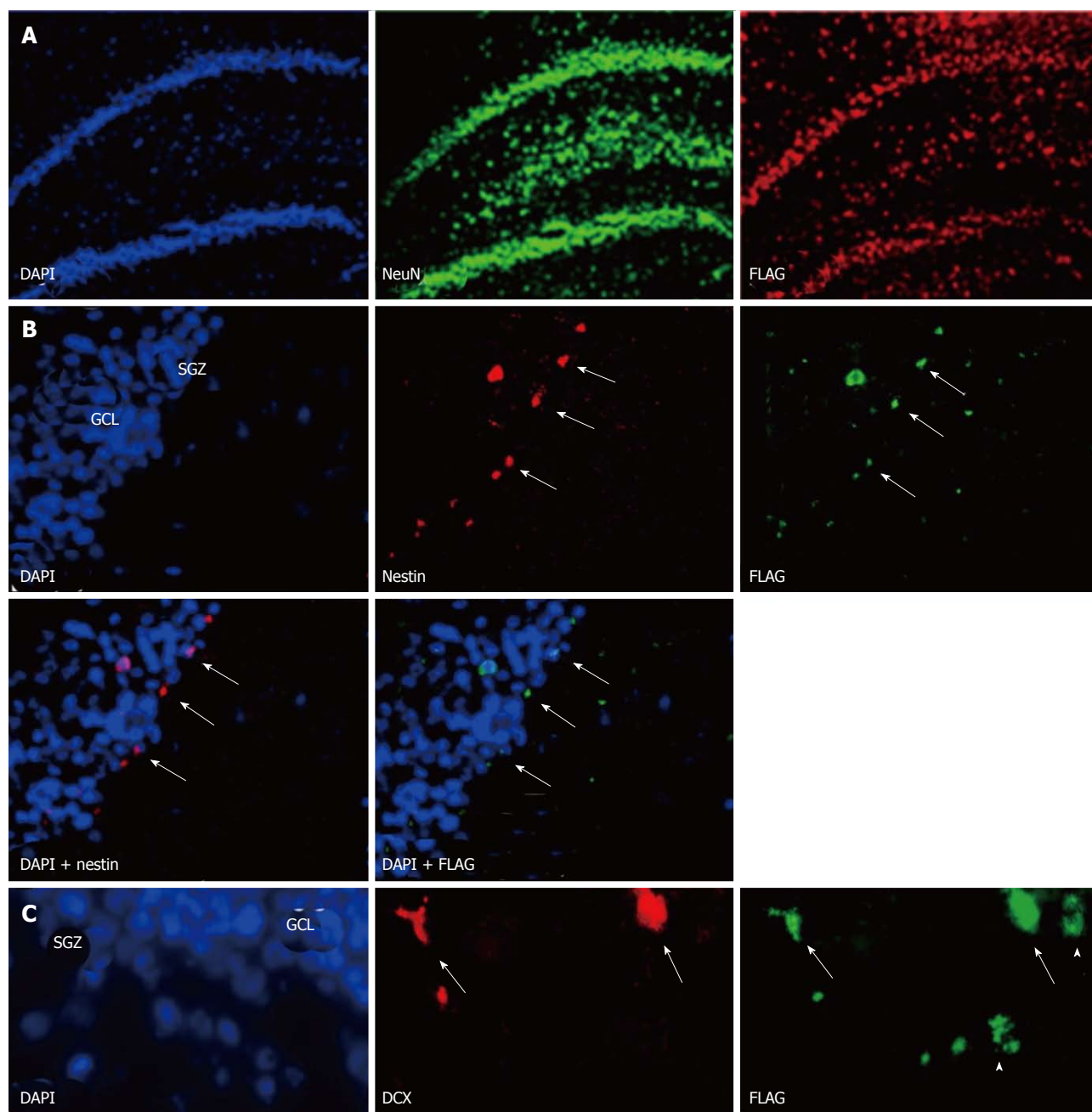


Figure 4 Bone marrow derived cells can migrate to the rat normal hippocampus. Sixteen months after injection of SV (Nef-FLAG) into the rat bone marrow (BM), transgene expressing cells were detected in the dentate gyrus (DG). A: FLAG-positive cells colocalized with NeuN, a marker of mature neurons. FLAG+/NeuN+ cells were located in the granular cell layer (GCL), as well as in the subgranular zone (SGZ) and the hilus. No FLAG-expressing cells were detected in the brain after injection of SV (BUGT), a control vector, in the BM; B: Nestin+/FLAG+ cells were detected mainly at the border SGZ/GCL, and more rarely in the GCL (arrows); C: DCX+ positive cells expressing FLAG were seen at the border SGZ/GCL and more rarely in the GCL (arrows). Note that some FLAG+ cells were DCX-, and were probably mature neurons (arrowheads). In all experiments, nuclei were stained in blue by Vectashield containing DAPI.

than the DG. Our results show that cells present in the adult BM can migrate to the CNS where they differentiate into cells of CNS lineages.

Consequently, it appears, that gene delivery to the BM induces transgene expression in brain cells of different lineages, in the DG and the SVZ. These results show that adult neurogenesis continues in the DG and SVZ during adult life, and that at least some of the cells generated that way derive from one or more populations of resident BM cells at the time of the administration.

Other hypotheses can explain these observations. rSV40 vectors can transduce CNS cells when directly administered into the brain^[53]. However, DG cells would unlikely be transduced at the time of the injection of the vector in the BM. Only 16 mo after the injection were transgene-positive cells seen in the DG. Transgene-positive cells were absent the first weeks after injection of the vector and were rare 4 mo after intramarrow injection of the vector. Moreover, transgene-positive cells were rarely detected in epithelial cells of other

organs usually transduced by intravenous rSV40 administration (e.g., liver, kidney), at all times after injection, suggesting that the vector did not diffuse significantly from the site of injection^[54,55].

Others also suggested that BM progenitor cells might differentiate into mature brain cells. It has been shown that endogenous neural stem cells are present in the DG and SVZ, and can generate different types of brain cells^[56]. These endogenous progenitor cells may be responsible for the constant remodeling taking place in the HC and OB^[15,16]. The apparent maturation and differentiation of BM progenitors seen in the present study is likely a physiological process because no insult was caused to the brain. Various types of injury can lead the adult rodent brain to repair itself by neurogenesis^[57-59]. Neurogenesis taking place in the HC and SVZ following such insults is based on the generation of new neurons that are able to migrate a considerable distance from their origin^[57]. Moreover, these regenerated neurons can replace dying cortical ones^[60,61].

It is still unclear if such process takes place in humans during physiologic or pathologic conditions. However, our results were observed without injury given to the CNS. Both experimental and human data showed that transplantation of male HSC coming from the BM into female recipients resulted in CNS neurons that were bearing Y chromosome^[45,46]. Cogle *et al.*^[46] detected neurons in the HC of women recipients coming from male BM transplants. However, this process took several months to occur. These data are coherent with our results^[46,62,63]. Similarly, BM progenitor cells from male mice, transplanted into immunocompromised female recipients, generated cells positive for Y-chromosome, that were detected in the brain and were positive for neuronal markers^[45,46].

Studies using gene-marked BM cells have shown that BM-derived cells may transdifferentiate into CNS cells of one or more lineages^[47]. Cell-cell fusion might support some of the reports^[62-64]. However, when they are exposed to certain growth factors (*i.e.*, brain-derived neurotrophic factor, epidermal growth factor), or when they are grown together with fetal mesencephalic or striatal cells, cultured BM stromal cells can express some glial and neuronal antigens^[65]. Similarly, when they are administered into brain ventricles of neonates, BM stromal cells can differentiate into cells immunopositive for astrocytic and neuronal markers^[66]. It has been suggested that BM progenitor cells can trans-differentiate into brain cells of different lineages^[45]. However, other reasons can explain these observations as well, and the mechanism(s) of such trans-differentiation are not clear as yet.

The first step in the migration of BM progenitor cells to the brain would be the homing of the BM-derived population(s) to their target. The mobilization pathway of HSC towards the brain involves a complex and intimate collaboration between adhesion molecules, cytokines, proteolytic enzymes, stromal cells, and HSC. This process can explain the regulation of HSC release, migration, and homing from the BM to the brain^[67].

Vascular and extracellular matrix molecules have an important role as well^[22,45,68-70]. Numerous reports involve mechanisms and factors inducing or promoting mobilization of HSC from BM into peripheral blood (PB). Several cell membrane proteins, including CXCR4 and its ligand, α -chemokine stromal-derived growth factor-1 (SDF-1), are involved in the mobilization of HSC to the PB^[71-74]. Different factors can increase the number of HSC migrating to the PB. Among these factors, some are related to tissue or organ injury, strenuous exercise and stress, local or systemic inflammation, and finally pharmacological agents such as CXCR4 small-molecule antagonist AMD3100 and granulocyte colony-stimulating factor (G-CSF)^[74-77]. Among these factors affecting HSC mobilization, G-CSF and SDF-1 are the best known. It must be noted that the activation of the complement cascade is activated by all these processes^[74,76].

BM niches are retaining HSC through the interaction between the chemokine CXCR4 receptor and $\alpha 4 \beta 1$ integrin. The respective ligands of CXCR4 and $\alpha 4 \beta 1$ integrin are α -chemokine SDF-1 and vascular adhesion molecule-1 (VCAM-1, also known as CD106), and they are present on cells in the BM niches (e.g., fibroblasts and osteoblasts)^[74-77]. One of the main factors affecting HSC mobilization, G-CSF, operates in two ways: First, it disrupts the anchoring relationship by decreasing the expression of SDF-1, thus reducing the binding of SDF-1 to CXCR4. Secondly, G-CSF enhances serum levels of other cytokines and growth factors^[67,78,79].

SDF-1 (chemokine CXCL12) is highly expressed in the BM where it is generated by osteoblasts in the endosteal region, as well as by endothelial cells and reticular cells located in the BM stroma. SDF-1 is a potent chemoattractant for HSC and it controls cell adhesion and survival as well. Synthesis of SDF-1 obeys to a circadian rhythm regulated by the sympathetic nervous system. Noradrenaline operates *via* $\beta 2$ -adrenoreceptors present on osteoblasts and *via* $\beta 3$ adrenoreceptors expressed in nestin-positive stem cells in order to decrease their production of SDF-1^[80,81].

CXCR4 and CXCR7 have been described as two chemokine receptors for SDF-1. The relationship between SDF-1 and CXCR4 in HSC is believed a key factor in the control of the traffic of HSC in the BM^[82,83]. AMD3100 is a powerful bicyclam CXCR4 antagonist that acts synergistically with G-CSF in humans. AMD3100 enhances mobilization of HSC in the BM^[84]. Once mobilized, HSC express decreased levels of CXCR4. CXCR7 is a second high-affinity receptor for SDF-1. However, CXCR7 is not linked to signaling pathways for migration of HSC. SDF-1 is internalized then degraded once bound to CXCR7; consequently, CXCR7 appears to act as a SDF-1 sink^[67,78]. CXCR4 and CXCR7 interact, but CXCR4 inhibition does not seem to modify the role of CXCR7^[67].

There are several factors influencing the interactions SDF-1-CXCR4 and $\alpha 4 \beta 1$ integrin-VCAM-1. For example, the sensitivity to SDF-1 depends on the incorporation of the CXCR4 receptor into membrane lipid rafts^[67,85,86]. As a consequence, the migration of HSC is influenced

by gradients of the bioactive lipids sphingosine-1 phosphate (S1P) and ceramide-1 phosphate (CP1). S1P and CP1 result from membrane lipid metabolism and are involved in stem cell trafficking.

Other important molecules involved in HSC mobilization are proteolytic enzymes released from activated granulocytes and monocytes that are present in the BM. These enzymes operate by attenuating SDF-1-CXCR4 and $\alpha 4\beta 1$ integrin-VCAM-1 interactions in the BM microenvironment^[74-77]. Another example of molecular intervention is the role of innate immunity in this process. One of the molecules involved in innate immunity, $\beta 2$ -defensin ($\beta 2$ -D), influences the sensitivity of HSC to SDF-1^[76]. Different proteolytic cascades such as the complement cascade, coagulation cascade, and fibrinolytic cascade, as well as several other proteolytic enzymes secreted by cells present in the BM might have a role as well^[87]. Different stress situations, local or systemic inflammation, and administration of pharmacological mobilizing agents (*e.g.*, G-CSF and AMD3100) can influence these proteolytic cascades^[76].

Activated complement stimulates oxidative stress, activation of platelets, and injury of erythrocyte membranes, interacts with different proteolytic cascades in the BM, and consequently, triggers mobilization of HSC^[67]. The third (C3a) and fifth protein components of complement (C5) have also an important role in the mobilization of HSC^[88]. The first cells migrating from the BM are neutrophils^[88]. C5b-C9 complex (or membrane attack complex) induces release of S1P, from red blood cells and platelets^[89]. Thus, inflammatory process and innate immunity will induce the migration of HSC from BM into PB^[76].

In another cascade involved in the mobilization of HSC, the fibrinolytic cascade, plasminogen binds to the BM extracellular matrix (ECM). Secondly, various proteins components of the ECM, including fibrin, laminin, are damaged by plasminogen after it converted into plasmin. Other proteases, such as metalloproteinases MMP-3, MMP-9, MMP-12 and MMP-13, are also activated by plasminogen in order to reduce the levels of other components of the ECM, such as collagen^[90].

Heme oxygenase 1 (HO-1) is also involved in the mobilization process of the HSC^[91]. Among other roles, HO-1 mitigates the inflammation linked to the complement by increasing the expression of complement inhibitors CD55 and CD59 on endothelial cells^[92]. HO-1 participates also in the regulation of the expression of SDF-1^[93], a major factor in the retention of HSC in BM niches^[74-77]. It has been shown recently that HO-1 influences actually negatively the adhesion and migration of neutrophils during acute inflammation^[94]. It has been suggested that negative regulators of the mobilization of HSC from BM exist. But few results have been reported so far. Serine protease inhibitors (serpins)^[95] and tissue inhibitors of metalloproteinases (TIMPs)^[96] seem to have such inhibitory effects on mobilization of HSC. HO-1 seem to influence negatively the migration of HSC^[74].

Concerning homing of progenitor cells to organs,

and not PB, other factors can be involved. For example, resveratrol enhances migration of mesenchymal stem cells to injured liver^[97].

After mobilization from the BM, the second step would be the differentiation into CNS cells of different lineages. However, mechanisms of differentiation are not totally clear yet^[98]. Neural cell adhesion molecules (*i.e.*, N-CAM), proteins regulating cell cycle transit^[99] and transcription factors^[100] have been implicated in this process, as well as molecules of the ECM^[68,69]. Ectopic expression of a specific set of transcription factors (c-Myc, Sox2, Oct4 and Klf4) can reprogram mouse embryonic and adult fibroblasts into embryonic stem-like cells^[101]. These cells were called induced pluripotent stem (iPS) cells. It has been reported that stem cells can be reprogrammed into iPS cells more effectively than into mature cells. Other types of cells can demonstrate multipotency. Among these cells, multilineage-differentiating stress-enduring (MUSE) cells^[102] and mesenchymal stem cells (MSC) have been described^[103,104]. MUSE cells are adult stem cells. They are characterized by differentiation into different lineage (mesodermal-, ectodermal- and endodermal) cells from a single cell. MUSE cells are tolerant to stress. They express markers of pluripotency, and are able of self renewal^[102]. They can be obtained from fibroblasts. Properties of MUSE cells resemble those of iPS cells, but they are devoid of tumorigenicity^[102]. MSC are a population of multipotent, self-renewing cells, mostly located in a bony niche, that regulate skeletal tissue and repair^[105].

High levels of sonic hedgehog and induction of Wnt signaling induce derivation of floor plate from pluripotent stem cells, increasing the quantity and quality of dopaminergic (DA) neurons consequently produced^[106,107]. These results led to the development of protocols for differentiation of mouse iPSCs into DA neurons^[108]. These newly generated cells expressed markers of DA neurons, such as the enzyme tyrosine hydroxylase, as well as the transcription factors Nurr1, and Pitx3. Once transplanted in a rat model of Parkinson's disease, these DA cells could repair lesions seen in the animal model^[108].

Transdifferentiation of fibroblasts to neurons has been reported. The introduction of three specific factors of neurodevelopment (Brn2, Ascl1 and Myth1l, or BAM) in mouse fibroblasts, directly generated neuronal cells, called induced neurons, or iNs^[109]. Similar approach was used with the same results by using human fibroblasts^[110]. Mouse and human iNs were characterized by immunopositivity for neuronal markers such as Tuj1, Map2, Tau and synapsin. Moreover, the combination of the iN factors with Lmx1a and FoxA2^[111] or a combination of Lmx1a and Nurr1^[112] in fibroblasts can directly generate cells with DA neuronal characteristics, named iDA neurons. The combination of the three neurodevelopmental factors (Brn2, Ascl1 and Myth1l, or BAM) with other factors implicated in the embryonic development of DA neurons enhances the transdifferentiation towards cells presenting characteristics of DA neurons^[113-118].

It is also possible to induce the transformation of

acinar exocrine cells from pancreas of cadavers into pancreatic β -cells. Cells generated that way can produce insulin, and are glucose-regulated. Once transplanted into immunocompromised diabetic mice, these cells can normalize glycemia^[119].

In our experiments, we examined the brain and other organs^[120] to assess the transgene expression outside of the BM in animals whose BM was injected with SV40-derived vector. We found that CNS cells of different lineages expressed the transgene several months after intramarrow administration of the vector, suggesting that transgene-positive cells were likely to have migrated from the BM. About 5% of DG cells were transgene-positive. BM progenitor cells were also migrating towards other organs (*i.e.*, spleen, lungs, liver). In the lungs, FLAG-expressing cells were mainly seen in the alveoli and were coexpressing markers of progenitor cells (*i.e.*, TTF-1) and of macrophages. Our studies do not determine the nature of the BM progenitors migrating to the CNS. These cells may be of hematopoietic, stromal or other origin. The hypothesis that BM cells can transdifferentiate into CNS cells of different lineages has been the topics of several conflicting reports. However, various animal models, different experimental paradigms and diverse methods have been used and could explain the discrepancies observed in these reports. In most of the studies against the hypothesis of BM to CNS transdifferentiation, the markers used are protein products of transgenes^[121-123]. Transgene delivered by numerous vectors, integrating or not, may disappear with time, particularly if they are of protein origin. By contrast, rSV40 vectors lead to long-term transgene expression^[54,124]. In other studies, DNA markers have been used to assess the engraftment of stem cells and the differentiation of donor cells in to host cells^[125,126]. DNA probes, such as the ones in FISH assays, were used in most studies sustaining the hypothesis of BM to CNS transdifferentiation. One of the important factors explaining the conflicting data appears to be the time between the transplantation of progenitor cells in the brain or in the periphery and their differentiation. Human cells transplanted into the mouse lateral ventricle differentiate into neurons in the OB only after several months^[61]. The number of transgene-positive neurons enumerated in the brain after injection of GFP-expressing HSC increases with age^[62,63]. The brief time intervals between the injection of the animals with progenitor cells and the harvest of the tissues might explain some negative results^[121]. However, these negative results might be due to the full maturity of the neurons, to the molecular marker used, and to the type of neurons as well. Our work suggest that BM-derived cells found in the DG are mature neurons, because expressing NeuN. They also have the shape of mature neurons in the considered area. However, we do not know at this point what is their physiological function and what type of synapses are established.

The settings of the experimental system might also explain the results observed. For example, some experiments include treating HSC *in vitro* with growth

factors that might modify their properties before their administration. Such treatment could potentially influence the homing of transplanted cells towards the brain or their differentiation into cells of CNS lineages. These experimental settings do not reproduce physiological conditions. By avoiding some of these treatments, we were able to transduce the BM directly without perturbing the different BM populations of cells. Thus, we can suggest that BM resident cells can transdifferentiate into cells of CNS lineages. However, additional studies would be useful to explore the functions of these cells.

Bone marrow progenitor cells express different markers of neuronal differentiation in the brain

We reported above that permanent BM-directed gene transfer using recombinant SV40-derived vectors led to expression of the transgene in mature neurons, and thus without CNS lesion indicating that BM progenitor cells can differentiate into cells of different CNS lineages. Most of transgene-expressing cells also expressed NeuN, a marker of mature neurons. However, it remained to be determined by what mechanism the cells from the BM come to be neurons. Although the observed gradual increase in transgene-expressing neurons over 16 mo suggested that the pathway involved differentiation of BM-resident cells into neurons, we could not rule out cell fusion as the principal route. Therefore, we tested here whether BM-derived progenitor cells migrating in the CNS could express markers of neuronal precursors or immature neurons. We injected SV40-derived vectors, carrying marker epitopes (FLAG or AU1), into the femoral cavities of rats or rabbits. Control animals received a control vector, SV (BUGT), injected into the BM as well. AU1- or FLAG-positive cells were seen in the DG 16 mo after injection of respectively SV (RevM10.AU1) or SV (Nef-FLAG) in the BM. In addition to cells expressing markers of mature neurons, transgene-positive cells were also positive for nestin and doublecortin, molecules expressed by developing neuronal cells. These cells were actively proliferating, as shown by short term BrdU incorporation studies (Figures 4B, 4C and 5). These results confirm that BM progenitors migrate to the CNS where they become neurons, by differentiating into neuron precursors and subsequently developing into mature neurons. Similar results were seen in the rat (Figures 4B, 4C and 5) and the rabbit (Figure 6).

This progression recapitulates the sequential stages of neurogenesis. Transgene-expressing cells positive for nestin and DCX were mainly localized at the border between SGZ and GCL, and less frequently in the GCL. This location is the one usually reported for immature neurons in the hippocampus. Cells in the SGZ have been shown to migrate into the GCL^[30].

In assessing the studies that report support for, or evidence against, BM cell transdifferentiation into other lineages^[121,122], the time between the administration of stem cells and harvest of the tissue is a critical factor^[61-63,66]. Thus, a brief time interval between injecting animals and collecting tissue may not identify markers

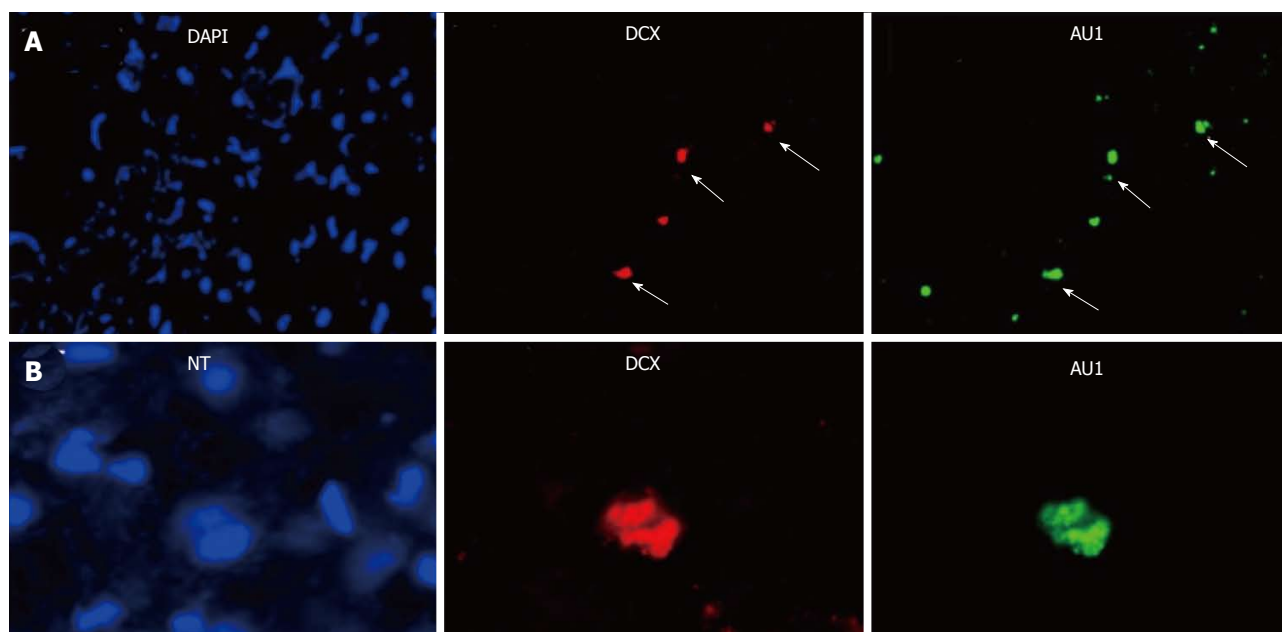


Figure 5 Transgene (AU1) expression in the hippocampus of rats whose bone marrow has been injected with SV (RevM10.AU1). A: Almost all DCX-positive cells were expressing AU1 (arrows); B: DCX+/AU1+ cells were of neuronal lineage, because they were stained by NT (arrows). No AU1 expression was seen after bone marrow injection of SV (BUGT), a control vector. DCX: Doublecortin; NT: Neurotrace.

belonging to the BM cells in the target organ studied. We have found that increases in neuronal and microglial cell populations expressing transgenes delivered to BM were gradual and protracted. No transgene-expressing neurons were seen 1 mo following BM injection, a few at 4 mo and many were at 16 mo^[51]. BM cells can undergo spontaneous fusion with other cell types, a process that is probably unlikely. Higher levels of transdifferentiation have been reported in some studies^[127]. The results observed in physiological conditions, as here, should also be compared to the ones seen during brain damage^[128-132] in order to determine the putative factors influencing the migration and differentiation of BM-derived cells in adult CNS.

FACTORS INFLUENCING NEUROGENESIS IN ADULT BRAIN AND ROLE OF SEIZURES

Factors influencing neurogenesis

Various physiological and pathological factors may increase neurogenesis in different areas of the brain. Neurogenesis is found to be increased in acute neurodegenerative disease^[28,133]. Hippocampal neurogenesis is enhanced during Alzheimer's disease^[134]. Brain injury such as stroke also leads to an increase in neurogenesis in humans^[135]. Neurogenesis may be increased following damage to the adult brain^[57,59]. Different classes of molecules have affected the rate of neurogenesis, including growth factor, hormones^[136] and neurotransmitters. Testosterone can increase generation of new neurons in the DG of adult male rodents^[136]. Blockade of neuroinflammation can restore adult neurogenesis in the hippocampus^[137].

Neuronal loss, inflammation, regeneration and gliosis following kainic acid-induced seizures

It has been reported that seizures can induce neuronal regeneration in the DG, but the extent of the process is hampered by the inflammation following the seizure. We first identified neuronal regeneration post kainic acid (KA)-induced seizure. For this purpose, we injected intraperitoneally 10 mg/kg KA in rats. KA causes tonic-clonic seizures. After the animals recovered from these seizures, they were rested for 7 d and their brains were examined for neuron loss in the HC, using either NeuN or NT as neuronal markers. We observed that neuron loss was most prominent in the CA1 and CA2/CA3 regions of the hippocampus, but loss of neurons was statistically significant, compared to control rats receiving vehicle only, in the hilus, SGZ/GCL and in CA1, CA2 and CA3.

At the same time, we demonstrated an inflammatory infiltration in the HC by enumerating macrophage/microglial cells using antibodies against Iba-1 (quiescent and activated cells) and CD68 (activated cells), and astrocytes using antibody against GFAP. Numbers of GFAP- and Iba-1-positive cells were increased in the HCs of KA recipients, compared to controls, indicating that brain injury related to KA administration led to infiltration of the affected area by macrophages and microglia, and to an astrocytic response. Neuroinflammation was associated with an increased expression of cytokines and chemokines, particularly regulated on activation, normal T cell expressed and secreted (RANTES) and macrophage inflammatory protein 1 α (MIP-1 α), as well as C-C chemokine receptor type 5 (CCR5) itself. RANTES and MIP-1 α are both ligands of CCR5 receptors. KA-induced increases in production of CCR5 ligands and ICAM-1

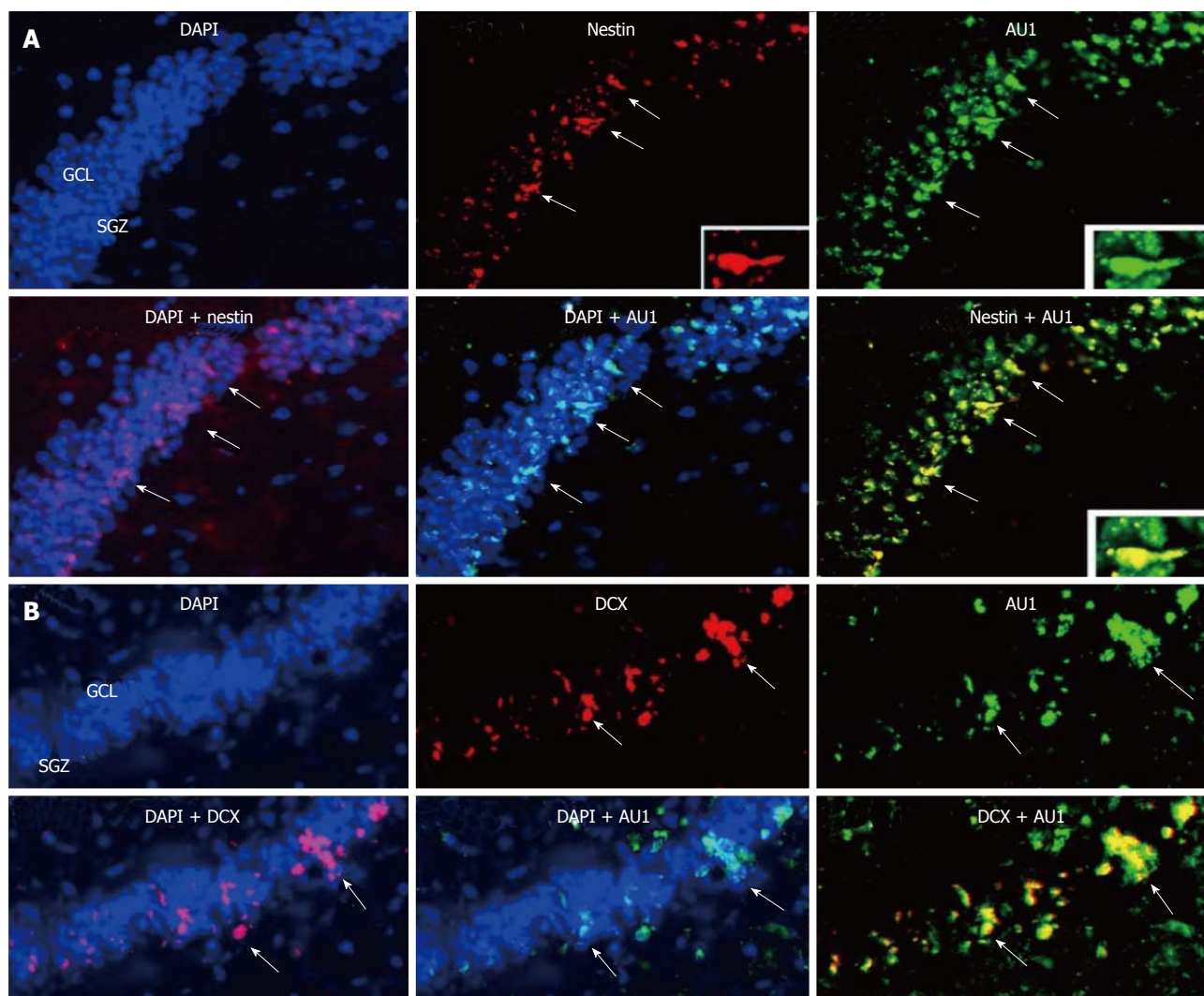


Figure 6 Migration of bone marrow derived cells to the rabbit hippocampus. Sixteen months after injection of SV (RevM10.AU1) into the rabbit bone marrow (BM), transgene-positive cells were seen in the dentate gyrus. Numerous AU1-positive cells colocalized with NeuN, a marker of postmitotic neurons (not shown). A: Nestin+/AU1+ cells were seen mainly at the border SGZ/GCL, and more rarely in the GCL (arrows); B: DCX+ positive cells expressing AU1 were detected at the border SGZ/GCL and more rarely in the GCL (arrows). SGZ: Subgranular zone; GCL: Granular cell layer; DCX: Doublecortin.

within blood vessels suggests that CCR5⁺ cells may be increased in the hippocampi of KA-treated rats, compared to control animals. We therefore enumerated CCR5⁺ cells in several areas of the HCs of rats treated with KA or saline. No CCR5⁺ cells were detected in control rat HCs. In contrast, CCR5⁺ cells were significantly more abundant throughout the hippocampi in KA recipients. In HCs of rats injected with KA, CCR5 was expressed mainly by lymphocytes, monocytes/macrophages, microglial cells, to a lesser extent by neurons, and rarely with astrocytes. This expression was seen not only in inflammatory cells in brain parenchyma, but in vessel walls as well.

KA-induced injury also elicits a regenerative response: Increased new neurons are formed following the insult. We assessed the level of cell proliferation and the populations of cells involved by labeling with BrdU. BrdU-expressing cells were seen in the GCL and SGZ of HCs from rats injected with KA. Very rare cells positive for BrdU were seen in HCs of controls. In KA recipients, 80.2% of these cells were neurons. In addition, proliferation of both

immature neurons and neural stem cells was involved, as evidenced by BrdU positivity in cells that also expressed doublecortin and nestin, respectively (Figure 7).

CCR5 regulates migration of bone marrow progenitors to the DG and limits neuroregeneration in response to injury

We reported above that KA elicits injury, inflammation and neuron regeneration in the HC. In the next step, we showed that decreasing CCR5 on bone marrow-derived cells reduces the number of KA-induced seizures, and related injury and inflammation. We used for that a bifunctional vector composed of a vector targeting CCR5 (RNAiR5) and a vector carrying a protein tag, used to evaluate transgene expression. The effect of *RNAiR5* gene (targeting CCR5) delivery to the BM on the migration of BM progenitors to the DG and their differentiation into neurons was assessed, with and without KA treatment.

Intraperitoneal injection of 10 mg/kg KA causes grade 5 tonic-clonic seizures (range of seizure duration,

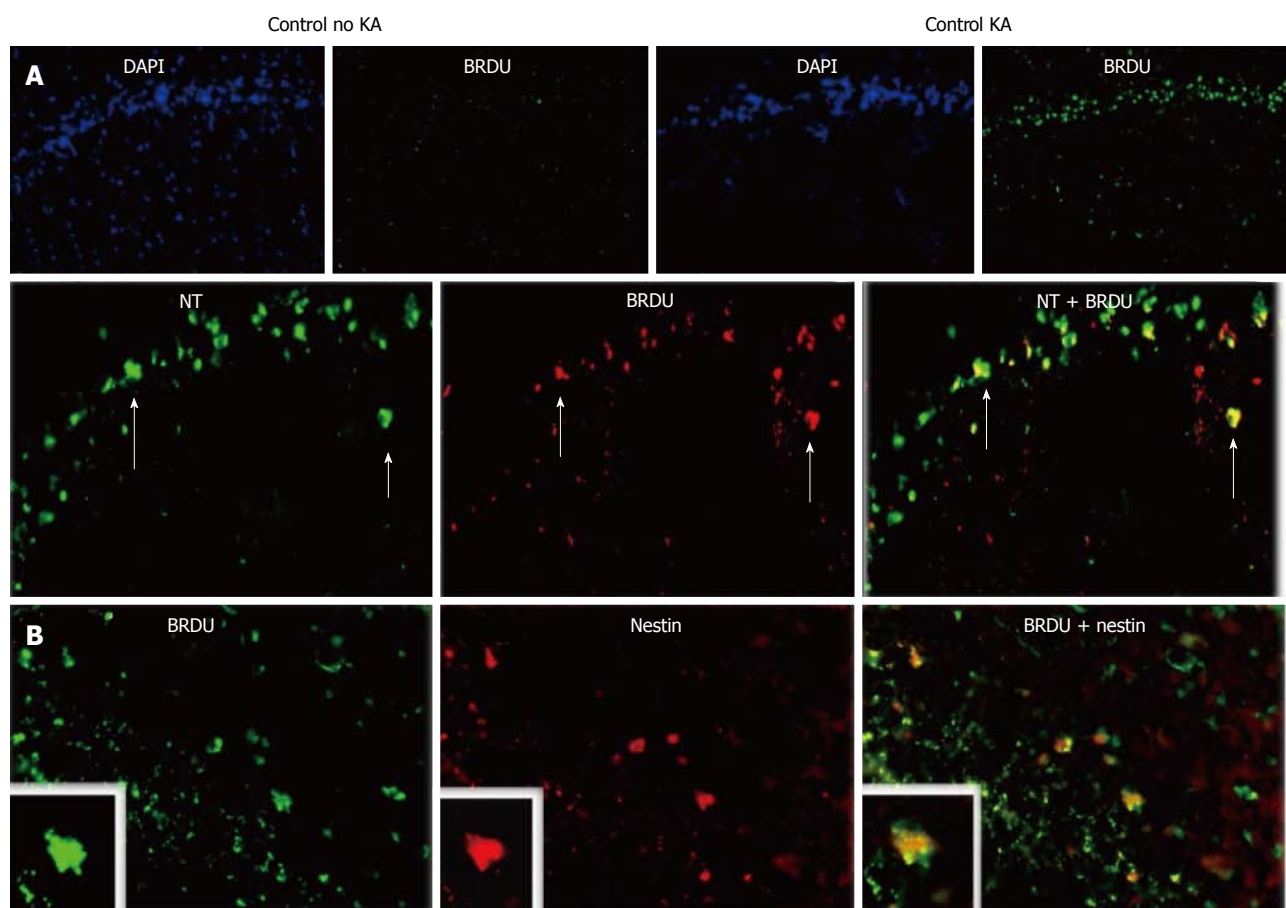


Figure 7 Kainic acid-induced regeneration in the hippocampus. Rats were injected with kainic acid (KA) intraperitoneally (*i.p.*), 10 mg/kg, and their hippocampi (HCs) analyzed by immunomicroscopy 7 d thereafter. A: Neurogenesis following KA treatment. Rats given KA or saline were injected with BrdU. Then, at 7 d post-KA, their DGs were immunostained for BrdU to visualize proliferating cells. DAPI counterstain is shown to facilitate interpretation. Arrows show neurons positive for BrdU. Double staining with Neurotrace (NT) for neurons is shown below; B: Double staining for nestin, a marker of proliferating and migrating neural cells (modified from Ref. [132]).

30–80 s) in normal rats beginning an average of 32.5 ± 4.7 min post-injection (range, 20–45 min). The average number of such seizures within a 4 h period after KA administration was 23.6 ± 3.4 (range, 21–28). Rats were given SV (RNAiR5-RevM10.AU1), the monofunctional vectors SV (RevM10.AU1) or SV (RNAiR5) intrafemorally. SV (BUGT) was used as control vector. Animals were challenged 4 mo later by *i.p.* injection of KA. Rats given RNAiR5-containing vectors were significantly protected from KA-induced seizure activity compared to rats receiving either SV (BUGT) or SV (RevM10.AU1), by all criteria applied: Time of seizure onset, number and severity of seizures and recovery time. The two RNAiR5-carrying vectors were comparable in protecting from KA-induced seizure activity, and were highly significantly different from the two control vectors [SV (RevM10.AU1), SV (BUGT)].

Four months after injection of the SV40-derived vector that does not target CCR5, and without KA administration, very few BM progenitors were seen in the DG. This is consistent with the observations reported about concerning the migration of BM-derived cells to the CNS. By contrast, in rats receiving a vector carrying the RNAi

that targets CCR5, there was a significant increase in the numbers of bone marrow-derived cells expressing neuronal markers. Thus, decreasing CCR5 led to increased bone marrow cell migration to the brain and increased DG neurons derived from those cells. However, in the absence of KA treatment, the number of cells originating from the BM was low. After KA treatment, and notwithstanding the ability of RNAiR5 to mitigate KA-induced injury, numbers of DG cells derived from the bone marrow was far greater in recipients of the vector targeting CCR5 than in recipients of an unrelated vector.

We examined, further, the influence of bone marrow-directed gene delivery of RNAiR5 on DG neuroregeneration. Proliferating cells were visualized using BrdU, and immature neurons were identified using doublecortin and nestin as markers. Again, despite the mitigating effect of bone marrow-directed RNAiR5 on DG injury and neuroinflammation, proliferation of neuronal precursors in response to KA treatment was approximately 3-fold that seen in recipients of the control vector only. Moreover, $72.2\% \pm 7.4\%$ of BrdU-positive cells were also transgene-positive, and $79.8\% \pm 6.9\%$ of transgene-positive cells were positive for BrdU. Most of these cells were of neuronal

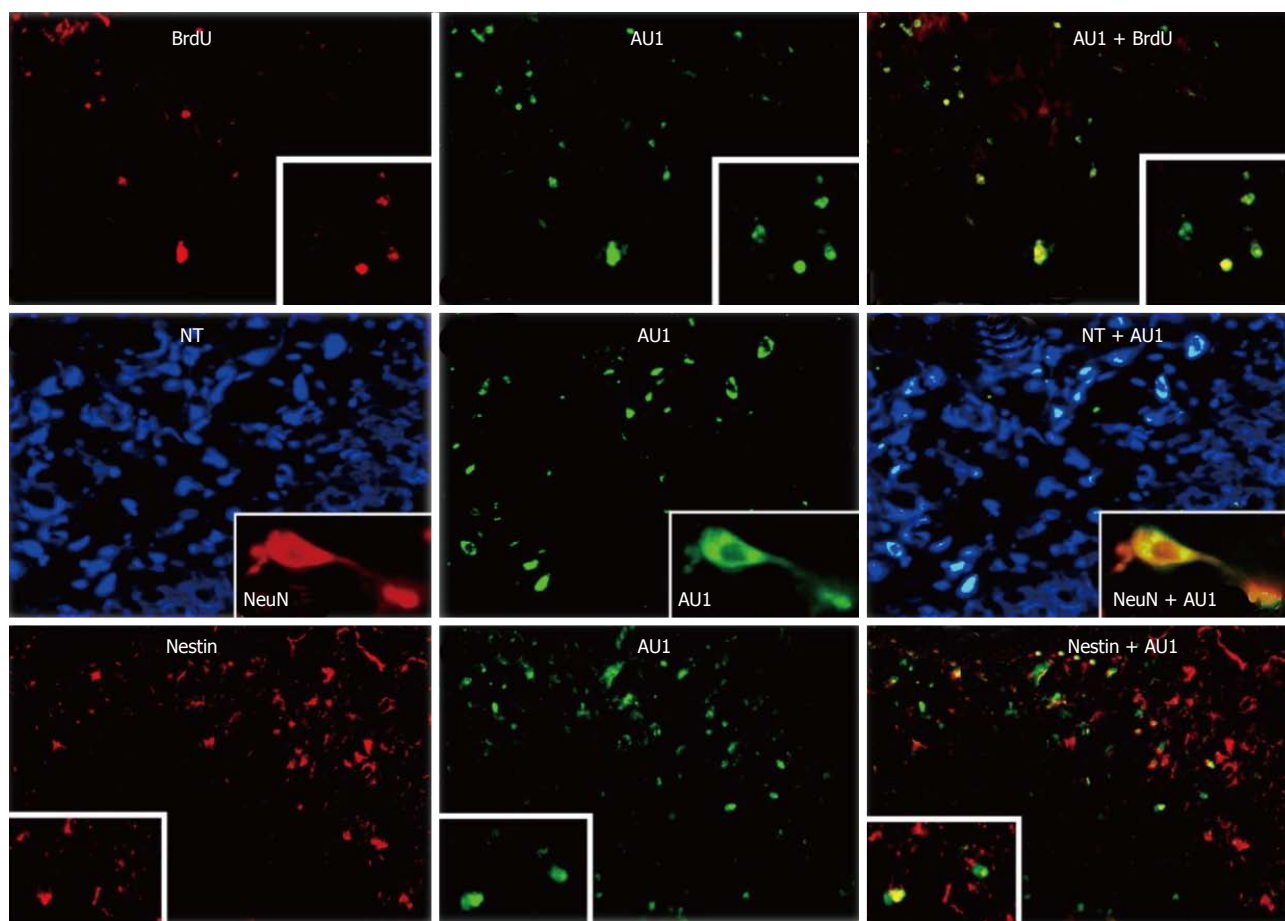


Figure 8 *RNAiR5* gene delivery to the bone marrow increases numbers of bone marrow-derived cells in the hippocampus and neuroproliferative activity in the hippocampus, in the resting state and in response to kainic acid treatment. Bone marrow-derived cells in the hippocampus (HC) were all neurons, and most proliferating cells in the HC were bone marrow-derived. Double immunostaining for AU1 plus BrdU (upper row) and nestin (lower row), and neuron identification using Neurotrace (NT) and NeuN (middle row) was performed. Shown are HC sections from kainic acid-treated rats, transduced with SV (RNAiR5-RevM10.AU1). Most of the AU1+ cells were also positive for BrdU (79.8%, \pm 6.9%) as a marker of cell proliferation, and most BrdU+ cells were also AU1+ (72.2%, \pm 7.4%). Numerous AU1+ cells were also positive for nestin, a marker of proliferating and migrating neurons, although there were as well many nestin-positive cells that did not express AU1. The equivalence of NT identification of neurons and NeuN immunostaining of neurons is illustrated in the insets in the middle row. Data are representative of 3 independent experiments (modified from Ref. [132]).

lineage, as demonstrated by immunopositivity for nestin and/or doublecortin or NeuN (Figure 8).

We reported that bone marrow-directed gene delivery of RNAi targeting CCR5 using a recombinant SV40-derived vector results in high levels of gene modification of bone marrow-derived cells, particularly Sca-1+ cells. As a consequence of the effectiveness of this approach to downregulating CCR5, our data suggest that the cell membrane chemokine receptor, CCR5, influences greatly the regulation of the traffic of BM progenitors towards the CNS, both in the basal state and in response to injury. Furthermore, reduction in CCR5 expression in circulating cells provides profound neuroprotection from, in this case, excitotoxic neuronal injury. CCR5 and its ligands enhance CNS inflammation and seizure activity, and may result in increased CNS injury as a result.

Inflammatory cell infiltration of the CNS entails adhesion of lymphocytes and monocytes in the blood to cerebrovascular endothelium, mediated by endothelial cell production of chemokines, clustering of integrins and migration of peripheral blood mononuclear cells

(PBMC) through the vascular endothelium into the brain. CCR5 and its ligands, RANTES and MIP-1 α , are known to be involved in this process^[138-144]. Thus, such transmigration is stimulated by RANTES but decreased by anti-CCR5 antibodies^[142,143]. Our data are consistent with these findings. Expression of MIP-1 α and RANTES on brain microvessels and endothelial cells is greatly increased after KA administration, and we showed that reducing cell membrane CCR5 probably decreases PBMC adhesion to CCR5 ligands^[145]. Lower levels of CCR5 on PBMC membranes following BM-directed gene delivery also decreases production of those chemokines by brain vascular endothelium. Migration of CCR5⁺ microglia and monocyte-derived macrophages is stimulated by CCR5 ligands and these cells, in turn, stimulate both endothelial activation and production of proinflammatory cytokines^[141]. Our results show also that the initial production of CCR5 ligands after KA administration is unaltered in rats injected with both control and rSV40s vectors targeting CCR5, but this changed over time between the recipients of control

vector and rSV40 vectors reducing CCR5. Thus, CCR5 is involved in a multiplicative effect of chemotaxis, stimulation of chemotaxis and then more chemotaxis. Altering this cycle by reductions in PBMC CCR5 may thus have neuroprotective and antiinflammatory effects that are disproportionate to the magnitude of the decrease in CCR5.

These results showing the interaction between CCR5 on PBMC and its ligands at the vascular level emphasize the role of vascular inflammation in KA-induced seizures. It has been reported that vascular inflammation and leukocyte-endothelial adhesion can participate in the development of seizures. $\alpha 4$ integrin and VCAM-1 antibodies can mitigate leukocyte-vascular interaction and prevent pilocarpine-induced seizures^[146]. It has been recently suggested that blood-brain barrier (BBB) breakdown can induce epileptiform activity^[147,148]. BBB disruption has been described after KA administration^[149-151]. During KA-induced seizures, disruption of the BBB is characterized by disappearance of tight junction ZO-1 and occludin, recruitment of neutrophils^[149], increase in the production of tissue plasminogen activator and NO^[150]. Activation of astrocytes, for example by glutamate agonists, can influence vessel permeability^[149]. Another chemokine, MCP-1 (CCL2), can induce BBB opening and KA-induced upregulation of MCP-1 mediates recruitment of macrophages and granulocytes^[151]. Increase of MCP-1 in blood vessels of HCs after seizures might lead to modifications of permeability of the BBB^[152]. Enhanced permeability of BBB can increase the access of KA to the parenchyma^[146]. In the pilocarpine model, it has been suggested that the effect of the drug can be to allow focal BBB leakage, which then synergizes with the CNS effects of pilocarpine to induce seizures. Leukocyte adhesion blockade prevented BBB opening in the pilocarpine model^[146]. In the present results, much less leakage of vascular contents from blood vessels was seen in HCs of rats given rSV40s vectors targeting CCR5, suggesting that the experimental reduction of the interaction between CCR5 on PBMC and CCR5 ligands on vessels limited BBB leakage.

BBB leakage is partly mediated by leukocytes through different mechanisms: Generation of oxygen free radicals, enhanced production of cytokines and chemokines, vascular alterations, release of cytotoxic enzymes. Adhesion of leukocytes to endothelium produces changes in small GTPases involved in cytoskeletal organization, and in calcium signaling, as well as in activation of kinases^[146,153]. Our demonstration that inhibition of interactions between CCR5 on PBMC and CCR5 ligands in vessels prevents BBB disruption after KA is consistent with prior observations about the effects of leukocyte adhesion on vascular permeability^[146]. Thus, our results show that CCR5 influences the synergistic interactions between leukocyte adhesion, endothelial activation, BBB leakage and seizure activity.

CCL3 (MIP-1 α)^[154,155] and CCL5 (RANTES)^[156,157] can be expressed by brain endothelial cells. Different mechanisms can be responsible for the induction of

CCL5 and CCL3 in the endothelium. Several studies have demonstrated the involvement of TNF- α and IL1- β in seizure activity^[129,158-160]. TNF- α and IL1- β can be rapidly produced by microglial cells^[161]. TNF- α and IL1- β stimulate expression of CCL5 in endothelial cells^[156,157,162]. TNF- α can also activate NF kappaB, the role of which has been underscored in seizure activity, including KA-induced neurotoxicity^[163]. TNF- α -induced CCL5 transcription involves cis-regulatory promoter elements, *i.e.*, NF- κ B, C/EBP β , NF-IL-6, NF-AT and the cAMP response element, CRE^[164]. TNF- α can induce NF- κ B, but apparently not AP-1, activity in endothelial cells^[165]. Other mechanisms upregulating CCL5 in endothelial cells involve HIF-1 α , JNK-2 and AP-1 (JunD). Lysophosphatidylcholine can induce rapid expression of CCL5 in endothelial cells by activating of multiple kinases^[166]. Increase of PLA2 activity has been reported in different models of epilepsy^[167] including kainic acid treatment^[168-170].

The type of benefit from decreasing CCR5 described in the present study has been seen in studies in which TAK-779, an inhibitor of CCR5, mitigates CNS damage due to ischemia^[171]. Targeting MIP-1 α may also protect from experimental autoimmune encephalomyelitis^[172] and other types of CNS injury^[173-176]. Another important consequence of BM-delivered, RNAi-mediated, decrease in CCR5 expression in blood cells is increased migration of BM progenitors to the DG, where they become neurons. These new neurons are generated in absence of lesions, but also can assist in repairing insults to the CNS^[177-179]. BM progenitors can migrate to the brain and become neurons by transdifferentiation or other processes^[44-48]. Inflammation may limit the ability of the brain to repair itself by neurogenesis, as the generation and survival of new neurons is inversely proportional to the magnitude of the inflammation^[128,180], and is facilitated by administration of inflammation inhibitors such as indomethacin^[137].

Our results underscore the relationship between ongoing neurogenesis and BM-derived cells. In those studies, we found almost no such cells in the DG at 4 mo after gene delivery to the BM, but numerous cells 15 mo after intramarow inoculation of the vector. Our current data show that decreasing CCR5 expression on bone marrow-derived cells both increased the basal level of neurogenesis from BM-derived progenitors and greatly amplified the regenerative response to excitotoxic injury. This observation is even more striking in light of the fact that recipients of the vectors targeting CCR5 had much less severe neuron loss than did controls. Furthermore, the level of neuron proliferation, as measured by BrdU incorporation, was also several-fold higher in recipients of vectors that contained RNAiR5 than in controls. It is likely, therefore, that targeting CCR5 by gene transfer or pharmacologic means may promote ongoing neurogenesis and neuroregenerative responses to injury. It might be due to an inhibition of inflammation, known for limiting neurogenesis^[128,137,180]. However, a direct role of CCR5 in regulating neuron regeneration cannot be excluded. It is not known whether the cell population(s) in the bone

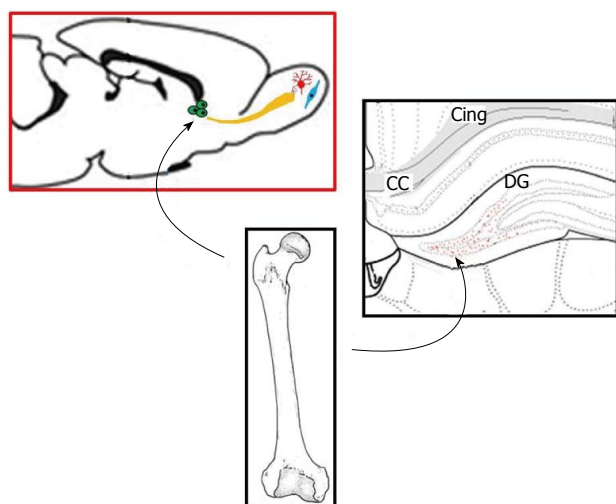


Figure 9 Figure suggesting the participation of bone marrow progenitor cells to neurogenesis in adults. DG: Dentate gyrus.

marrow that provide these neuron precursors are of hematopoietic or other origin.

Thus, the consequences of acquired deficiency in CCR5 highlights the role of CCR5 in neuroinflammation, excitotoxic injury, chemotaxis and astrocyte proliferation, and decreases in CCR5 may provide pronounced neuroprotection from such injury. In the present study, cells in the treated animals expressed less CCR5 (molecule per cell) than those from control animals. Even if the cells have not become negative for CCR5, the decreased expression of this receptor did affect their function. The distinction between a complete shutdown of CCR5 and reduced expression due to the RNAi is important, because if marginal reductions in a target can result in a disproportionate loss of function (due for example to reduced total avidity at the cell surface) there is greater hope for the use of such vectors in other *in vivo* applications.

In conclusion, our data demonstrate the centrality of CCR5 and its ligands in mediating injury-induced inflammation, and suggest that decreasing levels of CCR5 may have as its consequences neuroprotection and enhanced neuroregeneration. We confirm here that BM progenitor cells participate in neurogenesis in the adult brain, and migrate towards the DG and SVZ (Figure 9). SV40-based gene delivery of RNAi targeting CCR5 to the BM results in downregulating CCR5 in circulating cells. Consequently, the inhibition of interactions between CCR5 on peripheral blood mononuclear cells and CCR5 ligands in vessels prevents BBB disruption after KA treatment. The decrease of leukocyte-vascular interaction affects vascular permeability, thus, infiltration of parenchyma by inflammatory cells, and reduces neuroinflammation. Subsequently, our results imply that CCR5 influences the interactions between leukocyte adhesion, endothelial activation, BBB leakage and seizure activity. However, given the redundancy of cytokines and chemokines, CCR5 might be just one of the components implicated in the interaction between leukocytes and vessels, and

other chemokines, or other molecules, might be involved as well. For example, receptors for IL-1 and TNF- α are upregulated rapidly during seizures^[160]. The magnitude of seizure activity impacts on the inflammatory responses that follow seizures^[181-183]. Microglial activation, and production of IL-1 β , IL6, TNF- α and free radical species, directly affect the process of post-seizure neurogenesis^[181-184] and the survival of the neurons that are produced as a result^[159,182,185,186].

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Mucosal-associated invariant T cells from induced pluripotent stem cells: A novel approach for modeling human diseases

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Abstract

Mice have frequently been used to model human diseases involving immune dysregulation such as autoimmune and inflammatory diseases. These models help elucidate

the mechanisms underlying the disease and in the development of novel therapies. However, if mice are deficient in certain cells and/or effectors associated with human diseases, how can their functions be investigated in this species? Mucosal-associated invariant T (MAIT) cells, a novel innate-like T cell family member, are a good example. MAIT cells are abundant in humans but scarce in laboratory mice. MAIT cells harbor an invariant T cell receptor and recognize nonpeptidic antigens vitamin B2 metabolites from bacteria and yeasts. Recent studies have shown that MAIT cells play a pivotal role in human diseases such as bacterial infections and autoimmune and inflammatory diseases. MAIT cells possess granulysin, a human-specific effector molecule, but granulysin and its homologue are absent in mice. Furthermore, MAIT cells show poor proliferation *in vitro*. To overcome these problems and further our knowledge of MAIT cells, we have established a method to expand MAIT cells via induced pluripotent stem cells (iPSCs). In this review, we describe recent advances in the field of MAIT cell research and our approach for human disease modeling with iPSC-derived MAIT cells.

Key words: Mucosal-associated invariant T cells; Induced pluripotent stem cells; Differentiation; Adoptive transfer; Inflammatory diseases; Autoimmune diseases; Disease modeling; Infectious diseases; Immunocompromised mouse

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Core tip: Mucosal-associated invariant T (MAIT) cells, a novel innate-like T cell subset abundant in humans, play a pivotal role in immune-dysregulated diseases. However, MAIT cells are quite rare in laboratory mice and show poor proliferation *in vitro*. This makes it difficult to delineate their physiological functions in health and disease. Therefore, we developed a method to generate

human MAIT cells from induced pluripotent stem cells [redifferentiation of MAIT (reMAIT) cells]. Given that reMAIT cells harbor characteristics quasi-identical to those found in MAIT cells from human peripheral blood, they will be useful to model human diseases in animals.

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INTRODUCTION

T cells are distinguished from other lymphocytes, such as B cells and natural killer cells, by the expression of T cell receptors (TCRs) on the cell surface. T cells have been well-characterized as central players in adaptive immunity, so-called conventional T cells. The TCRs in conventional T cells consist of a heterodimer of α -chain and β -chain and are highly diverse owing to gene rearrangement together with insertion and/or deletion of nucleotides at the junctions between the gene segments, enabling them to recognize a wide variety of peptide antigens presented on major histocompatibility complex (MHC) molecules, which are also highly polymorphic^[1]. In recent years, however, non-conventional type T cells termed "innate-like" T cells have received keen attention in immune homeostasis and diseases^[2]. In contrast to conventional T cells, innate-like T cells express a limited set (semi-invariant) of TCRs and recognize nonpeptidic antigens presented on evolutionarily conserved non-classical MHC molecules^[3,4]. Innate-like T cells develop in the thymus, similar to conventional T cells^[5]. There is a time lag between the initial antigen exposure and execution of the maximum effector function in conventional T cell responses. Given that conventional T cells transit from naïve to effector/memory stage through the recognition of peptidic antigens, these T cells are ready to be activated and to expand upon receiving secondary stimuli to exert effector functions. In marked contrast, innate-like T cells have already acquired such immune competence when leave the thymus. This may be relevant to the fact that innate-like T cells, but not conventional T cells, express the transcription factor promyelocytic leukemia zinc finger (PLZF), which directs effector differentiation of these cells during thymic development^[5-7]. Thus far, it has been appreciated that the *raison-d'être* of innate-like T cells consists in filling a gap between innate and adaptive immunity^[8].

Mucosal-associated invariant T (MAIT) cells and natural killer T (NKT) cells are representatives of innate-like T cells expressing semi-invariant $\alpha\beta$ TCR in mammals^[2]. Because the discovery of NKT cell ligands has preceded that of MAIT cells, most of our knowledge on diseases has been made with NKT cells abundant in laboratory mice (but quite few in humans). NKT cells play a pivotal role in the suppression

of tumor growth and/or metastasis, and in ameliorating or aggravating autoimmune diseases^[9,10]. NKT cells produce a plethora of cytokines, including Th1-, Th2- and Th17-cytokines, upon stimulation, and MAIT cells also have a similar potential^[11,12]. Although they are different in many aspects such as antigens, restriction molecules for development and/or differentiation, and abundance, they are common in that they play a critical role in infectious diseases and autoimmune and inflammatory diseases. Regardless of their importance, it was not until recently that some information on MAIT cells has become available. In the last couple of years, there has been exciting progress regarding the functions of MAIT cells in the immunology field and in clinical settings. There are, however, some difficulties in studying MAIT cells, in that the frequency of MAIT cells is much lower in laboratory mice than in humans, and that MAIT cells show extremely poor proliferation *in vitro* with any T cell stimulants tested to date. Here, we provide an overview of recent advances in the study on MAIT cells and introduce our approach with induced pluripotent stem cell (iPSC) technology to overcome the experimental difficulties in MAIT cell study.

PHENOTYPIC FEATURES OF MAIT CELLS

MAIT cells are probably one of the most abundant T cell subsets in humans^[13]. However, until quite recently, MAIT cells had been hidden behind conventional T cells because they are indistinguishable from other T cell populations by standard T cell phenotyping using cell surface markers such as CD3, CD4 and CD8. MAIT cells are distinguished from conventional T cells and other T cell subsets such as NKT cells and $\gamma\delta$ T cells by the expression of an invariant TCR α chain, V α 7.2-J α 33 in humans and V α 19-J α 33 in mice, paired with a limited repertoire of TCR β chains; V β 13 and V β 2 are preferentially used in humans and homologous V β 8 and V β 6 in mice (Figure 1)^[13,14]. Together with invariant TCR α V α 7.2, human MAIT cells express a C-type lectin CD161 and interleukin (IL)-18 receptor α chain (IL-18R α) as specific markers^[15,16]. Primarily, MAIT cells are defined as CD3⁺, V α 7.2⁺, CD161⁺ and IL-18R α ⁺. MAIT cells can further be classified into CD8⁺ (most abundant), CD4⁻CD8⁻ [double negative (DN)] and CD4⁺ phenotypes (very few) in healthy human subjects^[13,17]. In addition, MAIT cells display CD45RA⁻, CD45RO⁺, CD95^{high}, and CD62L^{low} as their effector/memory T cell phenotype, and α 4 β 7 integrin⁺, CCR9^{int}, CCR7⁻, CCR5^{high}, CXCR6^{high}, and CCR6^{high}, suggesting MAIT cells home to the intestines and liver^[11,18,19]. High expression levels of CD161 in MAIT cells are accompanied by ROR γ t, IL-23R and IL-21R, markers associated with Th17/Tc17 type T cells^[11,19,20]. Furthermore, MAIT cells possess PLZF, indicating the capacity to promptly produce cytokines upon stimulation without priming^[7,17] and CD26⁺, a serine exodipeptidase, which processes chemokines in the extracellular matrix^[20,21]. Accordingly, MAIT cells have the potential to release a variety of cytokines under various conditions: Interferon (IFN)- γ , tumor necrosis factor (TNF)- α , IL-2, IL-4, IL-10, IL-17, IL-22, granzymes, and

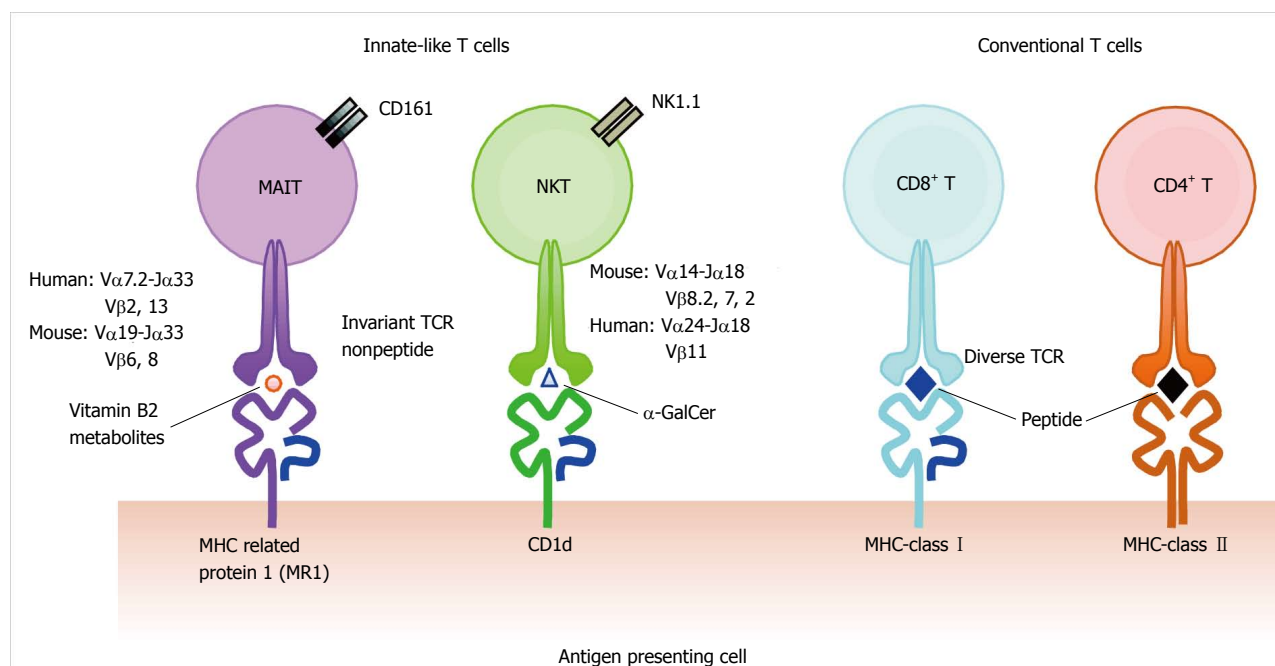


Figure 1 Comparison of the T cell receptors and the antigen presenting molecules among $\alpha\beta$ T cell subsets. Invariant T cell subsets consist of mucosal-associated invariant T (MAIT) cells and natural killer T (NKT) cells expressing invariant TCRs. MAIT cells and NKT cells recognize vitamin B2 metabolites on MR1, and α -galactosylceramide (α -GalCer) on CD1d, respectively. In contrast, conventional CD8⁺ and CD4⁺ T cells possess divergent TCRs and recognize a variety of peptides on major histocompatibility complex-class I and class II, respectively. TCRs: T cell receptors; MHC: Major histocompatibility complex.

others, which anticipates the multifaceted roles in health and diseases^[11,12,22].

MAIT CELLS AND MR1

The TCR of MAIT cells recognizes derivatives of vitamin B2 presented on the monomorphic MHC class-related molecule 1, MR1^[18,23] (Figure 1). MR1 mRNA is expressed ubiquitously in all types of cells, whereas the MR1 protein are not always on the cell surface but mainly in the endoplasmic reticulum^[24,25]. Although vitamin B2 derivatives are exogenous ligands from the biosynthetic pathway that some bacteria and yeasts possess, they are indispensable for the development of MAIT cells, because MAIT cells are absent in germ-free mice^[18]. TCRs for MAIT cells and MR1 are highly conserved during evolution, which suggests the functional and physiological importance of MAIT cells and MR1 in animals^[26]. Indeed, mouse and human MR1 molecules crossover part of the antigen presentation and activation in MAIT cells^[26].

MAIT cell development is dependent on MR1. Lymphoid progenitors derived from CD34⁺ hematopoietic stem cells in the bone marrow migrate to the thymus, wherein they undergo random rearrangement at the TCR loci. MAIT cell progenitors harboring the TCR V α 7.2-J α 33 are selected from CD4/CD8 double positive thymocytes that express MR1 loaded with unknown endogenous ligands^[18,27]. MAIT cells then egress from the thymus as naïve cells and further differentiate into effector/memory cells by recognizing commensal microflora-derived vitamin B2 metabolites bound to MR1 at mucosal sites^[18,19].

MAIT CELLS IN HEALTH AND DISEASES

MAIT cells consist of 1%-10% of T cells in the peripheral blood and of T cells in the intestinal lamina propria and 20%-50% in T cells of the liver, but they are at least 10 times less abundant in laboratory mice^[11,28]. MAIT cells are already present in the tissues of second trimester fetuses. Fetal MAIT cells exhibit a naïve phenotype but have potential functions in the activation and secretion of cytokines upon antigen stimulation^[29]. Although MAIT cells still showing a naïve phenotype and are low in frequency at birth, most of them have acquired a memory phenotype by 3 mo of age, and their frequency increases with age and reaches adult levels within 8-10 years after birth^[11]. This corresponds to the expansion and maturation of MAIT cells by commensal microflora colonizing after birth. The highest number of MAIT cells in PBMC is observed in adults aged 30-50 years, notably in females of reproductive age^[30]. MAIT cells, especially CD8⁺ MAIT cells as the most abundant subset, decrease drastically with age, implying an association with waning immunity in the elderly^[22,30].

The diseases in which a potential implication of MAIT cells has been reported are summarized in Table 1. A well-defined function of MAIT cells in disease settings is the control of infections with bacteria and/or yeasts. MAIT cells are activated by bacteria-infected cells in a MR1-dependent manner, followed by release of proinflammatory cytokines and cytotoxic granules, and eventually killing the infected cells^[16,31-33]. MAIT cells also express multidrug resistance transporter (ABCB1), which implies that MAIT cells are highly resistant to xenobiotics produced by bacteria^[11].

Table 1 Clinical relevance of mucosal-associated invariant T cells

Disease categories	Diseases or status	Features relevant to the diseases	Ref.
Infectious diseases	Pneumopathy	Decrease in frequency and absolute number of MAIT cells in peripheral blood	[16]
	Tuberculosis (<i>Mycobacterium tuberculosis</i>)	Decrease in frequency and absolute number of MAIT cells in peripheral blood Enriched in the lung	[16,37,92]
	HIV/AIDS (opportunistic infection)	Decrease in frequency of MAIT cells in peripheral blood, guts, and lymph nodes Failure of recovery of blood MAIT cells with successful cART Long-term cART restore colonic but not blood MAIT cell levels MAIT cells are depleted but retain functionality	[38-43,93,94]
	Sepsis (severe bacterial infection)	Decrease in frequency and absolute number of MAIT cells in peripheral blood of patients	[95]
	<i>P. aeruginosa</i> infection with cystic fibrosis	Decrease in frequency of MAIT cells in peripheral blood of cystic fibrosis patients with <i>P. aeruginosa</i> infection	[96]
	Cholera (<i>Vibrio cholera</i> O1)	Activation of MAIT cells in the acute phase No change of blood MAIT cell frequency in adult patients, but persistently decreased in child patients	[97]
Autoimmune diseases	Multiple sclerosis	Accumulation of MAIT cells in the central nervous system lesions Decrease in frequency of MAIT cells in peripheral blood Increased CD161 ^{high} CD8 ⁺ T cells in peripheral blood Accumulation of MAIT cells in the peripheral nerves	[44-46,48] [98] [44]
	Chronic inflammatory demyelinating polyneuropathy		
	Psoriatic and rheumatoid arthritis	Enrichment of CD161 ^{high} CD8 ⁺ T cells in the joints and secretion of IL-17 from those cells	[99]
	Rheumatoid arthritis		
	Inflammatory bowel disease	Decrease in frequency and absolute number of MAIT cells (in particular, in CD8 ⁺ and DN subsets) in peripheral blood Increased MAIT cell levels in the synovial fluid Decrease in CD8 ⁺ MAIT cells in peripheral blood of CD and UC patients Accumulation of MAIT cells in the inflamed ileon of patients with CD Reduced IFN- γ production in CD patients and increased IL-17 production in CD and UC patients Fewer MAIT cells in the inflamed ileon of patients with CD and UC Increased apoptosis in MAIT cells	[53] [49] [100]
	Psoriasis	MAIT cells reside in not only the dermis of patients but also that of health donors. MAIT cells may contribute IL-17 production in the dermis of patients	[51]
	Celiac disease	Decrease in frequency of MAIT cells in peripheral blood and guts of adult and pediatric patients	[52]
	Systemic lupus erythematosus	Decrease in frequency and absolute number of MAIT cells (in particular, in CD8 ⁺ and DN subsets) in peripheral blood Reduced IFN- γ production Elevated expression of PD-1 in MAIT cells	[53]
Inflammatory diseases	Asthma	Decrease in frequency of MAIT cells in blood, sputum, and endobronchial biopsy	[101]
	Diabetes type 2/obesity	Decrease in frequency of MAIT cells in peripheral blood Circulating MAIT cells display an activate phenotype MAIT cells are more abundant in adipose tissue	[55,56]
	Acute cholecystitis	Decrease in frequency and absolute number of MAIT cells in peripheral blood	[102]
	Fibromyalgia syndrome vs Spondyloarthritis vs Rheumatoid arthritis	Defined analysis of MAIT cell phenotype among three diseases that exhibit a similar clinical manifestation Decrease in frequency of MAIT cells in three diseases Three diseases are able to distinguish by surface marker expression	[57]
Tissue transplant	Cutaneous acute graft-vs-host disease	Infiltration of CD8 ⁺ T cells, CD161 ⁺ , CCR6 ⁺ , ROR γ t ⁺ in the epidermis and dermis of patients with GVHD	[103]
	Hemodialyzed and kidney transplant	Decrease in frequency of MAIT cells in peripheral blood Implication for the susceptibility to infections in the patients	[104]
Tumors	Kidney and brain tumors	Presence of MAIT cells in tumors	[58]
Physiological change	Fetus	Rare and immature in the thymus, spleen, mesenteric lymph nodes Mature and enriched in the guts, liver, and lung	[29]
	Neonate/infant	Naïve phenotype at birth. Acquisition of effector/memory phenotype and increase in frequency and number with age	[11,30]
	Adult	Maximum levels in the third and fourth decenniums Higher amounts in females with reproductive age than in males	[30]
	Aging	Decrease in CD8 ⁺ MAIT cells and increase in CD4 ⁺ MAIT cells with age Th2 shift in cytokine profile in elderly	[22,30]

CD: Crohn's disease; UC: Ulcerative colitis; MAIT: Mucosal-associated invariant T; HIV: Human immunodeficiency virus; AIDS: Acquired immunodeficiency syndrome; *P. aeruginosa*: *Pseudomonas aeruginosa*.

Although MAIT cells are extremely rare in laboratory mice, *Francisella tularensis*-infected mice revealed a massive expansion of MAIT cells in infected tissues earlier than the migration of conventional CD4⁺ and CD8⁺ T cells^[34], which suggests their unique function in host defense against bacterial infection. V α 19 iTCR (invariant TCR) transgenic mice (a MAIT-cell-enriched mouse model) and MR1 knockout mice (a MAIT-cell-deficient model), MAIT cells seemed to prevent the growth of bacteria such as *Mycobacterium abscessus*, *M. bovis* (BCG), *Escherichia coli* and *Klebsiella pneumoniae*^[16,35-37]. Accordingly, patients with bacterial infections such as tuberculosis and pneumopathies showed a decrease in MAIT cells in circulating blood, which might reflect their infiltration into the diseased sites^[16,37]. In HIV-infected patients, MAIT cells were also depleted from the circulating blood irrespective of the disease stage (acute or chronic infection), and even with combinatorial anti-retroviral therapy^[38-43]. Although it is believed that CD4⁺ T cell depletion causes immunodeficiency in HIV-infected patients, innate immune cells such as MAIT cells could play a crucial role in prevention of opportunistic infections with bacteria and/or fungi, which is a manifestation of AIDS.

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system caused by autoreactive T cells. Although it is suggested that myelin-specific CD4⁺ T cells might play a central role in MS pathogenesis, recent studies have indicated that MAIT cells accumulate in brain lesions concomitantly with a decrease in peripheral blood in MS patients^[44-48]. This evidence indicates that MAIT cells may play a pivotal role in MS pathology, but the underlying mechanisms are yet to be elucidated. An increase in IL-18 in the serum of MS patients could signify that MAIT cells tend to migrate into the brain^[46]. In conjunction with the high levels of IL-17 and IFN- γ secretion from MAIT cells in MS patients, one study has demonstrated that MAIT cells in MS exhibited proinflammatory profiles^[45], but another interpreted that these MAIT cells exhibited a regulatory function to suppress the pathogenic Th1 response^[48]. Accordingly, a novel animal model will be required to examine the direct contribution of MAIT cells in MS pathogenesis, as will be described later in this review.

Inflammatory bowel diseases (IBDs), such as Crohn's disease and ulcerative colitis (UC), are autoimmune diseases in which the potential contribution of MAIT cells is suggested owing to their anti-microbial activity, intestinal homing, and capacity to promptly induce both Th1- and Th17-cytokines. Similar to MS patients, a decrease in MAIT cells in the peripheral blood concomitant with an increase in MAIT cells in the injured ileal regions of IBD patients has been reported^[49]. In addition, peripheral blood MAIT cells from IBD patients showed more activated and proliferative state compared with that in healthy controls, suggesting that such alterations impinge on their functions. In fact, MAIT cells from the IBD patients produced significantly more IL-17 than from healthy donors, whereas there was no difference in IL-2 and TNF- α production^[49]. MAIT cells from UC patients produced more IL-22, a Th17-cytokine,

than controls. Upon binding to its cognate receptors on respiratory and gut epithelial cells, IL-22 evoked the expression of mucin and antimicrobial peptides, both of which play a critical role in the protection of epithelial cells from bacteria and/or fungal invasion^[50]. Expression of these proteins may in turn enhance the protection and accelerate healing of cellular damage, implying the tissue protective functions of MAIT cells^[49].

Numerous studies have reported possible implications of MAIT cells in psoriasis^[51], celiac disease^[52], systemic lupus erythematosus^[53], diabetes^[54,55] and obesity^[55,56]. In our recent study, MAIT cells were shown to be useful to distinguish diseases that manifest similar symptoms such as fibromyalgia syndrome, rheumatoid arthritis, and spondyloarthritis by measuring the expression of cell surface antigens, in particular, chemokine receptors associated with homing^[57]. MAIT cells tend to migrate toward peripheral tissues, particularly in inflammatory conditions, because they express a variety of chemokine and cytokine receptors. Most of these studies have implied that immune-mediated tissue damage is induced by the pathogenic proinflammatory features of MAIT cells. In contrast, MAIT cells may protect against the damage caused by inflammation, as described above for UC. Furthermore, a subset of MAIT cells (CD56⁻) accumulated in kidney and brain tumors and may operate in tumor immune responses^[58].

As detailed above, there is no doubt about the importance of understanding the functions of MAIT cells in health and disease. However, the following questions still remain to be answered: What are the underlying mechanisms in immune regulation, particularly in innate immunity? And what are the molecules that control the functions of MAIT cells other than vitamin B2 metabolites? Although laboratory mice are useful to model human diseases, the study of MAIT cells is quite limited owing to their paucity in mice^[18]. Furthermore, MAIT cells hardly propagate *in vitro*^[11]. A recent paper, however, has showed the potential for MAIT cells to proliferate in response to *E. coli* and to anti-CD3/CD28/CD2 antibodies^[33]. The expansion most likely depends on a precise balance between proliferation and activation-induced cell death, because MAIT cells are highly sensitive to activation-induced cell death^[33,38,59]. To overcome these difficulties, we attempted to produce human MAIT cells through iPSC technology.

GENERATION OF MAIT CELLS USING MAIT CELL-DERIVED iPSCS

iPSCs may be established from a variety of somatic cells^[60-62] and be differentiated into T cells, as can embryonic stem cells (ESCs)^[63-65]. Nonetheless, it is near-impossible to obtain a monoclonal T cell with an antigenic specificity. This is primarily due to the fact that iPSCs and ESCs carry the germline configuration of *TCR α* and *TCR β* , which are subject to random gene rearrangement during T cell differentiation, resulting in the generation of polyclonal T cells (Figure 2)^[65]. Although iPSCs have been

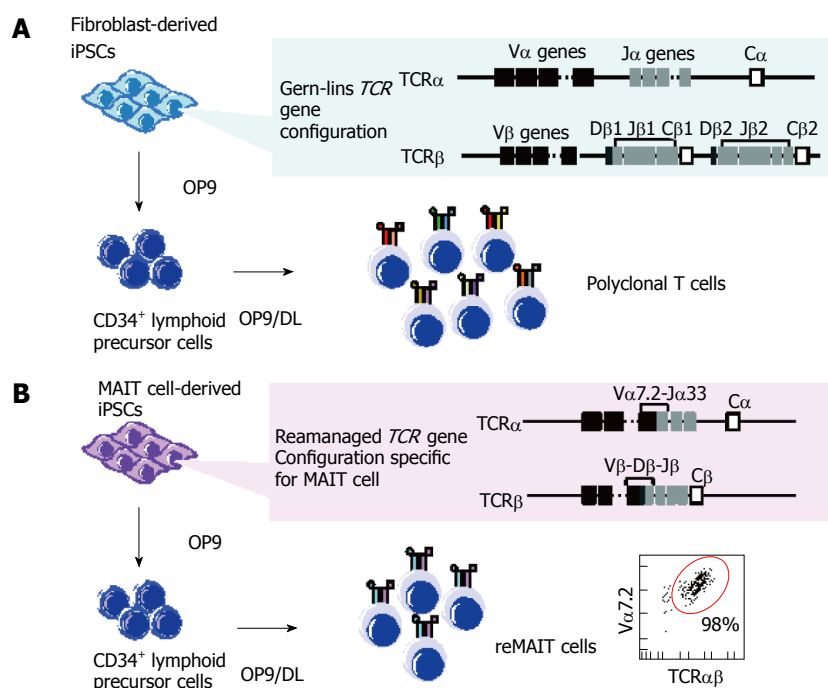


Figure 2 Scheme for T cell differentiation from induced pluripotent stem cells. Induced pluripotent stem cells (iPSCs) derived from normal somatic cells such as fibroblasts possess the germline configuration at T cell receptor (TCR) loci, whereas those from T cells harbor rearranged configurations (A); Upon differentiation in the T-cell-permissive conditions, the resulting T cells possess diverse sets of TCR repertoires; polyclonal T cells. In contrast, mucosal-associated invariant T (MAIT) cells-derived iPSCs exclusively confer MAIT cells in the same differentiation conditions. Note that MAIT cell-derived iPSCs possess a rearranged Vα7.2-Jα33 specific for MAIT cells in the genome (B).

established with terminally differentiated T cells in PBMC, the authors did not address whether or not differentiation of these iPSCs into T cells culminated in regeneration of an antigen-specific T cell clone^[66-68]. Recently, however, iPSCs have been established from tumor antigen-specific or HIV-specific CD8⁺ T cells with intention to rejuvenate T cells harboring the original epitopes, although the efficiency of such redifferentiation into the original clone remains unclear^[69,70]. Well before these reports, we have shown that the progeny of a cloned mouse from NKT cells possessed an in-frame rearranged *TCRα* (Vα14-Jα18) specific for NKT cells in the genome, and an increased number of NKT cells^[71]. This indicated that in-frame rearranged *TCRα* (Vα14-Jα18) had a strong impact on the destiny of T cells in the thymus. Such a notion has been explored further *in vitro*. ESCs prepared through nuclear transfer with hepatic NKT cells (ntESCs), harboring in-frame rearranged *TCRα* (Vα14-Jα18), gave rise to T lymphocytes exclusively comprising NKT cells (> 94%) when ntESCs were subjected to the OP9/OP9-DL system, which is well-known to promote T cell lineage differentiation from pluripotent stem cells^[64,65,72,73]. We have exploited a corollary that iPSCs derived from MAIT cells would efficiently redifferentiate into MAIT cells under the same conditions, because MAIT cells are innate-like T cells, and these iPSCs possess a rearranged *TCRα* (Vα7.2-Jα33), specific for MAIT cells^[21]. This turned out to be the case.

MAIT cells purified from umbilical cord blood (CB-MAIT) as TCR Vα7.2⁺ cells were reprogrammed with Sendai virus (SeV) vector harboring four reprogramming factors (*Oct4*, *Sox2*, *Klf4* and *c-Myc*) (MAIT-iPSCs) without any proliferative stimulation as used in reprogramming of antigen-specific CD8⁺ T cells^[69,70]. SeV is superior to other viruses, such as lentivirus, in that SeV

does not integrate into the host genome, thus leaving the genomic DNA free from interruptions^[21,67,69,74]. As expected, MAIT-iPSCs successfully redifferentiated into MAIT cell-like cells expressing Vα7.2, CD3, CD161, and IL-18Rα (reMAIT cells) with high efficiency (> 98%) in T-cell-permissive conditions (Figure 2)^[21]. reMAIT cells generally display a naïve phenotype, but express a high level of CCR6 (a receptor directing mucosal tissue homing and IL-17 expression), recapitulating that CB-MAIT cells that are still in an immature stage prior to exposure to commensal flora^[16,17,21,75,76]. Furthermore, reMAIT cells produce an array of cytokines, chemokines, and cytotoxic granules, such as granulysin, perforin and granzyme A, in an MR1-dependent manner. reMAIT cells also protect mice from Mycobacterial infection upon adoptive transfer, holding a promise to realize cell therapy with these cells^[21]. Taken together, reMAIT cells should function as innate-like T cells, although they are still immature^[16,17,21].

FUTURE PERSPECTIVES – DISEASE MODELING USING MAIT CELLS DIFFERENTIATED FROM iPSCS

reMAIT cells generated from iPSCs will be useful not only for deciphering their immunological functions *in vivo* but also for creating novel disease models in animals. Two types of genetically engineered mice, MR1-knockout mice and TCR transgenic mice, have been widely used to delineate the roles of MAIT cells *in vivo* (Tables 2 and 3). Originally, MR1-knockout mice (MR1^{-/-}) were generated to assess the roles of MR1 in the selection and expansion of MAIT cells *in vivo*^[18]. MR1-knockout mice possessed severely decreased TCR Vα19-Jα33

Table 2 Mice used in study for mucosal-associated invariant T cells

	Genotype	Characteristics	Ref.
Knockout mice	MR1 ^{-/-}	Impaired development of MAIT cells	[79]
Transgenic mice	V α 19 iTCR Tg	Enriched MAIT cells	[17,78-80]
	V β 6 V β 8 Tg	Increase of MAIT cells	[17]

MAIT: Mucosal-associated invariant T; iTCR: Invariant T cell receptor.

Table 3 Mucosal-associated invariant T cells in the diseases

Category	Mouse strains	Disease model	Phenotype	Ref.
Bacterial infection	MR1 ^{-/-}	<i>Escherichia coli</i>	Increase in the bacterial burden	[16]
	V α 19 iTCR Tg	<i>Micobacterium abscessus</i>	Repression of the bacterial burden	
	V β 6 V β 8 Tg			
	MR1 ^{-/-}	<i>Klebsiella pneumoniae</i>	Increased susceptibility to <i>K. pneumoniae</i> infection	[36]
	MR1 ^{-/-}	<i>Mycobacterium bovis</i> BCG	Enhanced bacterial growth at the early stage of infection	[35]
Autoimmune diseases		<i>Francisella tularensis</i>	Delayed adaptive immune reaction	[34]
	V α 19 iTCR Tg	Experimental autoimmune encephalomyelitis (model of MS)	Suppressed disease induction and progression	[78]
	MR1 ^{-/-}	Collagen-induced arthritis (model of rheumatoid arthritis)	Improved CIA score	[86]
	Adoptive transfer J α 33 ⁺ MAIT cells into BALB/c B10.R.III	TNBS induced colitis	Improved disease index	[105]
		Spondyloarthropathy by IL-23	Enthesitis induced by IL-22 produced from IL-23R ⁺ ROR γ t ⁺ CD4 ⁺ CD8 ⁺ T cells (MAIT cells?) in the entheses	[91]
Others	V α 19 iTCR Tg NOD	Non-obese diabetes	Delayed disease onset	[106]
	V α 19 iTCR Tg	Delayed-type hypersensitivity to sheep erythrocytes (type IV allergy)	Suppression of the disease	[106]

MAIT: Mucosal-associated invariant T; IL: Interleukin; CIA: Collagen-induced arthritis; iTCR: Invariant T cell receptor.

expression compared with their littermate controls^[18]. Thus far, MR1-knockout mice have been used as a model devoid of MAIT cells. MR1-knockout mice may have shed light on the roles of MAIT cells *in vivo*. However, the findings from the mice were too complicated to interpret, maybe because in part of the insufficient number of MAIT cells in the control, and the lack of an appropriate reagent to detect mouse MAIT cells. An MR1 tetramer that has been created recently is useful to detect MAIT cells in mice and humans^[77], but tetramer-positive cells may not always be functional cells. Three groups have independently reported V α 19 iTCR transgenic mice as a MAIT cell-enriched model^[17,78-80]. Two reports indicated that MAIT cells in V α 19 iTCR transgenic mice harbored an effector/memory phenotype; CD44^{high}CD69⁺CD25⁺ICOS⁺ and NK1.1^[78-80]. With the ligand-loaded MR1 tetramer, it was found that approximately 40%-50% of MAIT cells were CD4⁺, and the rest being comprised of DN cells and fewer CD8⁺ MAIT cells^[77], whereas in humans few CD4⁺ MAIT cells are present. Such a difference in CD4 or CD8 usage between mouse and human may reflect their physiological roles. In contrast, Martin *et al.*^[17] showed that MAIT cells from their V α 19iTCR transgenic mice were DN and CD8⁺ with few CD4⁺. Furthermore, NK1.1, CD25, CD69, and ICOS were not present in MAIT cells. Such inconsistency demonstrated that MAIT cells

are different in nature from those in transgenic mice. It is plausible that such an alteration stems from the differences in transgenes or commensal flora utilized. Should it be the case, the transgenic mouse may not be adequate to delineate the functions of MAIT cells^[17]. Therefore, it is indispensable to create a novel animal model to address the physiological roles of MAIT cells in health and diseases, and harnessing the results of animals for clinical applications.

In this context, use of humanized mice can be envisaged, because the human cells in question can be engrafted and their functions and development may be examined *in vivo*^[81]. To study the physiological roles *in vivo*, reMAIT cells were adoptively transferred to NOD/SCID or NOG (NOD/Shi-scid IL2R γ ^{null}) mice, both of which are devoid of mature B, T cells, and the later deficient in NK cells, functional macrophages, and dendritic cells^[21]. reMAIT cells migrated and engrafted in tissues such as the intestines, bone marrow, liver, and spleen, which probably mirrors the distribution of MAIT cells in humans^[18,71]. In addition, reMAIT cells dramatically changed the phenotype from naïve to mature concomitant with the expression of the chemokine receptors required for the tissue-specific homing. Moreover, reMAIT cells appeared to proliferate in mice, whereas they did not *in vitro*. These results indicated that reMAIT cells from iPSCs responded to external

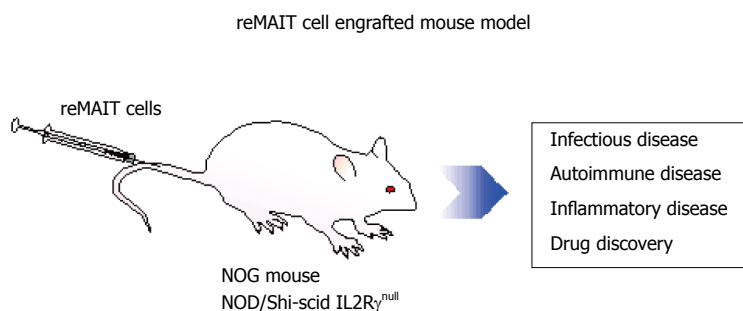


Figure 3 Utility of mucosal-associated invariant T cells from induced pluripotent stem cells (redifferentiation of mucosal-associated invariant T cells) for modeling human diseases. Severely immunocompromised mice received MAIT cells from induced pluripotent stem cells. reMAIT cells are useful for deciphering the physiological functions of MAIT cells in health and disease. MAIT: Mucosal-associated invariant T; reMAIT: Redifferentiation of MAIT.

cues, migrated to different tissues, and proliferated in mice. Such interactions most likely occur *via* chemokine receptors on reMAIT cells and *via* mouse MR1 bound with ligands from commensal flora or with an endogenous one. The data suggest that the function of reMAIT cells could be assessed *in vivo*, which opens up new horizons for modeling human diseases in mice.

Accordingly, the protective mechanisms of MAIT cells against bacterial infection have been examined using reMAIT cells^[71]. Upon adoptive transfer, reMAIT cells protected mice from *M. abscessus*, as demonstrated by a decrease in bacterial burden. Such a protective activity mirrors that observed with MAIT cells from PBMCs^[16]. Granulysin has been identified as an effector molecule in the control of mycobacterial infection. Granulysin is present together with granzymes and perforin in the cytolytic granules of cytotoxic cells such as CD8⁺ T and NK cells as well as MAIT cells^[32,82]. Granulysin plays a crucial role not only in the destruction of infected cells but also in killing pathogens^[83,84]. Given that mice are devoid of granulysin and its homologue^[85], mice harboring reMAIT cells could serve as a novel model to decipher the roles of human-specific factors.

There is accumulating evidence that MAIT cells play a pivotal role in inflammatory and autoimmune diseases. Nonetheless, delineating how MAIT cells are implicated in these diseases has to await the advent of appropriate animal models. Experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) are animal models for MS and RA, respectively. By using V α 19 iTCR transgenic mice and/or MR1-knockout mice, the implication of MAIT cells in autoimmune diseases has been investigated^[78,86]. In V α 19 iTCR mice, the severity of EAE was ameliorated in both induction and progression of demyelination compared with control littermates^[78,86]. In marked contrast, the severity of CIA was improved in MR1-knockout mice, whereas adoptive transfer of MAIT cells from V α 19 iTCR transgenic mice resulted in aggravation of the disease^[78,86]. EAE and CIA are intended to induce autoreactive T cells, especially focused on Th17 or Th1 responses, through hyperimmunization of putative target antigens (myelin basic proteins or type II collagen) with Freund's adjuvant. Induced T cells could migrate to target tissues and secrete proinflammatory or anti-inflammatory cytokines, which may further worsen tissue damage or help resolve the damage. It has been believed that such mechanisms recapitulate the etiology and pathology

of human diseases. Nonetheless, it is not appropriate to use such mice for disease modeling because MAIT cells do not react with peptide antigens, although they may respond to the components of adjuvant such as those from *M. tuberculosis*. Furthermore, the paucity of murine MAIT cells is another issue. Even though V α 19 iTCR transgenic mice can be used in a disease model, the nature of transgenic MAIT cells may be different from that present in the control. Given that MAIT cells are competent to produce a plethora of cytokines, a nature prerequisite for immunoregulatory functions, the above disease models may not be suitable for deciphering the etiology and pathology, in that such a crucial feature of MAIT cells is largely overlooked or distorted.

Exploring a disease model with reMAIT cells could further our knowledge of the etiology and pathology of MS. It has been reported that inflammatory demyelinating lesions are infiltrated by IL-17-expressing T cells in the mouse brain when they received cerebrospinal fluid from a progressive MS patients^[87]. A longitudinal study in MS patients indicated massive expansion of MAIT cells or MAIT cell-like cells, harboring canonical or atypical TCR V α and β chains but do not react with bacterial antigens, could play an important role in the onset and the formation of early active MS lesions^[47]. The above data implied the presence of yet-to-be-identified ligands responsible for the negative effects of MAIT cells in disease. Use of reMAIT cells could make it possible to examine whether or not sole ligands for MR1 or any epigenetic modifications of MAIT cells are responsible for disease. In either case, mice with reMAIT cells are useful to identify such ligands and to create a novel autoimmune disease model.

Should MAIT cells play a pivotal role in autoimmune diseases, it is tempting to anticipate that granulysin *per se* or in combination with granzymes and perforin exerts a cytolytic activity against the target tissue. In line with this hypothesis, granulysin may play crucial roles in transplant rejection and epidermal necrosis in toxic epidermal necrolysis and Stevens-Johnson syndrome^[85,88,89]. Furthermore, combined with the ectopic expression of human cytokines and/or chemokines, mice with reMAIT cells could be further fine-tuned to mimic human diseases by controlling tissue migration^[90,91]. Provided such an exquisite model is available, we can go to the next step of drug discovery and/or screening. Compounds that interfere either with the development of MAIT cells or the function of MAIT cells can be screened

in such a mouse model (Figure 3).

CONCLUSION

Recent studies have shed light on the unique properties of MAIT cells and on their possible involvement in a variety of human diseases, although MAIT cells have been overlooked behind conventional T cells and other innate immune cells for a long time. The paucity of MAIT cells in laboratory mice and their extremely poor proliferative capacity are the biggest obstacles to fully understand the function of MAIT cells in health and diseases. Reprograming and redifferentiation of MAIT cells from iPSCs have overcome these difficulties. Furthermore, mice with reMAIT cells will pave the way for unveiling the mechanisms underlying the diseases and open up new horizons in medical research.

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Retinoblastoma tumor suppressor functions shared by stem cell and cancer cell strategies

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Abstract

Carcinogenic transformation of somatic cells resembles nuclear reprogramming toward the generation of pluripotent stem cells. These events share eternal escape from cellular senescence, continuous self-renewal in limited but certain population of cells, and refractoriness to terminal differentiation while maintaining the potential to differentiate into cells of one or multiple lineages. As represented by several oncogenes those appeared to be first keys to pluripotency, carcinogenesis and nuclear reprogramming seem to share a number of core mechanisms. The retinoblastoma tumor suppressor product retinoblastoma (RB) seems to be critically involved in both events in highly complicated manners. However, disentangling such complicated interactions has enabled us to better understand how stem cell strategies are shared by cancer cells. This review covers recent findings on RB functions related to stem cells and stem cell-like behaviors of cancer cells.

Key words: Stem cells; Cancer; Retinoblastoma; Cancer stem cells

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Core tip: Carcinogenic transformation of somatic cells resembles nuclear reprogramming toward the generation of pluripotent stem cells. The retinoblastoma tumor suppressor product retinoblastoma (RB) seems to be critically involved in both events in highly complicated manners. This review covers recent findings on RB functions related to stem cells and stem cell-like behaviors of cancer cells.

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INTRODUCTION

The cell-of-origin in various types of cancers has been one of the most important areas of research in modern cancer biology, because deep understanding of this can help design of future cancer therapies^[1-4]. Many studies have indicated that distinct cells-of-origin give rise to distinct features of cancers, and can often predict the prognosis of patients^[5,6]. However, the debate on cell-of-origin of each cancer is often controversial, because cancer phenotypes do not stereotypically reflect phenotypes of their true cells-of-origin. This is at least partially due to the high developmental plasticity that is acquired after tumor initiation and during tumor progression.

The cancer stem cell hypothesis proposes a model in which like in normal tissues, cancer cells obey the hierarchy of development where stem cell-like cancer cells are placed at the top. This hypothesis does not always explain the cell-of-origin of a specific cancer; however, it has led us to the idea that cancer cells prefer to employ stem cell strategies in order to maintain tumor-initiating clones^[7,8]. This theory has been reinforced by many findings, including the switchable cell fates of cancer cells, the inseparable relationship between pluripotency and teratogenicity, the requirement for oncogenic elements for the generation of induced pluripotent stem (iPS) cells, and the oncogenic activities of many embryonic genes^[9,10].

Sophisticated gene-engineered and tumor-grafted mouse models have been used to trace the cells-of-origin for specific cancers, for instance in case of prostate adenocarcinomas, to basal or luminal cells^[2]. During these investigations, the existence of unexpectedly high levels of developmental plasticity became apparent, when comparing the cell-of-origin to its resultant tumor. In addition, researchers found switchable cell fates in cultured cancer cell lines that were induced by artificially altering the status of genes, such as tumor suppressor genes.

Although the significance of epigenetic alterations is still unclear, carcinogenesis results from the step-wise accumulation of readable lesions in the genome^[11]. Given the gain of developmental plasticity, even if it is transient, is essential for carcinogenesis, then the next question should be as follows: Which genes are mechanistically involved in this gain of plasticity?

This question is not satisfactorily answered yet, but a part of answer may be informed by the switchable cell fate of cancer cells. The retinoblastoma tumor suppressor gene (RB) is closely implicated in the change in developmental phenotypes of many types of cancers, including lung cancer, breast cancer, prostate cancer, osteosarcoma, and soft tissue sarcoma. This phenomenon has been attributed to the physical or genetic interaction between the RB gene product (RB) and tissue-specific transcription factors^[12]. However, emerging evidence indicates that inactivation of RB in particular genetic backgrounds or in certain contexts can lead cells to an undifferentiated state that resembles

that of most immature cells, such as embryonic stem cells^[13-15].

We also know that the targeted inactivation of RB, in combination with p53, provides strong experimental tools to determine the cell-of-origin of various types of cancers^[16-18]. Indeed, these two tumor suppressor pathways are the most commonly inactivated in human cancers, and simultaneous inactivation is sufficient to induce cancers from various types of somatic cells^[19]. Therefore, one of the optimal ways to understand RB function in the context of full carcinogenesis would be to determine RB functions in a p53-deficient genetic background.

This review briefly summarizes the well-established functions of RB in mammalian cells, presents cross-species evidence for the possible link between RB function and the control of stem cell activities, and describes findings that may explain the molecular mechanisms underlying this link. The RB locus was identified more than a quarter century ago; however, researchers are still providing new wineskins to new wines.

CELL CYCLE-DEPENDENT AND INDEPENDENT FUNCTIONS OF RB

Cell cycle control by RB

The RB gene was first identified as a tumor suppressor in the childhood malignancies retinoblastoma and osteosarcoma^[20]. Somatic RB loss typically causes unilateral retinoblastoma with no obvious risk for other types of malignancies. However, germline RB mutation often results in bilateral retinoblastoma, and carriers are at very high risk of various types of cancer over their lifetimes^[21]. Therefore, researchers proposed that RB might be involved in the core mechanisms of tumorigenesis. Indeed, unveiling the functions of RB in controlling cell cycle progression provided a big breakthrough to the field of cancer research^[22].

A primary RB function in cell cycle control is exerted at the G₁/S transition. RB undergoes dephosphorylation at the end of the M phase with the aid of protein phosphatases (PPs) and resumes its phosphorylated state during the G₁ phase by the action of cyclin D/cyclin-dependent kinase (CDK) 4 or 6 complexes^[23]. Most of cellular mitogenic signals converge on the transcriptional upregulation of D-type cyclins. This could be one reason that cells in the G₁ phase are most vulnerable to extracellular growth stimuli^[23,24].

Phosphorylation of RB alters its three dimensional (3D) structure. This results primarily in the loss of binding affinity to E2F family transcription factors^[25,26]. Among nine identified E2F family members (E2F1, 2, 3A, 3B, 4-8), RB was shown to bind to at least E2F1, 2, and 3A. Each of these three family members is able to positively transactivate genes, including cyclin E^[27]. Upregulation of cyclin E in cooperation with CDK2 further promotes RB phosphorylation. This enables cells to cross the boundary between G₁ and S. Further, with the aid of cyclin A, RB attains the maximal level of phosphorylation before cells enter the M phase^[23]. In addition, when bound to

hypophosphorylated RB, E2Fs form a transcriptional repressor complex that recruits histone deacetylase (HDAC) to epigenetically silence gene transcription^[28]. Therefore, the phosphorylation status of RB dramatically changes the expression of E2F-targeted genes. The function of RB in restricting the G₁/S transition is also mediated by its binding to SKP2, which destabilizes p27^{KIP1} by enhancing the ubiquitin-proteasome system when freed from phosphorylated RB^[29,30]. This represents one of E2F-independent functions of RB in the control of cell cycle progression.

RB plays pivotal roles also in M phase, which is most typically represented by the impact of RB inactivation on the chromosomal instability (CIN). E2Fs target a number of M phase genes including MAD2 which functions by inhibiting the anaphase promoting complex/cyclosome (APC/C)-cell division cycle 20 (CDC20) complex. This complex regulates spindle assembly^[31]. RB also controls the M phase by directly binding to cohesin and condensin II, two critical regulators of centromeric functions^[32].

"How many total RB functions are cell cycle-dependent?" is an intriguing question. RB mutants found in partially penetrant retinoblastomas (low grade retinoblastomas with limited genetic inheritance) or retinomas that failed to inhibit the cell cycle but retained the ability to promote terminal differentiation suggested that RB functions in cell cycle control and differentiation might be distinct^[33]. In addition, phenotypic analyses of *Rb*-deficient mice simultaneously lacking an *E2F* family member allowed at least partial discrimination of the E2F-dependent function from the E2F-independent function^[34]. However, since E2Fs target both cell cycle-related and cell cycle-unrelated genes, discrimination of cell cycle-dependent functions from cell cycle-independent functions of RB based on the E2F-dependency is difficult.

Artificial and acute alteration of RB status in a wild type genetic background often greatly affects the cell cycle. For instance, it induces cell cycle exit (quiescence or cellular senescence), or inversely cell cycle re-entry^[35,36]. This change in the cell cycle control is highly drastic, thus can mask cell cycle-independent phenotypes associated with altered RB activity. However, based on the experience of analyzing *Rb*-deficient mice, our group discovered several genetic backgrounds that allow mice or cells to exhibit cell cycle-independent phenotypes following RB inactivation^[14,15,37,38]. The cell cycle-independent phenotypes include gain of undifferentiated phenotypes and altered chemo-resistance^[15]. Therefore, we thought that control of the undifferentiated state of cells might represent at least a part of the cell cycle-independent functions of RB.

Cell cycle-independent functions of RB

Whole genome sequencing studies have revealed that the *RB* loci undergoes the fourth most frequent loss-of-heterozygosity (LOH) found in whole human tumors, following *CDK42A*, *PTEN*, and *SMAD4*. Thus, *RB* mutations definitely can be "driver mutations"^[39]. However, the type

of tumors in which *RB* mutations occur at their initiation is highly limited, including only retinoblastomas, small cell lung cancers (SCLC), osteosarcomas, and familial melanoma. In other types, in the vast majority of tumors, RB functions are largely maintained during initiation but these functions typically collapse while tumors undergo malignant progression^[40].

The question of why RB mutation is rare in the majority of cancers has yielded many interesting answers. Lack of RB in many cell types has already been linked to apoptosis through E2Fs, ARF, and p53^[41]. In addition, RB residing in mitochondria directly interacts with Bax, and thus regulates apoptosis in a completely E2F-independent and cell cycle-independent manner^[42,43]. These pathways represent a disadvantage in carcinogenesis upon RB inactivation when it occurs at early steps. The 3T3 cells lacking *Rb* are less susceptible to Ras-transformation, indicating that preservation of RB functions in the interaction of mitogenic signals and the cell cycle might be important for tumor initiation^[44].

Our group demonstrated that *Rb*-heterozygous mice generate adenomas or low-grade adenocarcinomas derived from calcitonin-producing cells (C cells) of neuro endocrine origin that exhibit whole evidence of DNA damage response and cellular senescence. However, the genetic background of a homozygous lack of *N-ras* allowed these *Rb*-deficient C cell tumors to progress to highly invasive and metastatic adenocarcinomas^[14,37]. RB appeared to regulate isoprenylation of the N-Ras protein. Isoprenylation (farnesyl moiety-transfer and geranylgeranyl moiety-transfer) is the chemical reaction that is essential for the initial maturation of this protein. RB loss causes intermediate level upregulation of N-Ras under particular culture conditions, which induces a DNA damage response and subsequently cellular senescence, thus antagonizing full carcinogenesis in a manner similar to oncogene-induced senescence (OIS). The mechanism whereby RB controls N-Ras isoprenylation involves E2F-dependent regulation of sterol regulatory element binding protein (SREBP) transcription factors and direct drive by E2Fs in some of mevalonate (MVA) pathway genes^[14]. This study indicated a case that RB influences intra-cellular signaling primarily by controlling metabolic pathways. Many reports, including ours, have directly implicated RB in the control of cellular metabolism^[36,45,46]. This may indicate that RB is simultaneously involved in the control of the cell cycle and metabolic regulation. This may further explain why cell cycle progression and cellular metabolism are tightly coupled. Cells never diminish their volume after rounds of cell division. This is because cells very strictly double biomass up to the time of mitosis, just as they strictly conserve genome size. From this point of view, cellular metabolism for biomass synthesis would not be passively controlled by the demands of cell cycle progression; rather, they are both actively and presumably simultaneously regulated by a common mechanism.

The outcomes of RB inactivation during malignant progression entail not only facilitated G₁/S transition, but

also many events critical for malignant cell behaviors. These include increased cell motility, angiogenesis, inflammatory response, metabolic rewiring, gain of undifferentiated developmental features, lineage change and altered drug resistance^[36,45]. Since acquisition of these abilities is not always associated with cell cycle progression, many of these, except G₁/S control, may more or less represent cell cycle-independent functions of RB. Particularly, gain of undifferentiated developmental features following RB inactivation and lineage changes presumably related to the developmental plasticity of tumors are central interests of this review article.

We need to be very careful in defining what is cell cycle-independent function of RB and what is not. E2F targets contain not only cell cycle-related genes. In addition, many LxCxE proteins that bind to RB are chromatin modifiers; hence, their roles in control of the cell cycle and differentiation are barely discernible. We later discuss the experimental system by which we addressed cell cycle-independent functions of RB in the context of regulation of undifferentiated cell states. In the next paragraph, we introduce accumulating biological evidence implicating RB in multiple stem cell systems. Later, we discuss possible cell cycle-dependent and independent mechanisms underlying RB functions in stem cells.

CROSS-SPECIES EVIDENCE THAT LINK RB TO STEM CELLS

Figure 1 shows a phylogenetic tree of genes in the RB family (Figure 1). RB gene orthologues do not exist in the genome of many of unicellular organisms, including yeasts, but appear in almost all multicellular organisms^[47]. In addition, the component including RB alone and/or E2F transcription factors and DP protein(s) are well conserved from plants to animals^[48]. This indicates that RB might be involved not only in cell-autonomous proliferation control but also in some machineries unique to multicellular organisms. Stem cell conservation attained by asymmetric cell division is apparently unique to multicellular organisms. Therefore, we present cross-species evidence that may link RB to stem cells.

Mammalian cells

Analysis of RB functions in embryonic development and embryonic stem cells provided a substantial amount of information regarding its potential roles in stem cells. The role of RB in various adult tissue stem cells was once thoroughly summarized by Sage^[19]. Our current review focuses on the cells-of-origins in which RB inactivation likely gives the first cue for clonal expansion of stem or progenitor cells during carcinogenesis.

Retinoblastoma develops from the retina composed of multiple lineages of cells. The cell-of-origin of retinoblastoma is quite controversial because of the complexity of retinal development and tumor phenotype. More in concrete, findings that contribute to the controversy include the

reported appearance of differentiated and undifferentiated developmental markers of mixed lineages within the same tumor derived from retinoblastoma patients and Rb-deficient mice^[49]. Human retinoblastomas show typically differentiated features^[50], and post-mitotic retinal cells can be the cell-of-origin in mouse models^[51]. These findings suggest that promoting dedifferentiation and increasing flexibility of fate determination are initially attained by RB inactivation in retinal cells. In other words, RB inactivation in retinal somatic cells endows them with the ability to differentiate to multiple types of cells. The dispute over cell-of-origin might be resolved, since cone precursors have been shown to be specifically vulnerable to Rb-deficiency-induced clonal expansion^[52]. However, given that the cell-of-origin has been truly traced to one lineage in retinoblastoma development, the findings on mixed and normally inconsistent developmental features co-existing in retinoblastoma tumor cells imply that RB-deficiency not only initiates tumors of a particular cell-of-origin, but also increases plasticity in lineage specification and probably induces dedifferentiation so that tumor cells employ multiple stem or progenitor cell strategies to fit to the retinal microenvironment.

There are several other types of cancers in which RB loss occurs prevalently at initiation: Osteosarcoma and SCLC. In these malignancies, discussions of the cell-of-origin and the role of RB in tumor initiation are inseparable similarly as in case of retinoblastoma. Mesenchymal stem cells or osteo-progenitors can be the cell-of-origin of osteosarcoma. The direct interaction between RB and osteoblast transcription factor Runx2 might at least partially explain the ability of RB to suppress tumor initiation in a lineage-specific manner. Rb deficiency in these cells often cooperates with p53 loss-of-function to generate osteosarcomas^[53]. p53-deficiency is in some case sufficient to induce osteosarcoma, and p53-deficient osteosarcomas can be converted to brown fat tumors (hibernomas) with subsequent RB inactivation^[12]. This has been attributed to increased expression of PPAR γ which is governed by E2Fs. However, this fate change might also be mechanistically supported by function of RB to influence on cell fate decision that may occur prior to the decision of commitment to terminal differentiation. Hence, in osteosarcoma, like in retinoblastoma, RB might function in mesenchymal stem cells or osteo-progenitors to contribute to suppress the plasticity in lineage specification.

There are also debates on the cell-of-origin of SCLC. Again, simultaneous inactivation of RB and p53 is sufficient to induce SCLC in mouse lungs^[3]. A study of cell type-specific deletion of RB and p53 indicated that neuroendocrine cells more often gave rise to SCLC than alveolar type II cells^[54,55]. From this and other evidence, neuroendocrine cells are believed to be the predominant cell-of-origin of SCLC. The differential roles of RB and p53 in the formation of SCLC will be discussed later (see below).

The role of RB in controlling pluripotency was first addressed by testing whether tumor suppressor depletion facilitates iPS induction^[56]. This study identified p53 but not RB to be an influential molecule in iPS induction.

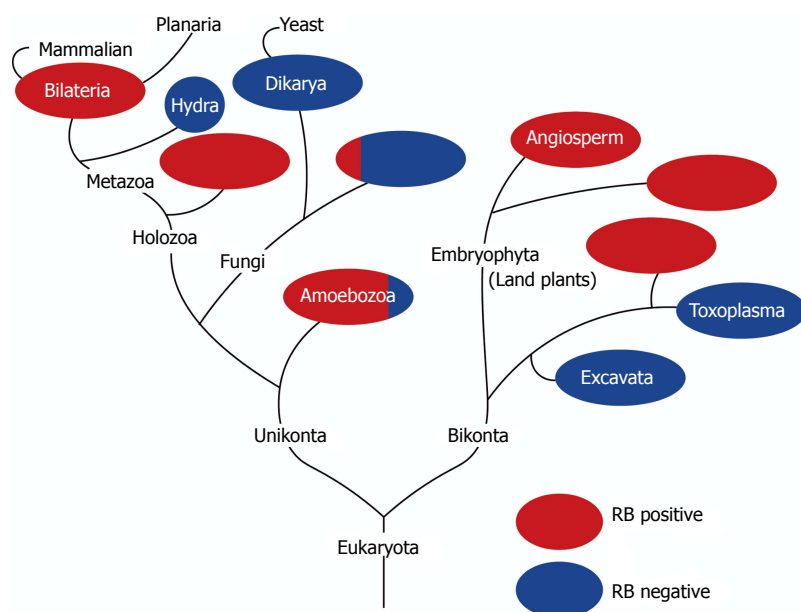


Figure 1 Evolution of the retinoblastoma gene in eukaryotic super-groups. Modified from Desvoyes *et al.*^[67]. RB: Retinoblastoma.

However, two later studies indicated that RB suppresses iPS induction from fibroblasts. A screen for short hairpin RNAs (shRNAs) that enhance iPS induction efficiency identified RB^[57]. Another study indicated that RB cleavage by caspase 3/8 is critical for iPS induction^[58]. More recently, RB was directly implicated in the transcriptional control of *Oct4* and *Sox2*^[59]. These findings indicate that RB inactivation leads to a state favorable for iPS induction by facilitating the induction of embryonic genes that induce a pluripotent state. This function of pRB may partly explain how stem cell functions can be shared by cancer cells. We indeed often observed increased expression of *Oct4* and *Sox2* in spheres induced by RB inactivation^[59]. The effect of RB inactivation on the cell cycle control is also contributable to iPS cell induction. We will discuss later many of the signals required for iPS induction are possibly controlled by pRB (see below).

MEFs lacking all members of the RB family can form embryoid body-like 3D structures in suspended culture conditions that express higher levels of embryonic genes and can form teratoma-like tumors when inoculated into immune-deficient mice^[13]. Since mostly tumor-derived spheres express higher levels of embryonic genes than when cultured under 2D conditions, this observation might result from carcinogenic change in MEFs.

Kareta *et al.*^[59] (our study) demonstrated that RB inactivation in *p53*^{-/-} MEFs give rise to sphere formation without carcinogenic conversion. Sphere formation in the absence of serum and in the presence of limited growth factors (bFGF and EGF) is thought to represent increased self-renewal/symmetric cell division. Preceding this observation, we analyzed C cell adenocarcinomas developed in *Rb*-heterozygous mice that simultaneously lacked *p53*, *Ink4a*, *Arf* or *Cdkn1a* (*p21*). *Rb*-heterozygous mice typically develop low-grade C cell adenocarcinomas. Simultaneous lack of an additional gene allowed *Rb*-deficient C cells to develop full brown tumors at seemingly similar levels. However, as compared to other

genotypes of C cell tumors that were all calcitonin-positive, thyroid tumors that developed in *Rb*^{+/-} *p53*^{-/-} mice showed virtually no expression of calcitonin. However, earlier neuroendocrine lineage markers, including synaptophysin, were expressed. These findings indicated that simultaneous inactivation of *Rb* and *p53* induced a highly undifferentiated status in neuroendocrine cells that were originally destined to develop into C cells. We then analyzed *Rb*^{-/-}; *p53*^{-/-} MEFs in comparison with *Rb*^{+/-}; *p53*^{-/-} MEFs. They showed insignificant differences in cell cycle progression; however, *Rb*^{-/-}; *p53*^{-/-} MEFs showed significantly higher self-renewal activity and increased expression of embryonic genes. This may represent a cell cycle-independent function of RB in controlling stem cell-like features. Lastly, we screened an FDA-approved drug library and found that some drugs reported to be effective as cancer stem cell therapies were also effective in cells lacking *Rb* and *p53*^[15].

Tumor cell fate

Here, we focus on four tumor types (SCLC, breast cancer, prostate cancer, and soft tissue sarcoma) in which RB inactivation during tumor progression presumably contributes to the increased developmental plasticity of tumor cells.

p53 is the primary gene mutated in SCLC (75%-90%)^[60]. *p53* mutations are found also in normal bronchioles of patients, suggesting that this mutation most likely occurs at tumor initiation^[61]. The second most frequent mutation occurs at *RB* loci. Recent comprehensive genomic profiling of SCLC revealed that bi-allelic losses at *p53* and *RB* loci were 100% and 93%, respectively^[62]. This signifies that the simultaneous inactivation in *p53* and *RB* occurs in almost 90% of all cases. This study showed a previously unexpected high frequency of *RB* inactivation in SCLC. In terms of determining *RB* function in SCLC development, other gene mutations relevant to *RB* loss in *RB*-intact SCLC (7% of *p53*-mutated cases) should be noted. Semenova *et al.*^[3] discussed possible *RB* functions in *p53*-mutated SCLC cells.

They pointed out that pluripotency genes such as *OCT4* and *SOX2* are frequently amplified in SCLC^[63] and that enhancers of zeta 2 (*EZH2*), which is implicated in the neural stem cell maintenance, are very often upregulated in SCLC following RB inactivation^[64,65]. Finally, they concluded that "RB loss (in SCLC) is associated with an increase in cell plasticity"^[63]. Although more evidence is needed to finally accept this notion, it is definitely an attractive hypothesis.

Recent publication from Engelman group demonstrated that gain of resistance to tyrosine kinase inhibitors in non-small-cell lung cancers (NSCLCs) harboring epidermal growth factor receptor (EGFR) mutation is associated with fundamental histological transformation from NSCLC to SCLC at a certain frequency (5%-15%). Surprisingly, in such subset of resistant cancers, RB was lost at 100% frequency^[66]. This report indicates that the developmental plasticity enhanced by RB inactivation is coupled to gain of drug resistance. The mechanism of drug resistance associated with NSCLC-to-SCLC conversion is currently unknown, but it would be definitely attractive to investigate RB functions in this context.

Breast cancers are frequently characterized by RB pathway inactivation, and low RB expression is a hallmark of basal-like breast cancers^[67-69]. RB inactivation in luminal type breast cancer induces tamoxifen resistance^[70], possibly owing to a gain-of-undifferentiated status following RB inactivation. Simultaneous inactivation of p53 and RB is prevalent in basal-like cancers^[71]. Basal-like and luminal type breast cancers have been recently suggested to stem from common luminal progenitor cells^[72]. Because p53 mutations are common in most breast cancer types^[73], RB status might be one of the determinants of basal-like or luminal type cancers. If so, RB could be implicated in determining the fate of breast cancers.

RB was also implicated in epithelial-to-mesenchymal transition (EMT) in breast cancer cells. Taya group demonstrated that RB depletion in a luminal type MCF-7 breast cancer cells induced EMT and overexpression of RB inhibited the EMT in MCF10A non-tumorigenic breast mammary epithelial cells^[74]. They also demonstrated that RB controls transcription of *SLUG* and *ZEB-1* in cooperation with the transcription factor activator protein 2 α .

RB is deeply implicated in prostate cancer development, especially during its progression. Although RB inactivation is observed in only 5% of primary prostate cancers, its rate rises to 40% in metastatic tumors. Furthermore, the RB signaling pathway is altered in 34% and 74% of primary tumors and metastatic loci, respectively^[75]. One study demonstrated a high rate of RB loci deletion and DNA methylation in the RB promoter in metastatic castration-resistant prostate cancers (CRPC)^[76]. RB depletion in hormone-dependent human prostate cancer cells induces androgen-independent cell growth through upregulation of androgen receptor (AR) in an E2F1-dependent manner^[77]. RB and p130 are involved in the regulation of *EZH2* transcription in prostate cancer cells, whose upregulation is often observed during prostate cancer progression^[78]. Loss of RB in prostate cancer

cells increases the expression of nucleolar and spindle-associated protein 1 (*NUSAP1*), which is associated with a poor prognosis in prostate cancer^[79]. Recently, we observed that RB depletion in androgen-dependent prostate cancer cells induces several lipid metabolism-related genes and some typical malignant features, including tumor spheroid formation. These observations indicate that inactivation of RB strongly promotes prostate cancer progression.

Simultaneous mutations in RB and p53 are frequently found in human soft tissue sarcomas^[80]. Conditional inactivation of both tumor suppressors by subcutaneous injection of AdCMVC into *p53^{fllox/fllox}; Rb^{fllox/fllox}* mice induced undifferentiated high-grade pleomorphic type sarcomas from locally resident cells. Inactivation of p53 but not RB is sufficient to induce well-differentiated sarcomas, such as rhabdomyosarcoma and leiomyosarcoma, but typically not sufficient to induce undifferentiated types of tumors^[81]. These findings indicate that RB inactivation does not directly contribute to the initiation of sarcoma development, but rather does contribute to converting well-differentiated types of tumors to undifferentiated types in a p53-deficient background. We recapitulated this finding in an *in vitro* culture system. We first developed poorly spherogenic p53-null soft tissue sarcoma cell lines from soft tissue sarcomas that subcutaneously developed in approximately 10% of *p53^{-/-}* mice with a C57BL/6 background. Additional depletion of Rb successfully induced less differentiated highly spherogenic and less differentiated sarcomas from "poorly spherogenic" p53-null soft tissue sarcoma cell lines. These findings indicate a possibility that in soft tissue sarcomas, RB directs both the self-renewal and plasticity of developmental features.

Planarians

Relying on a large population of pluripotent adult stem cells, planarians exhibit extraordinary high regenerative capacities. Zhu and Pearson presented a comprehensive study on the RB system in these organisms^[82]. Planarians possess unexpectedly few RB system components: A single Rb family member, single E2F (*E2F4-1*), and single DP. They are primarily expressed in planarian stem cells, and knockdown of any of these components significantly phenocopied the stem cell loss induced by irradiation or RNAi against stem cell-specific genes. The RB system was found to be indispensable for planarian stem cell self-renewal and survival; however, it was dispensable for late differentiation. Interestingly, planarians have 20 homologs to cyclin genes and none of them is homologous to cyclin E. An HDAC1 and a cyclin D homolog are expressed specifically in planarian stem cells, and knockdown of either of them induced deficiencies in stem cell functions. The simplicity of the RB system and ease of visualizing stem cell behaviors in planarians make this a valuable system for the RB research field, especially with regard to stem cell functions. Additionally, the molecular mechanism whereby the RB system contributes to the extraordinarily high regenerative capacity of this creature is great of interest.

Plant cells

RB family proteins and their binding partners had existed before multicellular organisms appeared on the earth, and are shared by plants and animals^[82]. *Arabidopsis thaliana* has one ortholog of an RB family protein, which was named retinoblastoma-related protein (RBR)^[83]. Inactivation of RBR led to the expansion of root stem cells without affecting the ability of progenitor cells (descendants) to self-renew and differentiate^[48]. The mechanism whereby RBR controls maintenance of root stem cells seems to involve two transcription factors shortroot (SHR) and scarecrow (SCR). SCR interacts with RBR through an LxCxE motif. Thus, surprisingly, the role of LxCxE motifs is well conserved between animals and plants. More surprisingly, the RBR status affects the function of the SHR/SCR complex to spatiotemporally control the expression of a plant homolog of D-type cyclin (CYC D6;1). CYC D6;1, in cooperation with its corresponding cyclin-dependent kinase (CDK), promotes phosphorylation of RBR^[83]. These findings indicate that in plants, the RB ortholog exerts the function of controlling stem cells through the regulation of well-conserved cell cycle machinery. Further investigation in this field might unveil many unexpected aspects of RB functions in the control of stem cells.

MECHANISTIC ASPECT OF RB FUNCTIONS IN CONTROLLING STEM CELL ACTIVITIES IN MAMMALIAN CELLS

Cell cycle

Analysis of the cell cycle status in embryonic stem (ES) cells provided valuable information on how its alteration might contribute to the acquisition of increased self-renewal and pluripotency. Conklin and Sage^[84] provided a concise perspective on the possible roles of RB in maintaining the ES cell functions. ES cells have a rapid cell cycle. Because of the prolonged or continuous expression of cyclin E and A family members and lower or no expression of many CDK inhibitors, human and mouse ES cells maintain high levels of activity in multiple CDKs. pRB is consequently hyperphosphorylated for longer periods in ES cells than in normally cycling cells. No obvious cell cycle phenotype in mouse ES cells following inactivation of all RB family members might be consistent with this view^[85,86].

An extraordinarily shorter G₁ phase as compared to the relatively prolonged S phase in ES cells would be beneficial to lessen the susceptibility to differentiate upon receiving stimuli to lender cells to do so. As G₁ as well is the period that is most vulnerable to mitogenic signals that directs cells to decide to proliferate, arrest or senesce, this phase could be the most critical one in the decision to differentiate upon various stimuli. Keeping the G₁ phase shorter could be the primary role of RB hyperphosphorylation in ES cells. Consistent with this view, overexpression of the constitutively active

(non-phosphorylatable) form of RB (RB^{7LP}) in human ES cells induced cell cycle arrest, followed by spontaneous differentiation and p53-dependent cell death^[87].

The difference in undifferentiated behaviors between *Rb*^{+/+}; *p53*^{-/-} and *Rb*^{-/-}; *p53*^{-/-} MEFs predicted that there could be cell cycle-independent functions of RB in controlling stemness. *p53*^{-/-} MEFs exhibited similar proliferation phenotypes regardless of Rb genotype; however, *Rb*^{-/-}; *p53*^{-/-} showed increased sphere forming activity relative to *Rb*^{+/+}; *p53*^{-/-} MEFs^[15]. The same phenomenon was observed in mouse soft tissue sarcoma cells and mouse mammary gland epithelial cells. Given that RB-deficiency has no or very little impact on cell proliferation in a *p53*-null genetic background, the mechanism whereby RB-deficiency effects stem cell-like features of multiple types of cells can be separated from that governs cell proliferation.

Quiescence and apoptosis

In contrast to mouse ES cells, inactivation of all RB family members in human ES cells exhibited abnormal quiescence, featured by G₂/M arrest and cell death^[87]. Cell death depended on the p53-p21 module, similar to RB overexpression. Thus, in human ES cells, both hypo- and hyper-activation of RB are counteracted by cell cycle arrest and p53 pathway activation, indicating a critical role for RB in the homeostatic control of ES cell activities. p53 is typically expressed in human ES cells at a low level^[88]. This might be beneficial for ES cells so that they are not too sensitive to alterations in RB activity status. Mouse hematopoietic stem cells lacking all RB family proteins exhibit impaired quiescence control and apoptosis in lymphoid progenitor cells^[89]. In MEFs, the absence of p53 endowed RB-deficient cells significant increase in self-renewal activity when cultured in the presence of limited growth factors^[15]. However, in a *p53*^{-/-} background, the RB status did not impact cell proliferation of MEFs under regular culture conditions. It should be noted again that in mouse ES cells, lack of RB family proteins generated no difference in the cell cycle^[85,86]. These findings indicate that RB may control stemness beyond its role in cell cycle control.

Cellular senescence

Cellular senescence is believed to be strongly inhibited in stem cells, otherwise irreversible growth arrest could lead to total elimination of a stem cell pool from tissues. RB plays pivotal roles in inducing and maintaining cellular senescence, not only by controlling required transcription of genes, but also by being involved in senescence-associated heterochromatic foci (SAHF)^[90]. Therefore, hyperactivation of RB can be harmful to stem cells, as was shown in a human ES cell study^[87].

Surprisingly, loss of RB function, as well, can induce cellular senescence. As mentioned above, thyroid tumors that developed in *Rb*-heterozygous mice were typically low-grade adenocarcinomas or adenomas of

C cell origin; however, the genetic background lacking *N-ras*^[37] or either *Ink4a*, *Arf*, *Suvh39*^[14], *ATM*^[38] or *p53*^[15] allow these tumors to develop into highly invasive and metastatic type medullary adenocarcinomas. The immunohistochemical observation of Rb-deficient C cell tumors lacking no other genes revealed whole evidence of cellular senescence, including increased expression of p16^{Ink4a} and HP-1, and positive β -galactosidase staining^[14]. Since a simultaneous lack of genes mediating DNA damage response (*ATM*) and cellular senescence (*Ink4a*) allowed malignant progression of C cell tumors, we concluded that these cellular responses prevented Rb-deficient premalignant cells from developing into malignant cells. Analysis of *Rb-N-ras* DKO mice revealed that the mechanism whereby RB loss induces a DNA damage response involved p130^[14]. Another system in which RB loss possibly induces cellular senescence under particular culture conditions is MEFs. *Rb* loss alone does not allow MEFs to escape senescence when cultured at low density. However, simultaneous loss of *N-ras* or *Ink4a* allowed MEFs to escape cellular senescence upon low cell density plating^[14]. The reason why *N-ras* loci are associated with susceptibility to senescence was explained by the E2F-dependent control of Ras isoprenylation^[14].

There are three other tumor suppressors whose loss of function can induce cellular senescence in particular contexts. These are PTEN, VHL and NF1^[90-93]. Somatic cells are protected from carcinogenesis when these tumor suppressor genes are inactivated at early steps of carcinogenesis. This could be the major reason that RB mutations are detected in only limited types of cancer at their initiation.

ES cells, iPS cells, and tissue stem cells seem to confer lower RB activity in order to accelerate self-renewal and to keep undifferentiated state. We do not know whether cellular senescence machineries are simultaneously suppressed during the period that RB function is suppressed in stem cells. Unlike *Rb*^{-/-}; *p53*^{-/-} MEFs, *Rb*^{-/-}; *Ink4a*^{-/-} MEFs did not form spheres^[15]. However, regarding iPS induction, the *Ink4a/Arf* locus appeared to be a barrier to reprogramming^[94]. Therefore, RB functions in controlling cellular senescence could be intimately involved in the regulation of stemness.

Chromosomal instability

Chromosomal instability (CIN) might be one of the events that are seemingly not shared by normal stem cells and cancer cells. In tumor cells, RB inactivation, especially when combined with p53 mutation, led cells to accumulate chromosomal aberrations^[95]. Inactivation of all RB family members in human ES cells causes CIN; however, clonal expansion of these cells was blocked by G2/M arrest and cell death^[87]. Presumably, then, normal stem cells are much less tolerant of CIN than cancer cells. RB status could influence CIN through its effects on Mad2 transcription and its direct interaction with cohesion and condensin II^[96]. The rapid cell cycle

in ES cells might increase the risk of accumulating DNA damage due to hyper-replication and nucleotide deficiency^[97]. How ES cells are protected from these risks and why cancer cells tolerate CIN needs to be clarified.

Epigenetics

Through LxCxE motifs, RB interacts with numerous chromatin modifiers, including DNMT1, SUV39H1, Suv4-20H1, BRN1, BRG1, HDAC, and KDM5A/JARID1A/RBP2^[36]. Among these, KDM5A might mediate RB function to control stemness. KDM5A demethylates tri- and dimethylated lysine 4 in histone H3. ES cells lacking KDM5A failed to maintain OCT4 and NANOG expression upon stimulation to promote differentiation^[98]. KDM5A controls RB-dependent myogenic differentiation, at least partially, through the regulation of mitochondrial function^[99]. Importantly, loss of KDM5A in Rb-heterozygous mice attenuated pituitary tumorigenesis^[98]. KDM5A was first identified in a screen to find proteins that bind to pRB mutants unable to bind to E2Fs^[100]. pRB mutants unable to bind to KDM5A failed to control differentiation. The mechanism whereby KDM5A controls mitochondrial function involves PGC-1/PPAR γ c1a^[99]. It is of great interest to determine whether other genes under the influence of RB-KDM5A axis control stemness based on the effect on their epigenetic status.

Our experience with the stem cell-like behaviors exhibited by RB-p53 double deficient cells indicated that some of stem cell-like features are not reversed by RB reconstitution. This suggests that the effect of RB deficiency on stem cell-like behaviors might depend on its role in epigenetic control. Many chromatin modifiers carrying LxCxE motifs may be involved in the epigenetic function of pRB; however, so far, only some of them have been characterized regarding their role in controlling stem cell functions. We demonstrated that Rb in mouse cells exerts its influence on the epigenetic control of *Ink4a*, *Shc*, and *FoxO6* through DNMT1; however, its significance in the control of stemness has not yet been elucidated^[38].

Tissue specific transcription factor

RB plays critical roles in the terminal differentiation of cells owing to its genetic and physical interaction with tissue-specific transcription factors, including MYOD, C/EBP, GR, GATA-1, PU-1, CBFA-1, PDX1, RUNX2, and NF-IL6^[36]. It is totally unknown whether the interaction between RB and these factors has any role in tissue stem cells. Myoblast regeneration induced by RB and ARF depletion in post-mitotic muscle cells^[101] may involve the elimination of MYOD functions because there is a physical interaction between RB and MYOD. Additionally, the physical interaction of RB and RUNX2 may explain dedifferentiation in osteoblasts that would occur upon development of osteosarcoma.

The interaction of RB with ID2, KDM5A, and EID1 may govern differentiation in a less tissue-specific manner.

ID2, when overexpressed in Nestin-expressing cells, induced precocious neural stem cell depletion^[102]. RB is to some extent dispensable in brain development during the embryonic stage since Rb-deficient embryonic brains exhibited almost normal development, except for ectopic cell cycle entry and cell death in cortical neurons^[103,104]. However, the role of RB in adult neural stem cells has not been sufficiently addressed yet.

Embryonic gene

There are reports that NANOG and SOX2 induce hyperphosphorylation of pRB through regulation of CDC25A and CDK6^[84]. OCT4, in cooperation with miR-335, induces hyperphosphorylation of RB by suppressing PP1 through NIP1 and CCNF^[105]. Following RB hyperphosphorylation, freed E2Fs transactivate OCT4-targeted genes. These findings indicate the general function of RB in orchestrating the embryonic gene network. The most surprising finding regarding the role of RB in the embryonic gene network was recently made by the Wernig and Sage laboratory^[59]. They discovered that RB is directly involved in the transcriptional control of OCT4 and SOX2. They also demonstrated that Sox2 deletion attenuated pituitary tumorigenesis in Rb-heterozygous mice. These findings clearly revealed the strong influence of RB on the embryonic gene network.

These findings also explain the upregulation of a series of embryonic genes in MEFs lacking all RB family members^[13] and also in those lacking Rb and p53^[15] when forming embryoid body or spheres under nutrition-restricted and floating conditions. However, there was no evidence of upregulation of these genes when cells were cultured under 2D culture conditions in the presence of serum. This implies that regulation of OCT4 and SOX2 by RB is influenced by the environment where cells are placed and other genes that play a critical role in allowing RB to control embryonic genes. It would be of great interest to survey such genes.

Metabolism

There are significant similarities between stem cell metabolism and cancer cell metabolic reprogramming (rewiring)^[106,107]. RB functions in various metabolic pathways have attracted attention from cancer researchers^[36,45,46,108]. Given that RB is the central molecule in cell cycle control, it is reasonable that RB also responds to demands for increasing biomass that is coupled to the cell cycle. It is well known that RB controls thymidine kinase 1 (TK1) and dihydrofolate reductase (DHFR), both of which are required for nucleotide synthesis^[109]. However, the biomass needed for doubling the cell size during the S phase contains not only nucleotides but also amino acids, lipids, and many other carbon metabolites. pRB undergoes post-translational modification by various nutrient signals involving SIRT1 and AMPK and by cyclin/CDK complexes stimulated by various mitogenic signals^[110,111]. RB regulates the transcription or the activity of wide range of enzymes, signaling molecules, and transcription factors, including OXPHOS genes, MVA pathway genes, UCP-1, SOD2, ASCT2, GLS1, PKA, AKT, mTOR, PDK4, PGC-1, ERR, FOXc2, HIF-1, BNIP3,

SREBP-1,2, PPAR and KDM5A^[12,14,112-124]. Genes encoding many of these factors are supposed to be driven by E2F transcription factors. Myc is also a well-established downstream molecule of RB, and is implicated in the transcriptional control of GLUT1, HK2, PKM2, and LDH-A^[45].

OXPHOS activity is significantly correlated to the efficacy of iPS induction^[125]. Besides its interaction with OCT4 and SOX2, RB might be implicated in nuclear reprogramming through its influence on OXPHOS.

HIF-1 α activation explains why hypoxia facilitates iPS induction and self-renewal of tumor cells^[126,127]. Additionally, this molecule was implicated in the long-term maintenance of hematopoietic stem cell (LT-HSC) populations^[128]. Thus, RB status presumably has a big impact on glycolysis and TCA activity through the functional interaction with HIF-1. We have recently identified an HIF-1-independent target of RB in the glycolytic pathway. It is of interest to address how RB and HIF-1 cooperate in the control of the glycolytic pathway.

Recent studies from two independent research groups highlighted the link between RB status and glutaminolysis in *Drosophila* and mammalian cells^[116,129]. Activation of the glutamine pathway not only fuels the TCA cycle, but also contributes to control of the cellular reactive oxygen species (ROS) level by regulating glutathione synthesis^[130]. This may give both stem cells and cancer cells an advantage in terms of the maintenance of stemness. Additionally, ASCT2 in cooperation with GLUT1 appears to regulate human hematopoietic stem cell lineage specification^[131]. Increased dependency on glutamine metabolism is frequently observed in cancers, and has been linked to the emergence of drug resistance^[132,133]. Glutamine metabolism could be an important link between stem cells and cancer cells.

While addressing the question of why Ras proteins are activated after RB loss, we discovered that RB controls a number of enzymes involved in the MVA pathway. This study has been extended to another study that addresses the role of the MVA pathway in controlling stem cell-like activity in prostate cancer cells. The mechanism that links RB to Ras include SREBP-1,2. The characterization of Rb-Srebp-1 DKO mice revealed that RB status has a big impact on the control of fatty acid quality. We also identified molecules that explain differential self-renewal activity between p53^{-/-} cells and Rb-deficient p53^{-/-} cells (see above). These include enzymes involved in the glycolytic pathway.

Inflammation

RB status has been linked to pro-inflammatory phenotypes in breast cancer cells^[68]. This study highlighted COX2 as a target of the E2F transcription factor. However, our reassessment of these data revealed that many pro-inflammatory cytokines and chemokines, including IL-6, CCL2, and CCL5, are upregulated when breast cancers express lower levels of RB. A similar observation was made in mice lacking Rb in the back skin of p21^{-/-} mice^[134]. The tumors derived from these mice exhibited high levels of pro-inflammatory cytokines and evidence of infiltration by

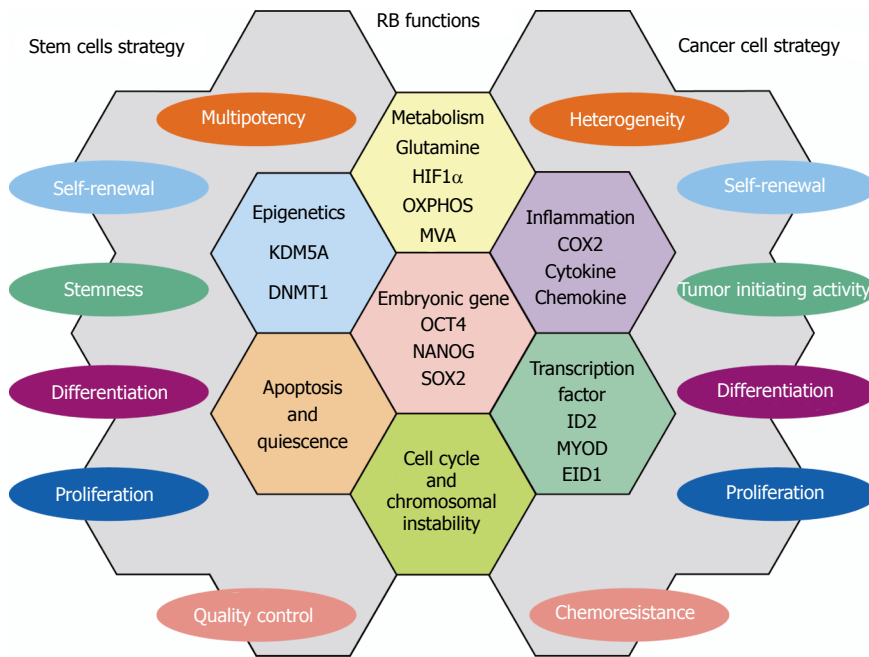


Figure 2 A schematic representation of molecular functions of retinoblastoma that may be shared by stem cells and cancer cells. RB: Retinoblastoma; MVA: Mevalonate.

immune cells. Indeed, many cytokines and chemokines, and even their receptors, are often regulated by E2F transcription factors^[135,136].

Among cytokines and chemokines, IL-6 and CCL2 are drawing attention as strong inducers of iPS cells. A study demonstrated that IL-6 is more potent than c-Myc in terms of their abilities to induce iPS cells^[137]. Another study demonstrated that CCL2 plays pivotal role in the maintenance of pluripotency in ES and iPS cells^[138]. There are many reports indicating that pro-inflammatory status is critical for cancer stem cells to evolve^[139-142]. Many of pro-inflammatory factors stimulates JAK/STAT3 pathway, thus contribute to enhance self-renewal of stem cells and possibly cancer stem cells. This could be one of core mechanisms that are shared by stem cells and cancer cells. Our recent efforts revealed that the RB status in soft tissue sarcoma, breast and prostate cancer cells significantly alter the pro-inflammatory status of these cells, and this significantly enhances self-renewal activity and chemo-resistance.

RB may control innate immunity as well. The RB-E2F1 complex appears to regulate toll-like receptor 3 (TLR3)^[143]. In *Drosophila*, RB, in cooperation with dCAP-D3, upregulates innate immunity^[144]. Several reports demonstrated that RB-deficiency in tumors attenuates the innate immune response, thereby promoting tumor development^[145]. There is also a report that showed a positive role for innate immunity in inducing iPS cells^[146].

CONCLUSION

In this manuscript, we described a part of numerous RB functions that are employed commonly as strategies to control stem cells and suppress cancer cells. These findings may help readers to understand how stem cell strategies are shared by cancer (stem) cells. Figure 2 demonstrates RB functions that may be shared by

stem cells and cancer cells. Although RB is one of tumor suppressors those have been characterized for long time and by big number of cancer researchers, our curiosity on its hidden roles in various biological events never fades away. In the near future, more number of stem cell and regenerative medicine researchers may stand up by this molecule. Although beyond the scope of this article, RB is implicated in the regeneration of many tissues/organs^[19]. Discovery of its target in the context of the undifferentiated state of cancer cells or in drug resistance may lead us to develop powerful tools in both cancer therapy and regenerative medicine.

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Stem cells sources for intervertebral disc regeneration

Gianluca Vadalà, Fabrizio Russo, Luca Ambrosio, Mattia Loppini, Vincenzo Denaro

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Abstract

Intervertebral disc regeneration field is rapidly growing since disc disorders represent a major health problem in industrialized countries with very few possible treatments. Indeed, current available therapies are symptomatic, and

surgical procedures consist in disc removal and spinal fusion, which is not immune to regardable concerns about possible comorbidities, cost-effectiveness, secondary risks and long-lasting outcomes. This review paper aims to share recent advances in stem cell therapy for the treatment of intervertebral disc degeneration. In literature the potential use of different adult stem cells for intervertebral disc regeneration has already been reported. Bone marrow mesenchymal stromal/stem cells, adipose tissue derived stem cells, synovial stem cells, muscle-derived stem cells, olfactory neural stem cells, induced pluripotent stem cells, hematopoietic stem cells, disc stem cells, and embryonic stem cells have been studied for this purpose either *in vitro* or *in vivo*. Moreover, several engineered carriers (*e.g.*, hydrogels), characterized by full biocompatibility and prompt biodegradation, have been designed and combined with different stem cell types in order to optimize the local and controlled delivery of cellular substrates *in situ*. The paper overviews the literature discussing the current status of our knowledge of the different stem cells types used as a cell-based therapy for disc regeneration.

Key words: Stem cells; Intervertebral disc degeneration; Spine; Tissue engineering; Cell therapy

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Core tip: This review paper aims to share recent advances in stem cell therapy for the treatment of intervertebral disc degeneration. The paper overviews the literature discussing the current status of our knowledge of the different stem cells types used as a cell-based therapy for disc regeneration. Intervertebral disc regeneration field is rapidly growing since disc disorders represent a major health problem in industrialized countries with very few possible treatments. Indeed, current available therapies are symptomatic, and surgical procedures consist in disc removal and spinal fusion.

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INTRODUCTION

Low back pain (LBP) is a common musculoskeletal symptom referred by more than 80% of the general population at least once in their life^[1]. It results in a relevant social and economic problem, affecting above all the productive population in developed countries. In fact, it presents a maximum rate of incidence in people between the ages of 45 and 64^[2] and it is the most frequent cause of disability and loss of days of activity in people under 45 years of age^[3,4].

The wide majority of LBP related to degenerative changes of the intervertebral disc (IVD). The IVD is a complex structure consisting of three specialized tissues: The annulus fibrosus (AF), the nucleus pulposus (NP) and the cartilaginous end-plate (CEP) which coats the adjacent vertebral body.

The AF is a fibro-cartilaginous ring providing the outer part of IVD. It is composed of collagen type I fibers, oriented radially and in opposite directions throughout concentric lamellae^[5], associated with an interlamellar matrix consisting of proteoglycans and non-collagenous proteins (such as elastin), in which mesenchymal cells, with a fibroblast-like morphology and phenotype^[6], are present. This matrix leads to an efficient interlamellar cohesion^[7].

The NP is a less structured gelatinous matrix rich in proteoglycans, mainly aggrecan, and type II collagen fibers randomly oriented. Aggrecan comprises a great number of negatively charged sulfated glycosaminoglycans that attract and imbibe water. The high level of hydration helps to maintain disc height and contributes to the load-bearing ability of the IVD^[8]. Small chondrocyte-like cells are scattered within the NP and are responsible for synthesizing and maintaining the matrix^[9].

The embryonic human IVD consists in two different structures: The NP, derived from aggregation of notochordal cells within a proteoglycan matrix, forming the gelatinous centre of the disc; the AF, which is derived from the perichordal mesenchyme, forming organized fibers surrounding the nucleus. During the sixth embryonic month, a mucoid degeneration of notochordal cells takes place in the NP and mesenchymal IVD cells invade the fibrocartilage. However, some notochordal remnants can be found in the IVD up to adulthood^[10].

The IVD provides support for vertebrae, shock absorber function and allows movements of flex-extension, lateral bending and rotation. The NP, surrounded by the annulus fibers, resists compressive stress, whereas AF resists primarily tensile, circumferential, longitudinal

and torsional stresses^[10,11].

Intervertebral disc degeneration (IDD) is an age-related chronic process characterized by a progressive decline of proteoglycans and water content in NP with loss of the disc ability to resist compressive loads^[12]. The first symptom of IDD is often LBP, that may lead to disc herniation, degenerative spondylolisthesis, instability and spinal stenosis associated with neurological symptoms such as radiculopathy and/or myelopathy. Current treatments for LBP and IDD range from conservative to surgical procedures^[13]. However, these treatment modalities have limited efficacy and do not produce predictable and reliable outcomes. In fact, they target the clinical symptoms instead of the pathophysiology involved in the degenerative process. An effective early treatment for LBP that may prevent, slow down or reverse the degenerative changes of the IVD is the goal of many researchers in the spine field. Exciting advances in tissue engineering have led spine researchers to develop novel regenerative techniques in order to alter the course of IDD and possibly lead to disc repair and recovery of function.

PATHOPHYSIOLOGY OF IDD

Although the increasing interest in biological treatments for IDD, its pathological basis is still not completely understood. The degenerative pathway is related to aging, starting from the second decade of life^[14], and to certain genetic profile expressions as well as environmental factors^[15]. Moreover, the IVD is the largest avascular tissue in the body, in which nutrition takes place by diffusion through the CEP, maintaining the viability of NP cells^[16].

The main structural changes in NP during IDD consist in a progressive reduction of proteoglycan content, first of all aggrecan^[17]. Morphological modifications are related to metabolic imbalance between anabolic and catabolic processes, regulated by multiple factors, such as anabolic growth factors [e.g., insulin-like growth factor-1 (IGF1)^[18], transforming growth factors β (TGF β), bone morphogenetic proteins (BMPs)^[19]] and catabolic enzymes [matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) resulting in changes in NP cells function^[20].

Extracellular matrix changes are associated with alterations of disc cell viability. The progressive reduction of cell density results in the inability of the IVD to revert degenerative changes by producing and maintaining a functional extracellular matrix^[21]. The decrease of NP proteoglycan content leads to progressive dehydration of gel-like nuclear matrix, decreasing disc height and altering its load-bearing capacity^[22]. The inefficiency of NP to absorb compressive stresses and to transmit forces circumferentially to the AF, leads to deterioration of the lamellar architecture of the AF itself, consisting of internal fissures spreading outward to the periphery^[23].

In addition to cracking and fissuring of the AF, disc herniation, subchondral sclerosis, CEP ossification and osteophyte formation^[24] may take place. The inherent avascularity, isolation, and low metabolic activity of the IVD may explain its apparent inability for self-repair following injury and degeneration^[25].

POTENTIAL BIOLOGICAL TREATMENTS

The therapeutic approach to treat or prevent disc degeneration could consist in recovering the disc ability to synthesize extracellular matrix, rich in proteoglycans, in order to re-establish disc hydration and NP visco-elastic features, thus restoring biomechanical properties of the IVD.

Several genes and growth factors have been found to influence the anabolic and catabolic processes, regulating the extracellular matrix homeostasis within the IVD. In this way recombinant growth factors or gene therapy technologies could be applied to treat the IDD^[26,27]. Intradiscal injection of growth factors, such as BMP-7, BMP-2 or IGF-1, has been shown to increase the proteoglycan level within the disc^[28-30].

The possibility to synthesize recombinant growth factors and to inject them with a percutaneous approach represents interesting advantages. However, the short half-life of exogenous growth factors has led to increasing interest for gene therapy in the treatment of IDD.

Gene therapy modifies the pattern of gene expression resulting in an *in situ* sustained production of specific gene products. In literature, numerous anabolic factors (such as TGF- β , BMP-2, BMP-7 or IGF-1), anti-catabolic factors (such as TIMP-1), and gene regulators (such as SOX-9 and LMP-1) have been found able to modulate the metabolic activity of disc cells, increasing proteoglycans disc content^[31-34]. However, side effects related to this emerging technology, such as inflammatory reactions of nerve roots and/or dura, have been described^[35]. More efficient and safe systems of transfection and transduction may be performed before clinical application of gene therapy^[27,36].

According to pathological findings in IVD aging and IDD, characterized by a progressive disc cell loss, cell therapy has been proposed in order to restore the disc cell population by introducing exogenous cells. A cell therapy approach can be performed by using different types of differentiated cells, such as NP cells^[37,38], AF cells^[39], cartilaginous chondrocytes^[40] and progenitor cells^[41-43]. The autologous disc-derived chondrocyte transplantation (ADTC) is a treatment based on autologous NP cells to replace the tissue loss caused by disc herniation and disc surgery^[44]. Although clinical data seems to report back pain improvement and prevention of disc height reduction after the treatment^[45]. ADTC procedure presents the following limits: (1) it is only applicable when discectomy is required; (2) it is a two-steps procedure, because discectomy and cell transplantation are performed in two different times;

and (3) disc cells lose their phenotypic characteristics when expanded in monolayer cell culture^[46].

Therefore, stem cell therapy is more attractive due to low harvest site morbidity, ease of *ex vivo* cell expansion, and favorable modulation of the cell phenotype before or after transplantation. In this review we will discuss about the potential use of different types of stem cells employed for disc repair.

STEM CELLS BASED THERAPY

Stem cells are unspecialized cells characterized by a high proliferation rate. They can reside in a quiescent state, in which they self-renew; during the proliferation process, they perform an asymmetric division producing two daughter populations: One of them constituted by identical stem cells and the second ones formed by progenitor cells committed to a lineage-specific differentiation program^[47]. Different types of human stem cells, ranging from embryonic to adult stem cells, have been found. Although embryonic stem (ES) cells are considered to be totipotent, legal and ethical controversies limit their use for clinical application in regenerative medicine^[48]. Adult stem cells represent a reservoir of progenitor cells harbored within specialized niches of the adult organism, suggesting the potential for therapeutic application in their host tissues. Adult stem cells have been discovered and characterized in tissues such as bone marrow^[49], adipose tissue^[50], periosteum^[51,52], synovial membrane^[53], muscle^[54], skin^[55], pericytes^[56,57], blood^[58] and trabecular bone^[59,60]. Their function consists in maintenance of the anatomical and functional features of each specialized tissue. Because they are committed to a lineage-specific differentiation pathway, these cells are able to produce a limited range of specialized cells according to the embryonic origin of the tissue itself. The application of adult stem cells in regenerative medicine does not raise any ethical problems, as they can be directly isolated from the patient.

The potential application in IVD regeneration has been described for some types of adult stem cells, including bone marrow-derived mesenchymal stem cells (MSCs)^[42,61,62], adipose tissue-derived stem cells (ASCs)^[41], muscle-derived stem cells (MdSCs)^[43], hematopoietic stem cells, olfactory membrane stem cells and synovial stem cells (Figure 1). Adult stem cell types are committed to differentiate following the lineage of mesenchymal tissues, including bone, cartilage, fat, and muscle^[49,50,54,63]. Moreover, according to recent findings^[64,65], MSCs, such as bone marrow MSCs, ASCs and MdSCs, seem to derive from the perivascular wall^[66] of the tissue the stem cells are from. Several therapeutic strategies for IVD regeneration based on stem cells have been described that direct injection into the IVD of undifferentiated or pre-differentiated cells (cell therapy). Application of constructs derived by conjunction of stem cells with a visco-elastic hydrogel (tissue engineering strategy); genetic

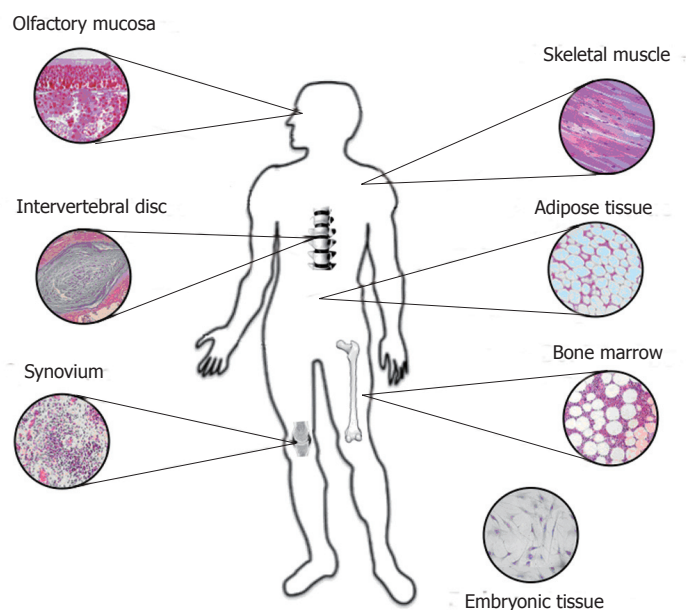


Figure 1 Major sources for harvesting stem cells used for disc regeneration.

modification by transfection of target genes followed by injection of transfected stem cells into the IVD (*ex vivo* gene therapy)^[27,67] (Figure 2).

Recently, a specific population of progenitor cells has been identified in the degenerated human IVD^[68]. This finding has been confirmed by Blanco *et al.*^[69], who demonstrated that progenitor stem cells are quite similar to mesenchymal stromal cells derived from bone marrow. Therefore, an alternative approach for treatment consists in recruiting endogenous progenitors to orchestrate IVD repair by administration of suitable drugs/growth factors.

BONE MARROW MSCS

In the past few years several *in vitro* studies have been conducted to evaluate the use of MSCs for the treatment of IDD.

The actual capacity of adult human MSCs to differentiate towards NP cells has been one of the first steps in the evaluation of their utilization as a cell source for IVD regeneration. MSCs can differentiate into chondrocyte-like cells phenotypically similar to NP cells in chondrogenic conditions^[68,70]. These methods have been considered a preconditioning system to direct MSCs into NP-like cells before they are implanted into the IVD.

The therapeutic effects of stem cells have been extensively studied *in vitro*. Several studies suggested that the regenerative potential of MSCs may result from MSCs and NP cells interactions that up-regulate extracellular matrix protein synthesis in terms of proteoglycans. Le Visage *et al.*^[71] cocultured bone marrow MSCs with NP cells at a 50:50 ratio in 3-dimensional pellet culture system for 2 wk showing that, although there was a trend of increase in

glycosaminoglycan (GAG) production, the difference was not significant. On the contrary, Sobajima *et al.*^[62] using a similar pellet coculture system at a different ratio, revealed a synergistic effect between NP cells and MSCs at 75:25 and 50:50 NP:MSC ratio, yielding a significant increase of proteoglycan synthesis rate and GAG content compared with culture of NP cell and MSCs alone. Recently, Svanvik *et al.*^[72] confirmed that coculture of MSCs with degenerated NP cells increases proteoglycan and collagen-type II production.

In order to delineate the effects, several studies have utilized the coculture model systems to understand whether the interaction between MSCs and IVD cells leads to MSC differentiation to an NP-like phenotype and/or whether MSCs promote regeneration through stimulation of native NP cells. Stem cells undergo differentiation under stimuli that come from the surrounding microenvironment. However, adult stem cells contribute to tissue repair and regeneration by not only differentiating into the phenotypes of the host cells^[49] but also creating a microenvironment that promotes the local regeneration of endogenous cells^[73]. Richardson *et al.*^[74] cocultured human MSCs with normal NP cells in monolayer with or without cell-to-cell contact. After 1 wk of culture, fluorescent-labeled MSCs separated by fluorescence-activated cell sorting and gene expression was evaluated. MSCs underwent a change in gene expression profile similar to NP-like cells as demonstrated by an increased expression of aggrecan and collagen type II genes^[74]. However, the low cell density in the NP^[75] makes direct cell-to-cell contact between MSCs and NP cells a rare event if stem cells are implanted into the disc. Therefore, our group studied the mechanisms of the interaction between human NP cells from degenerating discs and MSCs in 3-dimensional culture, a system that allows

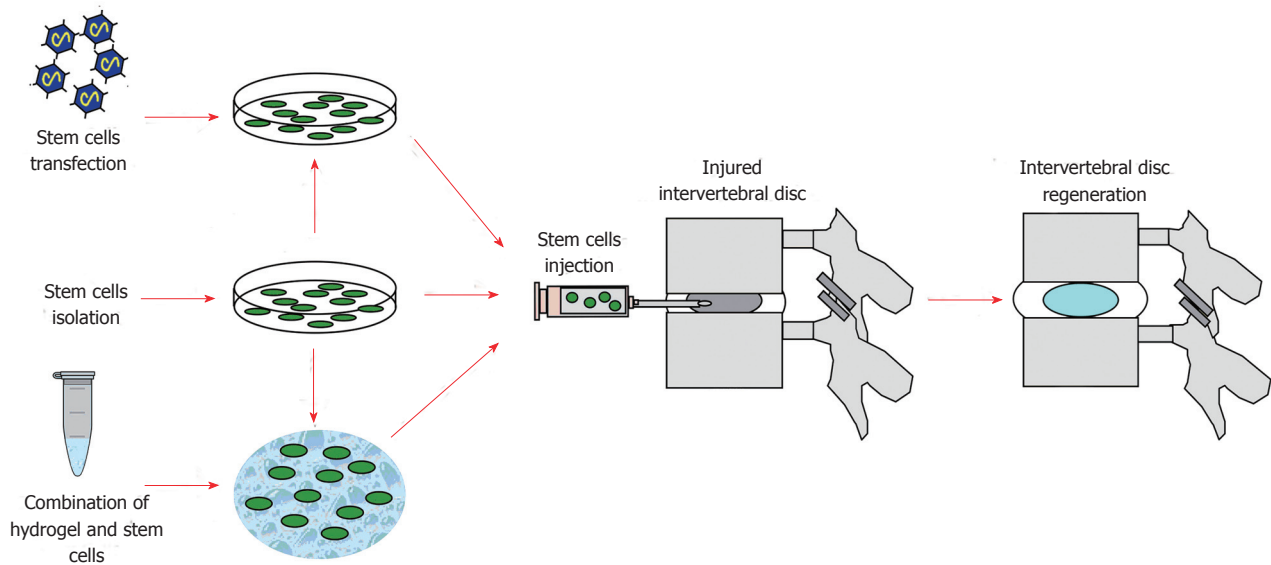


Figure 2 Flow chart summarizing the main steps of disc regeneration stem cell-based therapy. Stem cells can be either directly isolated and expanded, or combined with biocompatible carriers (e.g., hydrogels) or transfected with target genes, then injected into the nucleus pulposus of the injured intervertebral disc, potentially leading to disc regeneration.

short distance paracrine interactions typical of the NP tissue, hence miming its architecture^[76]. Using a double labeling cell system, changes in gene expression profile were analyzed on the MSCs or NP cells populations isolated from the coculture. MSCs acquired a more chondrogenic gene expression profile and influenced mRNA levels within the human NP cells.

Paracrine stimulation in the interaction between MSCs and IVD cells was also assessed in other studies. Yang *et al.*^[77] used a noncontact coculture system to elucidate the interaction between NP cells and MSCs mediated by soluble factors. These authors showed that secreted factors in the coculture were able to induce collagen type II expression by uncommitted MSCs when cultured with a higher number of NP cells. Korecki *et al.*^[78] studied the effect of conditioned media from notochordal cell cultures on MSCs showing differentiation toward a potentially NP-like phenotype with some characteristics of the developing IVD.

MSCs secrete a variety of cytokines and growth factors that are able to stimulate mitosis and tissue-intrinsic reparative potential of the host cells^[79]. Accordingly, Yamamoto *et al.*^[80] reported increased cell viability, proliferation and proteoglycan synthesis of rabbit NP cells induced by cell-to-cell contact with MSCs in a coculture monolayer system, supporting a trophic effect. Watanabe *et al.*^[81] further confirmed these data by using human cells. The trophic effect only partially reflected in our gene expression study. Although we observed an increase in collagen type II by NP cells after coculture with MSCs, aggrecan gene expression was down-regulated^[76]. This data reflected only a modest trophic effect of MSCs on degenerate NP cells. This finding is in agreement with Strassburg *et al.*^[82] who investigated differences in the interaction between human MSCs and NP cells from both nondegenerate

and degenerate discs during *in vitro* coculture with direct cell-cell contact. They concluded that, although both nondegenerate and degenerate NP cells are able to stimulate MSCs differentiation to an NP-like phenotype, MSCs were only able to stimulate degenerate NP cells to increase their matrix-associated gene expression to levels comparable to nondegenerate NP cells.

Cell fusion has been found to be potentially responsible for the plasticity and tissue regeneration potential of adult stem cells^[83,84]. The possible occurrence of cell fusion in the interaction between male MSCs and female NP cells has been studied by our group^[76]. Cell fusion was examined in a pellet coculture system to accentuate cell-to-cell interactions, a necessary condition for inducing cell fusion^[83]. Fluorescence *in situ* hybridization assay for the X and Y chromosomes demonstrated that cell fusion does not occur in the interaction between MSCs and NP cells^[76]. This result is in contrast with the data of Strassburg *et al.*^[82] who also studied cells fusion in the pellet coculture system. These authors were able to detect binucleated cells among cocultured cells during histologic observation, thus raising the possibility of cell fusion. Therefore, further studies needed to determine if the exposure of NP cells to MSCs leads to spontaneous cell fusion.

Organ culture systems were also used to study the potential effect of MSCs for IVD regeneration. Le Maitre *et al.*^[85] transplanted human MSCs in a bovine caudal disc and cultured them *in vitro* up to 4 wk. These authors showed that MSCs from older individuals differentiate spontaneously into chondrocyte-like NP cells upon insertion into NP tissue. Chen *et al.*^[86] studied the effect of porcine MSCs injected in an IVD degeneration model showing a regenerative potential. Therefore the *ex vivo* degenerative IVD organ culture system has the potential to contribute to a better

comprehension of alternative IVD regeneration strategies.

MSCs engraftment and long term survival in the harsh environment of the normal disc tissue has been demonstrated by several *in vivo* study in small animals^[62,87,88]. Crevensten *et al.*^[88] have shown that MSCs injected into rat discs with hyaluronan gel as a carrier, maintained viability over 28 d with cell proliferation. Zhang *et al.*^[87] have shown that allogeneic MSCs injected in a healthy disc increased the total proteoglycan content in the NP of rabbit discs. Sobajima *et al.*^[62] evaluated the long-term survival of allogeneic MSCs in healthy rabbit lumbar IVDs. The MSCs, retrovirally transduced with LacZ marker gene, were identified up to 6 mo after transplantation, observing MSCs migration from the NP to the inner AF.

However, in order to determine the efficacy of a stem cell therapy in preventing or delaying the progression of IDD, it is critical to rigorously test stem cell therapy in animal models of IDD. The first efficacy study published in the field is dated back to 2003: Sakai *et al.*^[89] elegantly showed that autologous bone marrow MSCs embedded in a collagen type II based carrier (Atelocollagen®) and injected in an NP aspiration model of IDD enhanced proteoglycan content and hydration. The same authors, using a similar study design, showed that the MSCs injected disc maintained disc height of 97% and magnetic resonance image (MRI) signal intensity of about 81% in a normal control group discs, while the degenerating disc group that did not received MSCs injection demonstrated a disc height value of about 67% and MRI signal intensity reduction of about 60%. Moreover, Sakai *et al.*^[90] demonstrated that undifferentiated MSCs transplanted into degenerated discs in rabbits proliferated and differentiated into cells expressing some of the major phenotypic characteristics of NP cells, suggesting that these MSCs may have undergone site-dependent differentiation.

Ho *et al.*^[91] studied the influence of the degenerative grade on the therapeutic potential of MSCs. These authors induced IDD in a rabbit model by stabbing and injecting MSCs at 1 or 7 mo. They observed that MSCs appear to be more effective in arresting degeneration at a relatively later stage of disc degeneration.

Jeong *et al.*^[92] used a xenogeneic transplantation model to study the effect of human MSCs injected into the coccygeal rat IVDs 2 wk after a blade stabbing. Disc height and MRI signal intensity of the MSCs transplanted disc increased compared to the degenerated control group at 2 wk after injection.

Yang *et al.*^[93] performed a study on a rabbit model of IDD induced by nucleus aspiration. These authors injected into the IVD a mixture of fibrin glue, TGF- β 1 and rabbit MSCs using as a control the carrier alone with the growth factor or the sham. These authors have shown that MSCs injection led to a reduced height loss associated with IDD and an increased quantity of collagen type II content and a decrease in the rate of cell apoptosis.

Hee *et al.*^[94] performed a study on a rabbit model of IDD induced through a compression device. Allogeneic MSCs embedded in Atelocollagen were transplanted into the compressed disc followed by unload or distraction. Controls underwent just distraction or unload period. This animal study showed that the transplanted IVDs performed better with respect to disc height, morphological grading, histological scoring and average dead cell count and that distraction increased the regenerative effect of MSC transplantation.

Allon *et al.*^[95] explored the potential of the use of bilaminar coculture pellets (BCPs) of MSCs in a shell of NP cells for IVD regeneration.

The pellets were transplanted *in vivo* in a rat tail nucleotomy model of disc degeneration.

BCPs were transplanted in a fibrin sealant (FS) carrier using as a control the FS with a pellet of just MSCs or NP cells, MSCs and NP cells randomly mixed or the FS only; and surgery only. This study showed that the proteoglycan and cytokine levels were not significantly different among groups. The BCP group had higher cell retention, disc height and increased disc grade over time than controls^[95].

Henriksson *et al.*^[96] performed a study using a xenotransplantation model in minipigs. IDD was induced in lumbar IVD by nucleosuction and 2 wk later human MSCs were injected in F12 media suspension (cell/med) or with a hydrogel carrier (cell/gel). The animals were sacrificed after 1, 3, or 6 mo. At MRI all injured discs demonstrated degenerative signs with fewer positive changes in the cell/gel group compared with cell/med discs and injured only discs. The authors concluded that transplanted human MSCs survived in the porcine spinal disc up to 6 mo and expressed SOX-9 and Collagen II, thus indicating differentiation. The hydrogel carrier has shown to facilitate the differentiation of transplanted hMSCs.

Recently, MSCs injection has tested in a larger animal model. Hiyama *et al.*^[97] evaluated the effect of MSCs transplantation on the suppression of IDD and preservation of immune privilege in a canine model of IDD. MSCs injection effectively led to the regeneration of degenerated discs and contributed to the maintenance of IVD immune privilege by the differentiation of transplanted MSCs into cells expressing FasL.

Serigano *et al.*^[98] used a canine IDD model to perform a dose-escalation study to assess the optimal number of cells to transplant into the degenerated IVD. Four weeks after nucleosuction, autologous MSCs transplanted at 105, 106, or 107 cells per disc. Unoperated and untransplanted disc were used as a control. MSCs-transplantation groups showed preservation of disc height and annular structure compared to the operated control group. The analysis of the survival rate of both transplanted and MSCs as well as NP cells demonstrated the better performance of 106 MSCs, when compared to 105 or 107, producing the best maintenance of the structure of IVDs and best inhibited IVD degeneration.

The goat study conducted by Zhang *et al.*^[99] per-

formed using a disc degeneration model induced by stabbing the disc with a number 15 blade. One month after injury, allogeneic MSCs were injected with a hydrogel into the IVDs. Degenerating IVDs injected with MSCs showed significantly increased proteoglycan content within the disc. However, collagen content, MRI imaging, and histology did not show statistically significant differences between the cell-treated and control IVDs.

Subhan *et al.*^[100] transplanted allogeneic MSCs embedded in a hyaluronan based hydrogel (HyStem) into degenerate discs by fluoroscopy assisted minimally invasive delivery in a rabbit model. Animals were divided into three groups: Group I treated with MSCs coupled with Hystem, group II injected with Hystem alone and group III was left without any intervention. At 8 wk after transplantation, histological assay and MRI T2 mapping of NP showed higher T2 signal intensity, disc height index and type II collagen and aggrecan content in group I compared to other groups; similar results were reported by Cai *et al.*^[101] as well.

In a pilot study, Orozco *et al.*^[102] injected autologous expanded bone marrow MSCs into the nucleus pulposus of 10 patients diagnosed with lumbar disc degeneration. One year follow-up investigated evaluation of back pain, disability and quality of life, whereas disc height and fluid content were assessed through MRI. Patients reported prompt improvement of pain and disability at 3 mo and increased disc water content at 12 mo, even if disc height did not restore.

In a similar study, Yoshikawa *et al.*^[103] harvested autologous bone marrow MSCs from the ilium of two patients diagnosed with spinal stenosis: Cells cultured in an autogenous serum media and then transplanted percutaneously into the stenosed spinal canal within a collagen sponge graft. At 2 years after surgery, patients reported symptoms improvement, while X-ray, Rontgen kymography and CT showed decreased instability and T2-weighted magnetic resonance indicated high moisture contents.

Overall, MSCs show a great capacity to differentiate towards the NP phenotype, especially when exposed to the chondrogenic molecular signaling within the injured disc, thus potentially restoring its physiological microenvironment and biomechanical properties. MSCs can be readily harvested from multiple sites, even if the procedure itself is not immune to secondary risks. Moreover, MSCs are the only stem cell type that have been transplanted in human disc proving to be safe and being able to reduce LBP in patient affected by IDD.

ADIPOSE STEM CELLS

Adipose stem cells (ASCs) are an alternative source of stem cells, instead of bone marrow MSCs, to regenerate the IVD. These cells can easily expand under standard tissue culture conditions and show a pluripotent mesenchymal differentiation capacity. Their therapeutic effect has been tested using coculture system with

other adult stem cell types^[104].

Li *et al.*^[41] evaluated changes in the gene expression pattern of rabbit fat-derived mesenchymal cells when exposed to NP and AF cells *in vitro*. Authors demonstrated an increase in expression of type II collagen and aggrecan genes from rabbit ASCs cocultured in 3-dimensional alginate beads with NP cells, compared to ASCs cocultured with AF cells and NP cells alone. These data have been also confirmed by Lu *et al.*^[105], who investigated the ability of ASCs to differentiate when exposed to stimuli secreted by NP cells *in vitro*. Authors performed a transwell co-culture system of human NP cells and human ASCs, employing both monolayer and micromass configurations, in order to evaluate the sole effects of soluble signals. Lu *et al.*^[106] demonstrated that the transwell co-culture of NP cells and ASCs, both cultured under micromass conditions, induces gene expression of both aggrecan and collagen type II, with concomitant down-regulation of osteopontin, collagen type I and PPAR- α in ASCs. Moreover, Lu *et al.*^[106] also evaluated the gene pattern expression of human ASCs cultured in collagen type I or type II hydrogels alone, or cocultured in transwells with micromass human NP cells. They demonstrated that ASCs differentiation along the cartilaginous lineage is characterized by up-regulation of collagen type IIA, type IIB and aggrecan gene expression and it closely related to cocultures with NP cells and type II hydrogel. Collagen type II represents an appropriate scaffold for the attachment of ASCs and a favorable microenvironment in combination with soluble factors secreted by NP cells inducing the differentiation along cartilage/NP lineage.

Disc regeneration strategies based on adipose stem cells have also evaluated in small and large size animal models. Jeong *et al.*^[107] investigated the adipose-tissue-derived stromal cell (ADSC) implantation to restore disc in a rat degenerated IVD model. The IVD damaged by needle injection and, after two weeks, injected with ADSCs or saline (as control). At 6 wk after transplantation, authors demonstrated the ability of ADSCs to restore degenerated IVDs, according to reduced disc height loss and restoration of disc signal intensity on MRI. The histological analysis with hematoxylin and eosin staining confirmed a greater IVD restoration in discs transplanted with ADSCs. In addition, positive findings in immunohistochemical staining for collagen type II and aggrecan have also revealed.

Ganey *et al.*^[108] investigated ADSCs-based cell therapy in degenerated IVD using a dog model obtained by performing a partial nucleotomy on lumbar discs. Six weeks after surgery, authors randomized discs to receive: ADSCs loaded in hyaluronic acid carrier (cells/HA) or HA without cells or nothing. Dogs were killed at 6 mo or at 12 mo. Disc analysis has performed with MRI, radiography, histology and biochemistry. No significant differences between the three different approaches have found in MRI signal intensity and radiographic disc height. However, gene expression of type II colla-

gen and aggrecan demonstrated a statistically significant increase of expression in discs transplanted with ADSCs when compared to discs receiving either the HA only or no treatments. ADSCs are able to provide a regenerative stimulation in the injured IVD. Moreover, the histological analysis showed an abundant extracellular matrix surrounding the cells and cell clustering or cloning within NP. AF fibers were tight and laminated according to the normal IVD morphology. Because of the evidence of injected ADSCs survival, the histology suggested their responsibility for the observed morphology resembling the healthy IVD. Ganey *et al.*^[108] reported that ADSCs were effective in promoting disc regeneration in an animal injured disc model.

Sun *et al.*^[109] assessed the influence of ADSCs on NP cells in a compressive load culture: Unphysiological mechanical stimulation was set in order to mimic the stressful conditions leading to IDD. ADSCs protected NP cells from apoptosis through caspase-9 and caspase-3 inhibition, increasing ECM gene expression while diminishing metalloproteinases synthesis inhibiting production of pro-inflammatory factors.

ADSCs have proven to give rise to a chondrogenic lineage and to increase aggrecan and type II collagen synthesis, hence favoring disc regeneration. While they can easily be harvested without significant risks, ADSCs actual efficacy has not been established yet.

SYNOVIAL MSCS

In the last years, synovial MSCs aroused an increasing interest about their application in cell therapy strategies for disc regeneration. They could be a potential source of stem cells because they present a proliferative rate greater than other types of MSCs, such as bone marrow MSCs^[110]. In addition, they show a high chondrogenic potential, demonstrated by the ability to synthesize extracellular matrix after transplantation into articular cartilage defects in a rabbit model^[111].

Miyamoto *et al.*^[112] assessed the effect of intradiscal transplantation of synovial MSCs by using an IVD degeneration rabbit model. After allogeneic synovial MSCs transplantation, researchers performed imaging analyses, including X-ray, MRI and histological analysis. Moreover, they performed an *in vitro* study in order to investigate the interaction between synovial MSCs and NP cells, by producing a co-culture system of human synovial MSCs and rat NP cells. The results showed that synovial MSCs injected into the disc were able to stimulate the remaining NP cells to synthesize type II collagen and to inhibit the expression of matrix degradative enzymes and inflammatory cytokines. These data were confirmed by *in vivo* findings, showing that IVD height in the MSCs group was higher than disc height in the degeneration group.

Synovial MSCs exhibit a notable proliferative and regenerative potential, which is confirmed by pilot studies in articular and disc degenerative models. Further studies are needed to support these findings, in order to

plan an appropriate therapeutic protocol.

MDSCS

MdSCs have shown to reside within skeletal muscles and to be characterized by typical stem cell features, such as self-renewal and multilineage differentiation. Indeed, they are capable of giving rise to other mesodermal cell types, including hematopoietic, osteogenic, chondrogenic, adipogenic and skeletal myogenic cells^[113].

As Adachi *et al.*^[114] reported appreciable healing of cartilage defects using muscle-derived cells embedded in collagen gels in a rabbit model, it has been hypothesized that the chondrogenic lineage commitment of MdSCs might therefore provide a prompt source for generating and expanding NP cells as well.

In this regard, our group investigated the role of MdSCs as a source of chondroprogenitor NP cells using an *in vitro* coculture system. NP cells were isolated from human IVD specimens and then cocultured with MdSCs harvested from the hind limb skeletal muscle of three mice. Proteoglycan synthesis and total GAG content were subsequently analyzed to assess eventual changes in extracellular matrix production, while DNA content measured as an index of cell proliferation. Each of these parameters was significantly increased in the coculture compared to NP cells monoculture, hence suggesting a promising role of MdSCs for disc regeneration^[115].

MdSCs have been only recently discovered as a novel stem cell population residing within muscles: As mesodermal progenitors, they can differentiate into different cell types, including chondrocytes, thence showing a potential role in disc regeneration. *In vivo* studies are needed to evaluate the factual MdSCs regenerative potential for disc regeneration cell-based therapy.

OLFACTORY NEURAL STEM CELLS

Human olfactory neural stem cells are multipotent stem cells showing the ability to differentiate along both neural lineage, leading to neurons, astrocytes and oligodendrocytes formation^[116], and non-neural lineages^[117].

Murrell *et al.*^[118] investigated the differentiation of olfactory stem cells (OSCs) into NP chondrocyte-like cells both in *in vitro* and *in vivo* settings. The *in vitro* study has performed coculturing OSCs derived from rat olfactory mucosa with rat IVD biopsies. The *in vivo* study consisted in transplanting genetically engineered OSCs, which were able to express green fluorescent protein, into a rat model of injured IVD, without any pre-differentiation *in vitro*. Authors showed that olfactory mucosa-derived progenitor cells could induce to differentiate into NP chondrocyte-like cells, as demonstrated by cellular morphology at the microscopy and by expression of proteins suggestive of NP chondrocyte phenotype (collagen Type II - CT2

and aggrecan - CSPG) at the immunochemistry. These findings have been confirmed by both OSCs induced *in vitro* with medium conditioned with NP environment and OSCs transplanted into injured rat NP.

OSCs can surprisingly differentiate into NP-like chondrocytes when exposed to the injured IVD environment and produce NP matrix constitutive elements. However, major concerns related to the invasive approach to harvest OSCs from the olfactory bulb can not be disregarded.

INDUCED PLURIPOTENT STEM CELLS

Induced pluripotent stem cells (iPSCs) are somatic cells which have been genetically reprogrammed in order to forcedly express such genes and factors that lead to an embryonic stem cell-like state. Mouse iPSCs were firstly reported in 2006: These cells have been proven to act like pluripotent cells, given that they express stem cells peculiar markers, generate tumours containing cells from all three germ layers and are able to differentiate into different cytotypes when injected in mouse embryos^[119]. Human iPSCs, isolated in 2007 for the first time, seem to show similar properties^[120,121].

Due to their pluripotency and patient-specificity, human iPSCs have been proposed as a source for generating notochordal cells in order to re-establish disc homeostasis.

Using a mouse model, Chen *et al.*^[122] isolated autologous embryonic fibroblasts which were then epigenetically reprogrammed into iPSCs through a polycystronic lentiviral vector. CD24⁺ iPSCs subpopulation was further detached using magnetic activated cell sorting and subsequently cultured in a laminin-rich media in order to drive NP-like cell differentiation and matrix synthesis which appreciably resembled native NP tissue. Culturing iPSCs in a hypoxic notochordal cell-conditioned medium (NCCM) led to similar outcomes.

Liu *et al.*^[115] harvested porcine NP tissue, which was pulverized and added to a culture plate loaded with human iPSCs, in order to induce notochordal cell-like differentiation, which was highlighted by the expression of three notochordal marker genes: Brachyury T, cytokeratin-8 and cytokeratin-18. Most notably, these cells showed the ability to generate NP-like tissue *in vitro*, which was characterized by NP phenotypic markers such as type II collagen, aggrecan and GAGs.

In spite of their capacity to induce chondrogenic differentiation, iPSCs might potentially lead to tumorigenesis due to their pluripotency. In addition, genetic engineering reprogramming techniques are characterized by notable costs that might be unlikely borne.

HEMATOPOIETIC STEM CELLS

Adult bone marrow includes two different kinds of stem cell populations: Non-hematopoietic stem cells [non-hematopoietic stem cells (HSCs)], including MSCs, which do not express CD34 and HSCs which express

CD34. Wei *et al.*^[123] evaluated xenogeneic transplantation of human bone marrow cells, both non-HSCs and HSCs, in a rat degenerated disc model in order to find out which population could be used to obtain disc-like cells. The human bone-marrow (CD34⁺ and CD34⁻) cells have been injected into rat coccygeal discs, after isolation and labeling with a fluorescent marker. Authors performed histological analysis, immunochemistry and survival rate analysis in all groups at different time points (at 1, 10, 21, and 42 d). They demonstrated that CD34⁻ cells were able to survive in the NP of host discs until 42 d, whereas CD34⁺ cells detected only up to 21 d. Moreover, only CD34⁻ cells presented a gene expression pattern similar to chondrocyte cells (positive for Collagen II and SOX-9).

Wei *et al.*^[123] registered data providing evidence that HSCs should not be used to treat IDD, because they are not able to differentiate in chondrocyte-like cells and restore degenerated NP.

The inefficacy of HSCs transplantation in the regenerative cell-based strategies to treat disc degeneration was also demonstrated by Haufe *et al.*^[124] in a clinical study, in which autologous HSCs derived from pelvic bone marrow were injected into degenerated discs of patients affected by low back pain. This study presents an important methodological bias, because any evidence, both *in vitro* and *in vivo*, supporting HSCs transplantation in degenerated disc, has been reported in literature. In fact, authors concluded that HSCs transplantation do not produce any clinical improvement in treated patients^[124].

DISC STEM CELLS

To date, several studies have successfully reported the isolation of disc stem cells from the IVD, namely cartilage end plate-derived stem cells (CESCs), annulus fibrosus-derived stem cells (AFSCs) and nucleus pulposus-derived stem cells (NPSCs), according to their localization within the disc.

These cells exhibit typical stem cell markers and are able to differentiate *in vitro* along the mesengenic pathway into various cytotypes belonging to osteogenic, chondrogenic and adipogenic lineages. In addition, disc stem cells notably resemble bone marrow MSCs immunophenotype, gene expression profile and self-renewal capacity^[125,126]. It is thought that these cells are remnants of multipotent mesendodermal embryonic cells, while they might, in a smaller proportion, derive from adjacent vertebrae bone marrow^[127].

A real stem cell niche was identified in the pericondrium and in the ligament side of the AF: Henriksson *et al.*^[128] proposed that cells from the niche are promptly recruited to NP and AF, along with MSCs from bone marrow, to undergo differentiation in case of tissue injury.

This assumption is further confirmed by the expression of migration and epithelial-mesenchymal transition markers (*e.g.*, SNAIL, SLUG, ITGB1) within the niche

itself^[129].

However, lack of standardization in both characterization and isolation methodology makes disc stem cells inner potential difficult to be evaluated. Nonetheless, as reported by Sakai *et al.*^[130] disc progenitors number seems to decrease progressively with aging and degeneration, thus limiting the possibility to perform autologous re-implantation in older patients. The same study individuated hTIE-2 and GD2 as markers of disc stem cells committed to differentiate into NP progenitor cells. Quantification of these markers might thus correlate with the actual number of progenitor cells present in the disc, in order to assess the extent of disc degeneration.

Wang *et al.*^[131] compared human bone marrow MSCs, AFSCs, NPSCs and CECs regenerative properties in a rabbit model of IDD. The abovementioned cells harvested from patients undergoing posterior lumbar interbody fusion and isolated from iliac crest bone marrow and discectomy specimens, respectively. Stem cells were then cultured, expanded and seeded in alginate gel to subsequently injected into rabbit IVDs after NP aspiration. At 6 mo after implantation, animals sacrificed and discs analyzed; morphological evaluation demonstrated that CECs yielded the highest regenerative potential, followed by NPSCs, BM-MSCs and AFSCs, that showed the lowest potency.

To date, eleven preclinical animal studies investigated outcomes subsequent to IVD tissue or cells transplantation, which demonstrated to delay disc degeneration and, in some models, to favor NP regeneration. However, some of the aforementioned studies reported an increased synthesis of type II collagen while proteoglycan production - and correspondingly disc hydration - was not restored to physiological rates^[132].

Disc stem cells seem to reside within the inner disc niche, as remnants of mesendodermal embryonic stem cells. According to conflicting results, further studies needed to assess their actual usability for disc degeneration cell-based therapy and to establish standardized protocols regarding harvesting techniques, isolation and identification.

EMBRYONIC STEM CELLS

Embryonic stem cells (ESCs) represent another possible source of stem cells for disc regeneration, basing on their ability to differentiate along different cell lineages, including notochordal cells.

Notochordal cells are the first cells forming the NP during the embryogenesis of IVD. Moreover, it is also known that the adult NP host chondrocyte-like cells. According to their ability to differentiate along the chondrogenic lineage with opportune culture conditions^[133], they could differentiate into cells able to produce extracellular matrix restoring the inner disc material. Sheikh *et al.*^[134] investigated the ESC-derived chondroprogenitors transplantation into a degenerated disc in a rabbit model. Researchers performed a pre-

conditioning culture of murine ESCs in order to induce differentiation toward a chondrocyte lineage. In addition, ESC-derived cells have been labeled prior to implantation with a green fluorescent protein. After 8 wk from the implantation, H&E staining, confocal fluorescent microscopy and immunohistochemical analysis have performed on disc samples. Comparing with control non-punctured discs and control degenerate punctured discs, IVDs subdued to implantation of chondroprogenitor cells showed islands of notochordal cells at H&E histological analysis and immunofluorescence staining. These authors demonstrated the proliferation of notochordal-like cells, which are responsible of proteoglycan matrix production, into degenerated IVDs transplanted with ESCs.

However, ESCs show notable tumorigenic properties: They characterized by high telomerase activity (which leads to potentially infinite proliferation) and formation of teratoma. Nonetheless, ESCs handling is surrounded by several ethical issues due to their embryonic provenance, thus making improbable their use in IDD treatment^[135].

TISSUE ENGINEERING APPROACHES

The choice of a suitable scaffold for stem cells remains an important issue in the development of this new therapy. Injectable viscoelastic scaffolds are more desirable for IVD tissue engineering to minimize the annular damage and to favor the implantation in a high-pressure structure. Sakai *et al.*^[42,89,90] compared cell viability after injection into rabbit NPs of a pure cell suspension compared to a soluble cell-augmented polymer such as fibrin glue that can polymerize *in situ*, providing the evidence that matrix-assisted cell transfer allows efficient augmentation of IVD. Besides fibrin glue, other scaffolds have been used in IVD tissue engineering such as collagen gels, hyaluronan gel^[88], and genipin cross-linked chitosan^[136]. The architectural and mechanical properties of the scaffold are also important. New micro- or nano-scale dimension scaffold as well as new signal release technologies may provide new perspective in IVD stem cell based tissue engineering.

Mercuri *et al.*^[137] explored the use of a chemical stabilized elastin-glycosaminoglycan-collagen hydrogel as a scaffold capable of resembling NP resilient, mechanical and hydrophilic properties. This material proved to induce chondrogenic differentiation of human-derived adipose tissue stromal cells (hADSCs), resulting into increased aggrecan and type II collagen synthesis *in vitro*. *In vivo* evaluation performed by transplantation of the hydrogel into subdermal pockets of the dorsal mid-line of rats; at 4 wk after injection, the material showed full biocompatibility, cell infiltration and evident remodeling.

Tsaryk *et al.*^[138] investigated the use of a collagen-low molecular weight hyaluronic acid semi-interpenetrating network loaded with gelatin microspheres in order to resemble NP main features, such as gel-like consistency,

Table 1 Summary of studies sorted by stem cells types and experimental setting

Cell type	Ref.	Years	In vitro study		Pre-clinical study		Clinical study
			Cell culture	Organ culture	Small animal	Large animal	
Bone marrow MSC	Sakai <i>et al</i> ^[42]	2003			x		
	Crevensten <i>et al</i> ^[88]	2004			x		
	Yamamoto <i>et al</i> ^[80]	2004	x				
	Risbud <i>et al</i> ^[61]	2004	x				
	Zhang <i>et al</i> ^[87]	2005			x		
	Steck <i>et al</i> ^[70]	2009	x				
	Sakai <i>et al</i> ^[90]	2005			x		
	Sakai <i>et al</i> ^[89]	2006			x		
	Richardson <i>et al</i> ^[74]	2006	x				
	Le Visage <i>et al</i> ^[71]	2006	x				
	Ho <i>et al</i> ^[91]	2008			x		
	Vadala <i>et al</i> ^[67]	2008	x				
	Hiyama <i>et al</i> ^[97]	2008				x	
	Yang <i>et al</i> ^[77]	2008	x				
	Sobajima <i>et al</i> ^[62]	2008	x		x		
	Jeong <i>et al</i> ^[92]	2009			x		
	Henriksson <i>et al</i> ^[96]	2009				x	
	Le Maitre <i>et al</i> ^[85]	2009		x			
	Chen <i>et al</i> ^[86]	2009		x			
	Wei <i>et al</i> ^[123]	2009			x		
	Svanvik <i>et al</i> ^[72]	2010	x				
	Watanabe <i>et al</i> ^[81]	2010	x				
	Yoshikawa <i>et al</i> ^[103]	2010					x
	Hee <i>et al</i> ^[94]	2010			x		
	Korecki <i>et al</i> ^[78]	2010	x				
	Yang <i>et al</i> ^[93]	2010			x		
	Strassburg <i>et al</i> ^[82]	2010	x				
	Serigano <i>et al</i> ^[98]	2010				x	
	Allon <i>et al</i> ^[95]	2012			x		
	Zhang <i>et al</i> ^[99]	2011				x	
	Di Martino <i>et al</i> ^[143]	2012			x		
	Subhan <i>et al</i> ^[100]	2014			x		
	Cai <i>et al</i> ^[101]	2015			x		
	Tsaryk <i>et al</i> ^[138]	2015	x		x		
	Orozco <i>et al</i> ^[102]	2011					x
	Vadalà <i>et al</i> ^[147]	2015		x			
Adipose tissue MSC	Lee <i>et al</i> ^[54]	2000	x				
	Lu <i>et al</i> ^[105]	2007	x				
	Lu <i>et al</i> ^[106]	2008	x				
	Jeong <i>et al</i> ^[107]	2010			x		
	Ganey <i>et al</i> ^[108]	2009				x	
	Mercuri <i>et al</i> ^[137]	2014	x		x		
Muscle-derived SC	Vadalà <i>et al</i> ^[76]	2008	x				
Olfactory SC	Murrell <i>et al</i> ^[118]	2009			x		
iPSCs	Chen <i>et al</i> ^[122]	2013	x		x		
	Liu <i>et al</i> ^[115]	2014	x			x	
Synovial MSC	Miyamoto <i>et al</i> ^[112]	2010			x		
Hematopoietic SC	Haufe <i>et al</i> ^[124]	2006					x
Disc stem cells	Liu <i>et al</i> ^[125]	2011	x				
	Sakai <i>et al</i> ^[130]	2012	x				
	Wang <i>et al</i> ^[131]	2014	x		x		
	Liu <i>et al</i> ^[139]	2015	x				
	Shi <i>et al</i> ^[126]	2015	x				
Embryonic SC	Sheikh <i>et al</i> ^[134]	2009			x		
Wharton's Jelly SC	Liu <i>et al</i> ^[125]	2011	x				

MSC: Mesenchymal stem cells; SC: Stem cells; iPSCs: Induced pluripotent stem cells.

high hydration rate and appreciable biomechanical strength. Moreover, this fully biocompatible material has proved to easily inject inside the NP and to favor proliferation and chondrogenic differentiation of bone marrow MSCs and nasal chondrocytes, both *in vitro* and *in vivo*.

Liu *et al*^[139] designed aligned fibrous polyurethane

scaffolds using electrospinning to culture rabbit AFSCs in order to perform functional AF replacement. Random fibrous scaffolds were used as a control: Both showed comparable cell attachment and proliferation features, while AFSCs cultured on aligned scaffolds exhibited more natural morphology, higher gene expression activity and increased type I collagen and aggrecan synthesis.

Peroglio *et al.*^[140] designed a thermoversible hyaluronan-based hydrogel [hyaluronan-poly(N-isopropylacrylamide)] to induce human MSCs differentiation into the disc phenotype and to evaluate the effects of preconditioning. Cells conducted in the hydrogel or alginate for 1 wk under hypoxic conditions in a chondropermissive media alone or with TGF- β 1 or GDF-5. Then, the cells suspended *ex vivo* in the gel and supplied to bovine IVDs. The HA-pNIPAM gel led to disc phenotype differentiation more promptly than alginate: Higher GAG/DNA ratio, type II collagen, SOX9 and other markers reported *in vitro*. In addition, preconditioning seemed to induce a lower degree of differentiation if compared to direct combination of the cells with the gel into the disc environment.

TOWARDS CLINICAL TRIALS

Though many issues remain unresolved^[141-143], exciting progress certainly has been made toward the realization of a stem cell therapy as a potential therapeutic option for the treatment of IDD.

In a systematic review and meta-analysis, Wang *et al.*^[144] assessed the efficacy of cell therapy in IDD treatment in 22 animal controlled trials: Stem cells transplantation was significantly associated with increased disc height index, T2 weighted MRI signal intensity, type II collagen synthesis and diminished degeneration grade. Therefore, these promising results provide a solid basis for testing the effects of cell therapy on humans.

In order to move toward successful human clinical trials, it is critical to rigorously test the long-term effects of stem cell-mediated strategies in animal models of disc degeneration that closely simulate the human condition on disc biology, nutrition and biomechanical functions such as larger animal models or primates. In fact, animal models of IDD are all of relatively short duration, induced in young and previously healthy animal discs rich in notochordal cells, where the effects of the induced degeneration on disc nutrition are unknown^[145-147].

The patients that could expect to benefit from stem cell-mediated therapy are those with mild or moderate grades of IDD, in whom the structural integrity of the disc remains preserved. Because nutrition supply to many degenerated discs is poor^[25], there is theoretical concern over the added nutritional demands arising from the increased number of metabolic active cells into the disc after transplantation. Therefore, evaluation of the nutrition transport into the IVD, using microelectrodes able to evaluate oxygen or nitrous oxide diffusion^[148,149], may be useful in order to select the patients that could benefit from the treatment (Table 1).

CONCLUSION

Thanks to the recent research efforts aimed at further developing our knowledge of the biology

and biochemistry of the IVD, our understanding of the process of IDD is rapidly growing. While there is still much to learn, some key factors involved in disc breakdown have become evident. Identification of the importance of cell loss within the disc has led to a focus on novel treatments aimed at regenerating the degenerating tissue. With its unique ability to differentiate into different cell types and to secrete a wide range of trophic cytokines, adult stem cell therapy has received considerable interest showing much promise with regard to treating chronic conditions such as IDD. Multiple studies have determined the feasibility of adult stem cell therapy for IDD, and recent studies have demonstrated proof of efficacy of autologous bone marrow MSCs transplantation in reproducible animal models as well as to be safe in human clinical trials. Other stem cells populations are still under evaluation with few proofs of efficacy in animals. Nonetheless, adult stem cell therapy has shown promise in becoming a powerful tool in the future treatment of IDD.

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Endometrial mesenchymal stem cells as a cell based therapy for pelvic organ prolapse

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Abstract

Pelvic organ prolapse (POP) occurs when the pelvic organs (bladder, bowel or uterus) herniate into the vagina, causing incontinence, voiding, bowel and sexual dysfunction, negatively impacting upon a woman's quality of life. POP affects 25% of all women and results from childbirth injury. For 19% of all women, surgical reconstructive surgery is required for treatment, often augmented with surgical mesh. The surgical treatment fails in up to 30% of cases or results in adverse effects, such as pain and mesh erosion into the bladder, bowel or vagina. Due to these complications the Food and Drug Administration cautioned against the use of vaginal mesh and several major brands have been recently been withdrawn from market. In this review we will discuss new cell-based approaches being developed for the treatment of POP. Several cell types have been investigated in animal models, including a new source of mesenchymal stem/stromal cells (MSC) derived from human endometrium. The unique characteristics of endometrial MSC, methods for their isolation and purification and steps towards their development for good manufacturing practice production will be described. Animal models that could be used to examine the potential for this approach will also be discussed as will a rodent model showing promise in developing an endometrial MSC-based therapy for POP. The development of a preclinical large animal model for assessing tissue engineering constructs for treating POP will also be mentioned.

Key words: Endometrium; Mesenchymal stem cells; Endometrial mesenchymal stem cells; Pelvic organ prolapse; Mesh; Tissue engineering; Regenerative medicine

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Core tip: Pelvic organ prolapse is the herniation of pelvic organs into the vaginal cavity and affects approximately 25% of all women. Traditional mesh-augmented surgical treatments cause complications such as pain and mesh erosion. A tissue engineering approach using endometrial mesenchymal stem cells seeded on new composite mesh show promise in animal models through their modulation of the chronic inflammatory response and promotion of physiological and biomechanically compliant neotissue.

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INTRODUCTION

The repair of damage to tissues and organs constitutes almost half of all medical expenses^[1]. In the early 1990s in the United States alone, \$400 billion was spent per annum treating conditions linked with tissue and organ failure^[1]. Despite both this enormous cost and high demand for tissue and organ repair, therapies currently available are unable to fully restore tissues and organs. With an ageing population and increasing demand for organ and tissue replacement the emerging field of tissue engineering and regenerative medicine offers hope for a possible solution for many intractable clinical problems^[2].

TISSUE ENGINEERING

Tissue engineering combines both biological sciences and engineering to develop treatments that restore, maintain or improve tissue function^[1,3,4]. Though similar to regenerative medicine, an important distinction resides in the potential use of synthetic and semisynthetic materials in tissue engineering^[4-6]. This separation can be better understood by considering the three major components of tissue engineering: Metabolically active cells^[7], polymeric micro-carriers or scaffolds^[8] and bioreactors to produce the tissue engineered construct for implantation^[9].

The application of stem cells to tissue engineering applications has been a major recent advance in the field. Although a variety of stem cell types exist, including human embryonic cells and induced pluripotent stem cells, this review will focus on mesenchymal stem/stromal cells (MSCs). The potential for using MSCs for clinical purposes is an expanding area, for both their relative ease of acquisition and their versatility although many utilize their immunomodulatory and anti-inflammatory properties rather than generating new tissue^[10-12]. Polymeric micro-carriers, hydrogels and scaffolds are essential components for supporting

the reconstitution of damaged tissue. Seeding a scaffold with viable adult stem cells enables their differentiation into the cells desired when implanted into the body^[13]. One key question in the tissue engineering field is the choice of polymer, particularly whether to use synthetic or biodegradable polymers. Bioreactors are generally defined as devices in which biological and/or biochemical processes for generating the tissue engineering construct are developed under closely monitored and tightly controlled environmental and operating conditions, *i.e.*, Good Manufacturing Practice^[14]. In modern tissue engineering, bioreactors are powerful tools to support and direct *in vitro* development of stem cell populations into functional tissues by simulating an appropriate biological, physical and mechanical environment. In essence, bioreactors are the means by which the desired tissue is generated *in vitro* and directed in its development for transplanting into the patient.

PELVIC ORGAN PROLAPSE

Pelvic organ prolapse (POP) is the herniation of pelvic organs into the vagina (Figure 1)^[15,16]. Symptoms of POP include bowel and urinary incontinence, pain, voiding, bowel and sexual dysfunction, severely affecting the quality of life of affected women^[17]. POP is a common condition, affecting approximately 25% of all women in the United States and Western countries, and is particularly prevalent in post-menopausal women. The main risk factor is vaginal birth and age. However, obesity is also a contributing factor, particularly in regard to POP recurrence^[18]. Though not as well understood, a genetic predisposition to POP is a factor in some cases, particularly in genes regulating collagen and elastin synthesis in the pelvic floor and vaginal walls^[19-21]. Given that the United States, Europe and Australia face increasing obesity rates and an aging population, the prevalence and severity of POP will only increase over the coming years. The economic and healthcare costs are considerable, approximating US\$1 billion each year^[22].

Surgical reconstruction for treatment of POP

Currently the standard treatment for POP is native tissue repair conducted transvaginally (colporrhaphy) or abdominally (sacral colpopexy). This surgical treatment has a high failure rate with 30% of patients requiring one or more further surgeries due to recurrence of POP^[23]. Additionally, reconstructive procedures in older women have complication rates from 15.5% to 33%, with the majority related to urinary tract infections, febrile morbidity and blood loss requiring transfusion^[24]. Indeed, the mortality from urogynecological surgery increases with each decade of life, with the most common complications occurring in women 80 years or older^[25].

The first generation of augmented treatments for POP involved the implantation of polypropylene mesh into the vaginal walls to alleviate the herniation

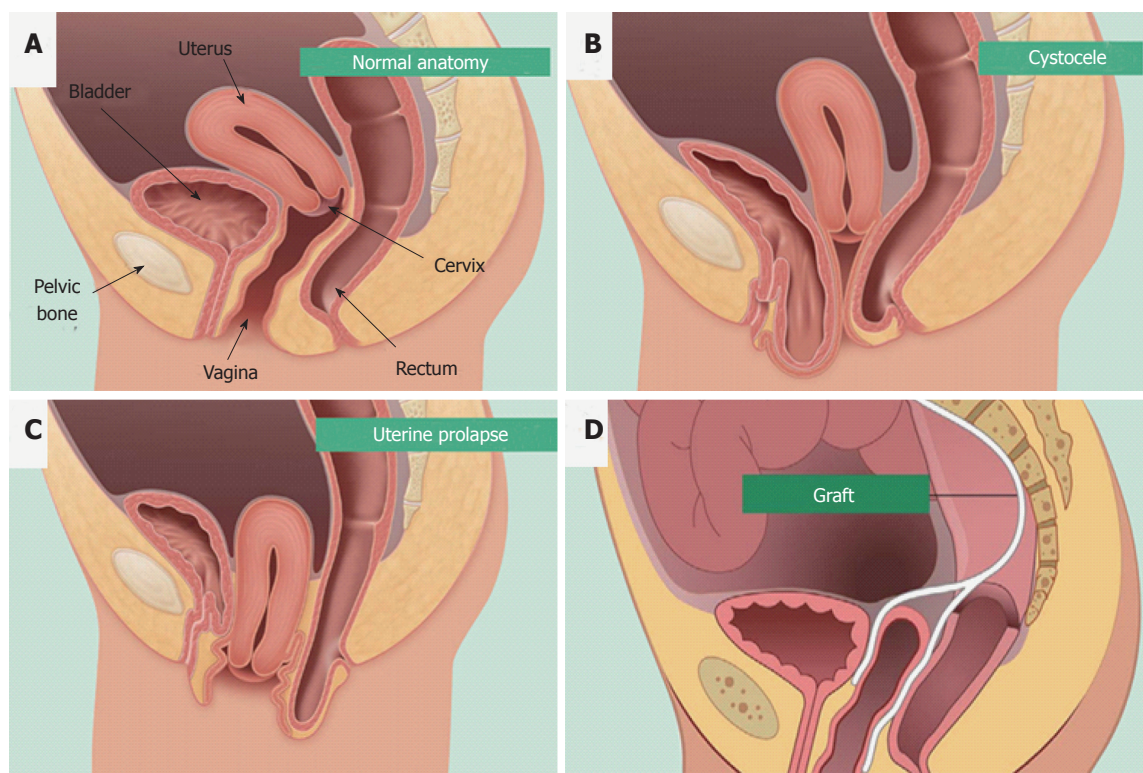


Figure 1 Pelvic organ prolapse mesh treatment. Normal pelvic anatomy (A) and herniation of the bladder (B) and uterus into the vagina (C). Synthetic mesh augmentation of vaginal walls as a colporrhaphy treatment for pelvic organ prolapse (D). Hysterectomies are also used to treat uterine prolapse (reproduced with permission from BARD medical).

and support the pelvic organs (Figure 1D)^[26]. Mesh has been available since the 1950s for the repair of abdominal hernias^[26]. Though successful for many women, up to 30% will require subsequent surgery while also enduring other complications such as fibrosis, mesh erosion into the vagina, bladder or bowel, chronic inflammation and mesh shrinkage^[24,26,27]. This resulted in worldwide recalls of many of the leading brands of meshes for vaginal surgery, leaving women with fewer options for treatment once again.

CANDIDATE CELLS FOR TISSUE ENGINEERING APPLICATIONS FOR POP

Skeletal muscle derived stem cells

Skeletal muscle has been identified previously as a potential source of progenitor stem cells capable of differentiating into myogenic and osteogenic cell lineages in rat models^[28-33]. The use of skeletal muscle stem cells to deliver gene therapy is being explored for treating muscular dystrophy and stress urinary incontinence, another pelvic floor disorder involving the urethra^[28]. In addition, they are being used to regenerate both skeletal and cardiac muscle, bone and cartilage. As a potential source of cells for treating POP, muscle-derived stem cells (MDSC) are particularly attractive as they can now be isolated from human skeletal muscles and differentiated into skeletal myo-

tubes, *in vitro* and *in vivo*^[33]. In rat models MDSC have been used to treat fibrosis. The ability of MDSC to promote vaginal epithelial regeneration and vaginal wall repair in a rat model makes them candidates for treating POP^[34]. However to avoid the risk of immune rejection from allogeneic sources, MDSC are better derived from the patient's own muscle tissue. Such an autologous procedure is expensive and invasive, causing significant pain and morbidity for the patient. An alternative source of cells for POP treatment could prove more beneficial and practical for the patient.

Fibroblasts and myofibroblasts

As major producers of collagen and an essential cell for the formation of connective tissue, fibroblasts have also been suggested as an alternative cell source for POP treatment^[35]. Vaginal myofibroblasts from nulliparous women have higher contractile strength compared to those from parous women, suggesting that vaginal delivery and overstretching of the vaginal wall affects myofibroblast function^[36]. However, the use of autologous vaginal fibroblasts from patients for treating their pelvic floor disorders raises concerns about the quality of cells utilised. Other studies have observed that vaginal fibroblasts derived from prolapsed tissues have impaired function, such as delayed fibroblast-mediated collagen contraction and lower production of collagen synthesising enzymes^[21]. This could be avoided if women have a vaginal biopsy to collect and cryopreserve fibroblasts before childbirth in order to

obtain better quality cells, however long-term planning and storage facilities are not available to most women. The invasive method of acquiring human vaginal fibroblasts and subsequent morbidity is unfortunately an obstacle in their use as the main source of cells for a tissue engineering-based approach to treating POP.

Buccal mucosal fibroblasts (BMF), however offer a readily available and plentiful source of cells and could prove an alternative to human vaginal fibroblasts. BMF are harvested from the inside of the cheek lining and express the typical MSC/fibroblast surface markers but do not function as MSC^[37]. They produce important components of the extracellular matrix, collagen I and elastin, both of which are required for strengthening the vaginal walls to alleviate and prevent herniation^[35,38]. The interaction of BMF with various biodegradable scaffolds has been examined *in vitro* for potential treatment of PFDs including POP^[38]. Although BMF offer a potential candidate for the treatment of POP, they currently remain untested for this purpose in animal models and their ultimate suitability remains unknown.

MSCs

MSC have been extensively used as cell-based therapies predominantly for their anti-inflammatory and immunomodulatory non-stem cell properties^[39,40]. However they also have potential for tissue engineering purposes for regenerating new tissues or promoting the activity of endogenous stem cells^[10,13,41]. MSC populations have the capacity for self-renewal, high proliferative potential and differentiate into a variety of mesodermal and other lineages^[42]. Recent advances in cellular identification using more specific markers has shown that MSC can be extracted from most tissues including bone marrow, umbilical cord, placenta, adipose tissue and endometrium, although not all of these sources have demonstrated clonogenicity for their MSC populations^[43-47]. Typically, MSC actively respond to stress or injury in a similar manner to the way cells of the innate immune system respond to pathogen exposure. When supplied systemically, exogenous MSCs home to sites of injury in response to inflammation^[48]. Here MSCs operate in a paracrine manner secreting large amounts of diverse proteins, growth factors, cytokines and chemokines that promote a variety of effects including neo-angiogenesis, tissue regeneration and remodelling, immune cell activation, suppression of inflammation and cellular recruitment^[13,41,49-51].

The potential of MSC as a cell-based therapy has recently been explored in numerous clinical applications. The ability to direct bone marrow MSC differentiation into other cell types and lineages has shown that these cells maintain a phenotype lacking tissue-specific characteristics until exposed to signals in damaged tissues^[52]. MSC obtained from dental pulp have been used to repair related tissues such as periodontal ligament, dental papilla and dental follicle^[53]. The ability of adipose tissue and bone marrow MSC to act as precursor cells has also been exploited by directing

their differentiation toward the chondrogenic lineage in order to produce cartilage-synthesising chondrocytes^[54]. Although MSC show promise as cell-based therapies, more understanding of their mechanism of action and utilising their potential is needed. Early use of MSC has not always met expectations, often producing inconsistent results^[55]. This may be due to lesser refined methods of isolating and cultivating MSC resulting in the administration of fibroblasts and myofibroblasts rather than undifferentiated MSC^[56]. Until recently, production of significant numbers of MSCs posed a challenge, as the regenerative potential of MSC declined during culture expansion^[57,58], which is required due to the small numbers of perivascular MSC present within tissues^[59]. For tissue engineering applications and tissue repair following ischaemia (*e.g.*, cardiac muscle), local rather than systemic delivery is desirable and will likely result in greater local concentration of MSC at the desired tissue site, even when the mechanism of action is paracrine^[60]. A further consideration is allogeneic vs autologous. Seeding MSC onto scaffolds, such as polyamide/gelatin (PA + G) for POP or polylactic-co-glycolic acid nano-fibers appears to produce better outcomes in preclinical studies^[57,61]. MSCs are a versatile and promising stem/stromal cell which can be used for a variety of regenerative medicine applications. Additionally, MSC have greater capacity to regenerate tissues from which they are derived^[39]. With this in mind, MSC obtained from the lining of the uterus could be useful in the development of treatments for other regions of the female reproductive tract, *e.g.*, vaginal wall tissue in cases of POP.

ENDOMETRIUM AS A NOVEL SOURCE OF MSC

Regenerative potential of endometrium

The endometrial lining of the uterus serves as the site of embryo implantation, placentation and the development of the embryo and foetus during pregnancy^[62]. The upper functional layer of the human endometrium undergoes extensive growth, differentiation and shedding each menstrual cycle under the influence of sex steroid hormone fluctuations^[63]. Following menstruation, the remaining basal layer regenerates the new functional layer, which undergoes rapid cellular proliferation followed by differentiation (Figure 2). If an embryo does not implant, the terminally differentiated epithelium and stroma is shed during menstruation^[64]. Much like the continuously renewing small intestinal mucosa, the endometrial mucosa undergoes many cycles of regeneration during a woman's lifetime, indicative of its highly dynamic and regenerative capacity.

Endometrial MSC

The existence of stem/progenitor cells within the endometrium and their role as progenitor cells for regenerating endometrial tissue has only recently been

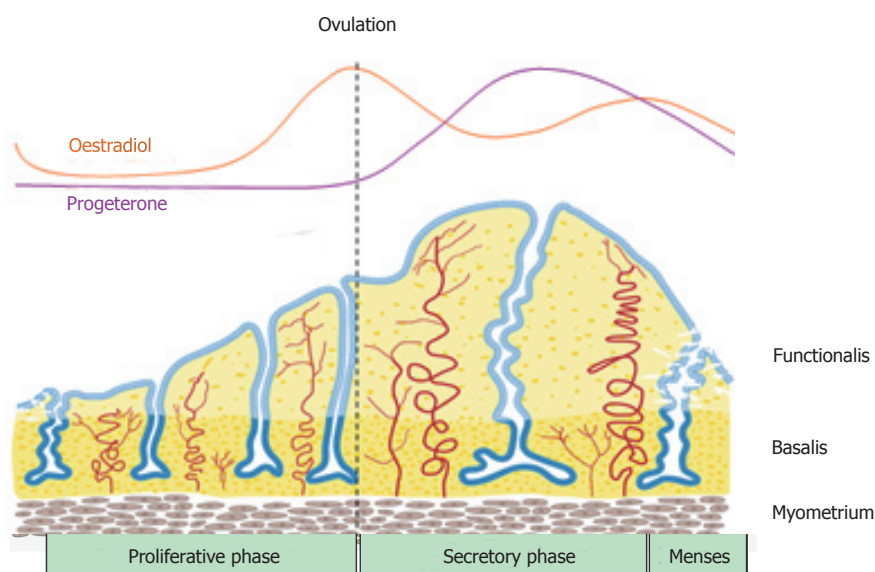


Figure 2 Schematic of changes in the human endometrium during the menstrual cycle, illustrating the growth, differentiation and shedding of the functionalis layer. The functionalis layer regenerates 4-10 mm during the proliferative phase (10 d) as cells proliferate in response to rising circulating estrogen levels. During the secretory phase, progesterone induces differentiation of the epithelium and stroma to generate an endometrium receptive to implantation of an embryo. This entire process occurs over 400 times during a woman's reproductive life indicating the regenerative potential of human endometrium (reproduced from ref.[63] with permission).

reported. Endometrial MSC (eMSC) are clonogenic, multipotent, differentiating into four mesodermal lineages: Osteoblasts, chondrocytes, smooth muscle cells and adipocytes *in vitro* (Figure 3) and expressing the typical pattern of MSC surface markers^[44,65,66]. Endometrial side population (SP cells) also demonstrate MSC properties^[67,68]. Serial clonal culture shows that clonogenic eMSC undergo self-renewal *in vitro* and have high proliferative potential^[44]. The population of clonogenic eMSC within human endometrium is small approximating 1.3%, necessitating the identification of specific surface markers to allow their prospective isolation and enrichment from endometrial biopsies^[69,70].

Prospective isolation of eMSC

In order to exploit the regenerative ability of eMSC, they must first be isolated from the heterogeneous population of cells obtained from dissociated endometrial tissue. Ideally this requires the identification of unique surface markers on eMSC that will identify their *in vivo* niche and separate them from undesired stromal fibroblasts and other cells. Indeed several sets of specific surface markers have been identified on eMSC^[70-73]. Almost all clonogenic human endometrial stromal cells with MSC properties are found in the CD140b⁺CD146⁺ population, comprising 1.5% of the stromal fraction^[70]. These markers revealed a perivascular niche for eMSC adjacent to endothelial cells suggesting they are pericytes (Figure 4). The transcriptome of the co-expressing CD140b⁺CD146⁺ cells indicates they are distinct from CD140b⁺CD146⁺ endothelial cells, but more similar to endometrial CD140b⁺CD146⁻ stromal fibroblasts^[73]. To obtain these co-expressing cells, a flow cytometry sorter must be used, which limits the utility of this marker set, given the damaging effects of

automated cell sorting on cell viability^[70]. To overcome this problem a single perivascular marker was sought for isolating eMSC. The W5C5 antibody identified a population of perivascular endometrial stromal cells with typical MSC properties that also reconstituted stromal tissue *in vivo* when transplanted beneath the kidney capsule^[72]. The W5C5⁺ cells comprised 4.4% of endometrial stromal cells. The epitope recognised by the W5C5 antibody is the Sushi Domain-containing 2 (SUSD2) adhesion molecule^[74]. A single marker enables magnetic bead sorting, a gentler protocol than using a cell sorter as evidenced by increased clonogenicity of SUSD2⁺ cells compared to CD140b⁺CD146⁺ cells^[72]. TNAP (tissue non-specific alkaline phosphatase) is another single marker that identifies eMSC, but has less utility as the epitope is also expressed by endometrial epithelial cells^[75]. Another perivascular marker (AOC3) identified by RNA sequencing SUSD2⁺ and SUSD2⁻ cells may have utility for isolating eMSC^[76], but the common bone marrow MSC marker Stro-1 does not enrich for endometrial stromal cells with MSC properties^[69]. All these markers revealed that the perivascular eMSC were found in both the functionalis and basalis layers of human endometrium, indicating that eMSC will be found in menstrual blood and can be isolated from biopsies and curettage as well as hysterectomies^[56,77].

eMSC can also be obtained from post-menopausal women following short term (8 wk) estrogen replacement which regenerates their atrophic endometrial tissue^[78]. Collection of menstrual blood or an endometrial biopsy are convenient sources not requiring anaesthesia, with the latter available as a simple office based procedure. Such tissue sources are ideal for cell-based therapies (Figure 5). Despite their great promise, eMSC and menstrual blood MSC have yet to

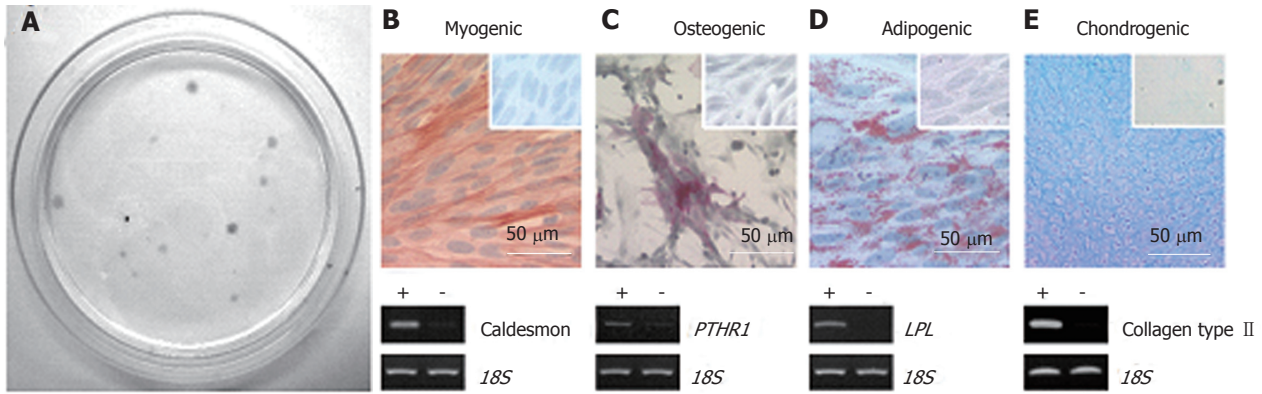


Figure 3 Endometrial mesenchymal stem cells. Clonogenic (A); and differentiate into 4 mesodermal lineages from a single clonogenic cell (B-E); myocytes (B); osteocytes (C); adipocytes (D); chondrocytes (reproduced from ref. [44] with permission) (E). PTHR1: Parathyroid hormone 1 receptor; LPL: Lipoprotein lipase.

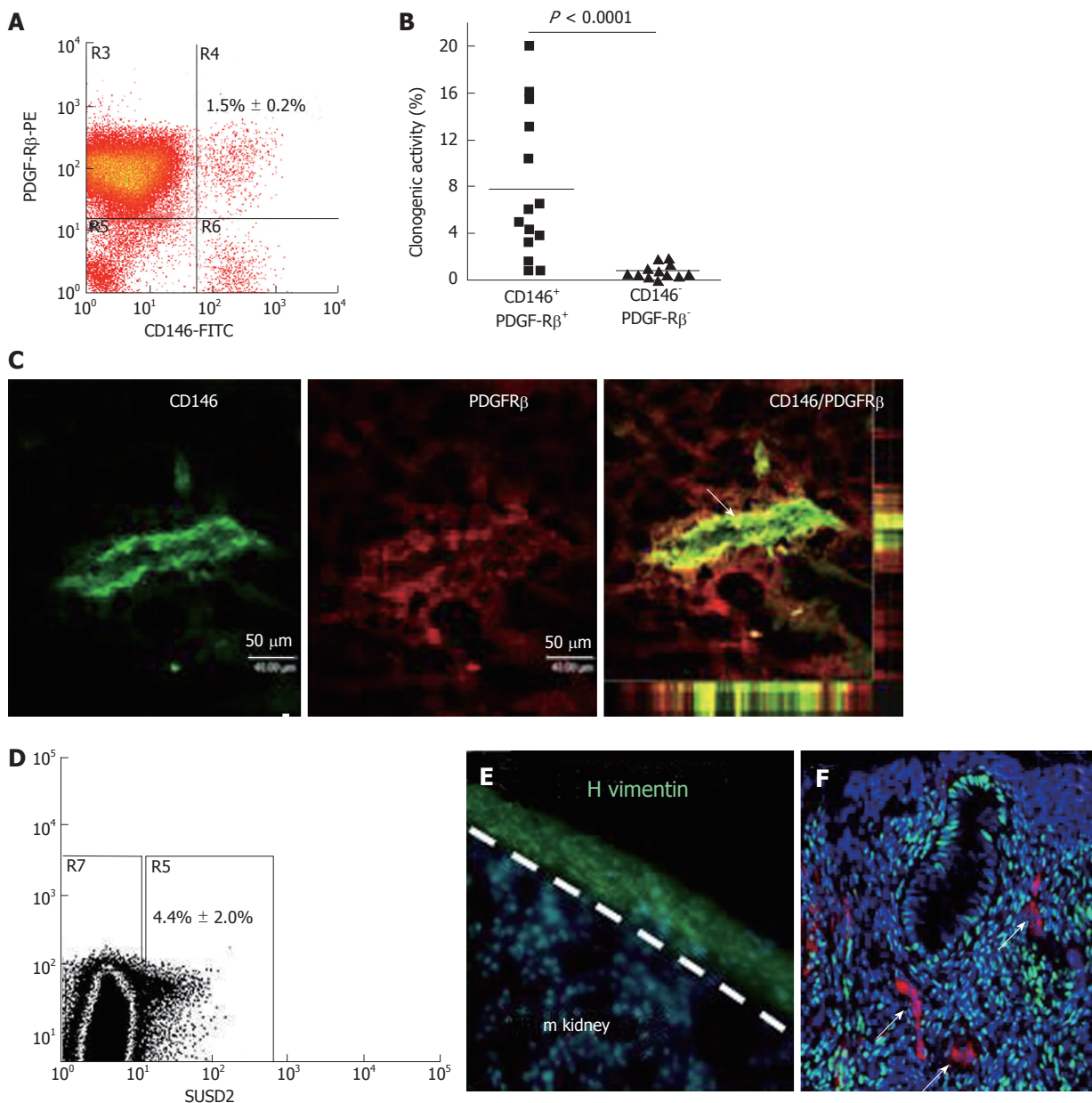


Figure 4 Specific enriching for endometrial mesenchymal stem cells. Flow cytometry plot of CD146⁺PDGFRβ⁺ fraction (A) which contains most of the clonogenic stromal cells (B) and reveals their pericyte identity *in vivo* (C); SUSD2⁺ cells in endometrial cell suspensions (D) which E reconstitute human vimentin⁺ stromal tissue when transplanted under the kidney capsule of NSG mice, and F have a perivascular location in human endometrium. SUSD2⁺ cells (red) do not express estrogen receptor-α (green), but endometrial stromal cells do (DNA blue). The white arrow indicates perivascular SUSD2⁺ cells (reproduced ref. [70,72,78] with permission).

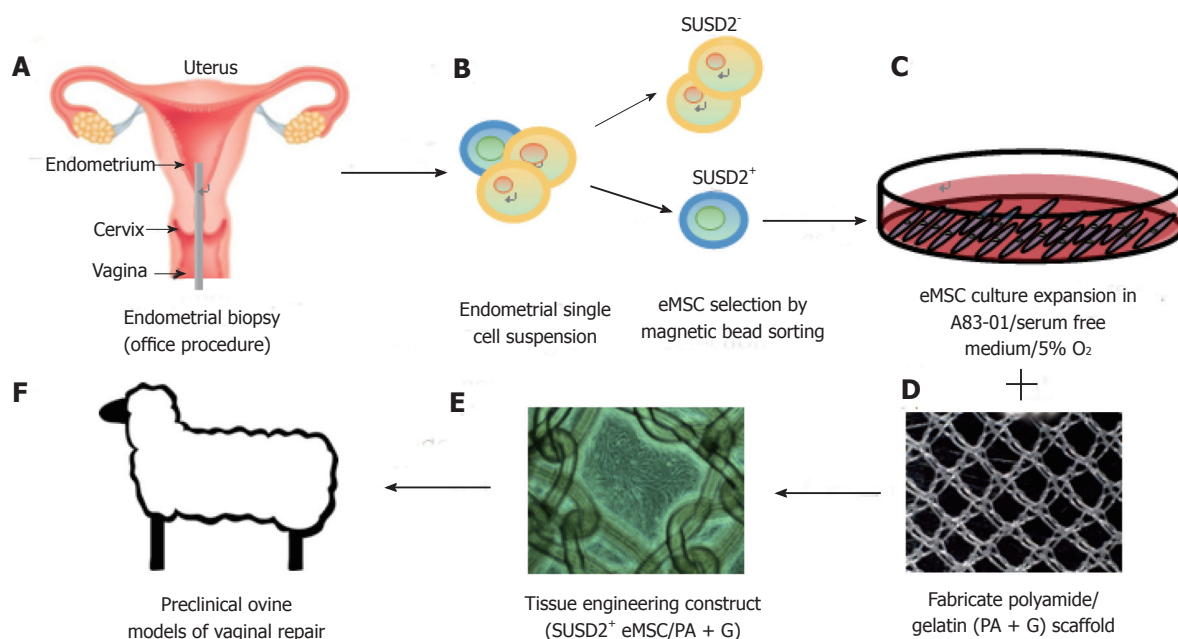


Figure 5 Isolation and application of e-mesenchymal stem cells in pelvic organ prolapse vaginal repair. (A) simple office based endometrial biopsies can be used to obtain patients' tissues, which are dissociated, then (B) eMSC selected using SUSD2 magnetic bead sorting, followed by (C) culture expansion in A83-01/serum free medium in 5% O₂ to generate large numbers of undifferentiated SUSD2⁺ eMSC (90%-95%) for (D) seeding onto fabricated scaffolds which will create an (E) eMSC/PA-G tissue engineering construct for implantation into (F) a large animal preclinical model to assess their efficacy in vaginal repair of parous ewes with evidence of POP (reproduced with permission from ref.[57,103] with permission). POP: Pelvic organ prolapse; MSC: Mesenchymal stem cells.

be significantly explored as therapeutic agents for stem cells therapies. There are certain endometrial disorders where caution maybe required eg endometriosis. However this disorder affects young infertile women who will not have the opportunity to develop POP. Indeed, it will be important to ensure no underlying uterine or other pathology (e.g., malignant tumour) in identifying suitable patients for cell harvesting to treat their POP. For example, should a woman have uterine cancer, it would not be possible to use her eMSC for cell-based therapies. Similarly, it would also be contraindicated to use another source of autologous MSC in case tumour cells have spread to organs such as bone. These important issues should be considered in developing the potential of eMSC as cell-based therapies.

Large animal models are usually required to provide data for regulatory bodies prior to translating potential cell-based therapies into the clinic. If autologous applications are being evaluated, it becomes necessary to derive MSC from species such as ovine, porcine, canine, equine and non-human primates^[79,80]. Often antibodies used as biomarkers to derive MSC from human or mouse do not cross react with these species. For example, neither CD140b, CD146 nor SUSD2 cross react with ovine endometrial tissue^[81]. However, the bone marrow MSC surface marker CD271^[82] cross reacts with ovine endometrial stromal cells enriching for eMSC demonstrating clonogenicity, *in vitro* self-renewal and the ability to differentiate into adipogenic, myogenic, osteogenic and chondrogenic lineages^[81]. The CD271⁺ ovine eMSC were identified in a perivascular

niche around arterioles and venules *in vivo*, but unlike human eMSC which have a pericyte location, ovine CD271⁺ stromal cells occupied the adventitia in the periphery of these vessels (Figure 6). In human bone marrow and adipose tissue, vascular adventitial cells show similar MSC properties as those located in the pericyte position^[83].

eMSC phenotype and gene profile

Cell fate decisions made by somatic stem cells to self-renew or undergo differentiation depends upon the cellular microenvironment or niche from signals emanating from cells and extracellular matrix that comprise this niche^[84]. In this context, understanding both the extrinsic and intrinsic gene regulation pathways operating in undifferentiated eMSC and their more differentiated progeny could shed light on their function in endometrial regeneration. Gene expression profiling comparing purified endometrial cell populations of CD140b⁺CD146⁺ eMSC, CD140b⁺CD146⁻ stromal fibroblasts and CD140b⁻CD146⁺ endothelial cells showed that eMSC differentially expressed 762 and 1518 genes, respectively^[73]. The eMSC gene expression profile was typical of stem cells, showing upregulation of self-renewal genes of the *TGFβ*, *FGF2*, *WNT*, *IGF* and Hedgehog signalling pathways in comparison with the endometrial stromal fibroblasts and endothelial cells. The expression of SUSD2 was also elevated in the double positive eMSC population. G-protein coupled receptor- and cAMP-mediated signalling were also upregulated in the CD140b⁺CD146⁺ population, similar to genes involved in maintaining the undifferentiated state of

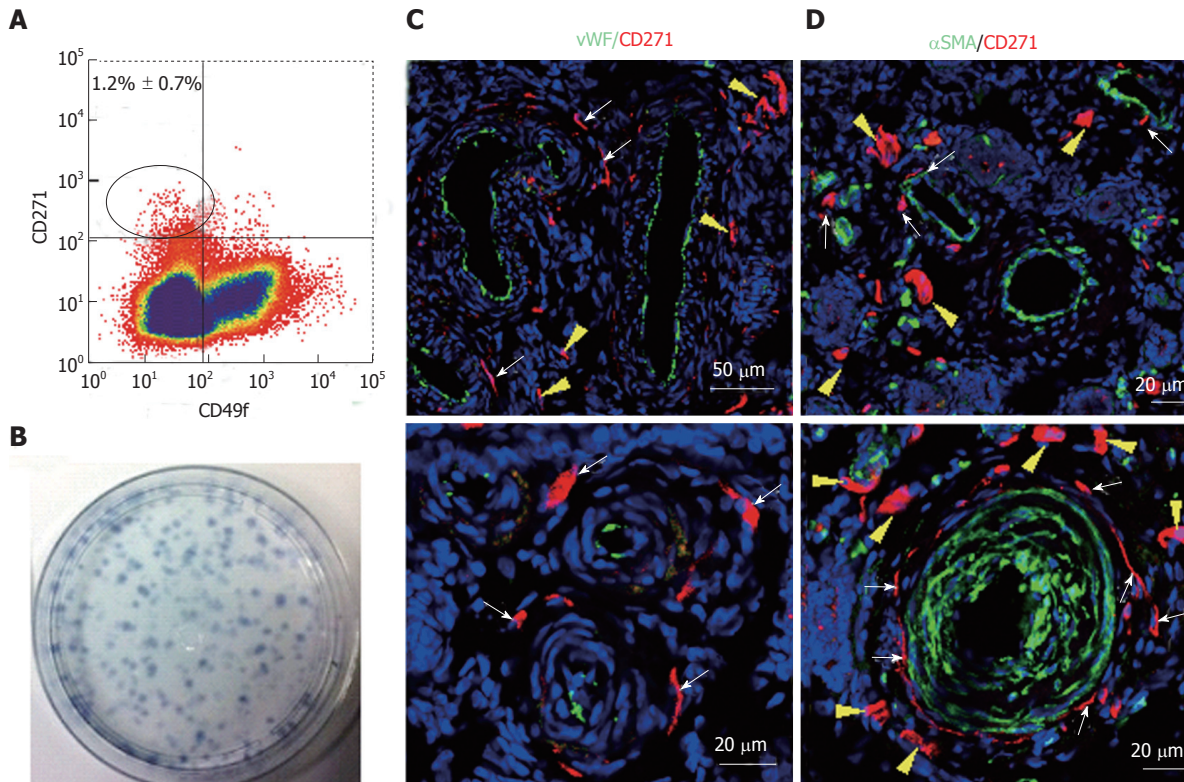


Figure 6 Specific markers for ovine e-mesenchymal stem cells. Flow cytometry plot of ovine endometrial cells immunolabelled with CD271 and CD49f antibodies (A). The CD271⁺CD49f⁺ population enriches; Clonogenic stromal cells (B); Immunofluorescence images of ovine endometrium stained with CD271 (red) and vascular markers reveals their *in vivo* perivascular location in the adventitia of veins and arteries (C, D); vWF an endothelial marker (green), showing CD271⁺ cells are perivascular but not pericytes (C); α SMA, a perivascular marker (green) showing CD271⁺ cells located adjacent to α SMA⁺ cells in the adventitia of vessels rather than expressing α SMA themselves (D). White arrows: perivascular CD271⁺ cells; yellow arrows: CD271⁺ cells not associated with vessels (reproduced from ref.[81] with permission). vWF: Von Willebrand factor; α SMA: Alpha smooth muscle actin.

bone marrow MSC. The CD140b⁺CD146⁺ population also showed upregulation of immunomodulatory and immunosuppressive genes^[73]. eMSC displayed increased expression of Cyclin D1 (CCND1) which ensures their progression through the G1 phase of the cell cycle^[73]. Gene profiling has confirmed human eMSC as pericytes, while RNA sequencing of cultured endometrial SUSD2⁺ and SUSD2⁻ cells revealed 134 differentially expressed genes, with many of those in the SUSD2⁺ population characteristic of perivascular cells^[76]. The *in vivo* differentiation pathway for eMSC is to decidualised perivascular cells and decidual cells of the endometrial stroma, a process mediated by the post-ovulation sex steroid hormone, progesterone, *via* production of cAMP. The perivascular location of eMSC in the spiral arterioles renders them well situated to participate in the regeneration of the uterine lining and formation of the placenta during embryo implantation and subsequent pregnancy^[76].

Tissue engineering for POP repair

Given the problem associated with mesh implantation for POP repair, and the need for physical support, a tissue engineering approach may provide a more durable treatment. The ideal treatment for POP would be an implantable autograft that alleviated herniation

and regenerated the damaged tissue within the vaginal wall.

In vitro studies

For a cell based treatment to be practical, methods for procuring and expanding the necessary cells need to be developed. Culturing and expanding eMSC *in vitro* has been optimised in serum-free conditions, showing that fibronectin is the optimal substrate for attachment^[85]. Additionally, hypoxic conditions of 5% O₂ increased the proliferation rate and yield of eMSC, whilst maintaining multipotency and their expression of CD140b, CD146 and SUSD2. Culturing eMSC on a polyamide/gelatin composite scaffold with exogenous TGF β 1 and PDGF-BB induced their differentiation into smooth muscle cells expressing SM22 α and SM-myosin heavy chain^[86]. Incubation with connective tissue growth factor induced the eMSC to differentiate into collagen-producing fibroblasts. The differentiated smooth muscle cells and fibroblasts no longer expressed the eMSC marker SUSD2, confirming their differentiation into these desired cell types for POP repair^[86]. Although these *in vitro* studies show promise, it is also essential to confirm smooth muscle and fibroblast differentiation *in vivo* to gain mechanistic understanding prior to transferring this technology into clinical applications.

Methodology has now been developed for culture expansion of eMSC in serum free medium containing A83-01, a TGF β 1 receptor inhibitor, that maintains eMSC stemness and SUSD2 phenotype^[87]. TGF β 1-mediated apoptosis and senescence is prevented and proliferation promoted in A83-01-treated eMSC cultures maintaining the percentage of SUSD2⁺ cells to more than 90% for all samples. This effect of A83-01 is mediated *via* Smad2/3 phosphorylation. A83-01 treated eMSC are more clonogenic than untreated control cells and retain their MSC properties^[87]. A major advantage of this culture method is that a reproducible percentage of SUSD2⁺ eMSC is achievable for all patient samples, an important consideration for scale out culture expansion of autologous cells.

In vivo studies

As outlined earlier there are substantial problems with current mesh augmentation of POP surgery. The use of autografts increases morbidity at the donor tissue site, biological materials often fail due to their rapid and unpredictable degradation^[16], and the synthetic PP mesh currently used is biomechanically too stiff and often erodes into adjacent organs^[56]. A better solution may be to combine the advantages of both the synthetic and biological approaches. This could utilise a synthetic mesh as a scaffold to not only support the prolapsed tissue but also provide a vehicle upon which to seed eMSC for delivery to sites of vaginal damage^[26,88]. The eMSC could serve by modulating the inflammatory and immune responses and perhaps more importantly incorporating into the vaginal wall to regenerate the lost or damaged tissue or promoting endogenous stem cell populations to initiate repair which mesh alone cannot do.

Small animal rodent models

Recent efforts to test this possibility show potential utility. A non-degradable, polyamide (PA) mesh with biomechanical properties more closely matching vaginal tissue was coated with gelatin^[88] to provide a substrate for seeding with SUSD2⁺ eMSC. This tissue engineering construct was then implanted into a fascial defect on the dorsum of immunocompromised rats and assessed following necropsy at several time points over 90 d^[57]. In the explanted eMSC/PA + G tissue complexes, greater neovascularisation was observed early on at 7 d compared with PA + G controls. Initially there was a greater influx of M1 inflammatory macrophages around the eMSC-seeded mesh. At 60 d these macrophages had changed to a M2 wound healing phenotype and by 90 d there were fewer CD68⁺ macrophages around the cell-seeded PA + G filaments in comparison to PA + G alone, indicating a milder chronic inflammatory response in the long term. Importantly in these studies the cellular response at the mesh interface was assessed quantitatively in 50 μ m increments around individual filaments using image analysis rather than subjective scoring^[57,88]. Similar quantities of new collagen were generated around the PA + G

mesh filaments, irrespective of the inclusion of eMSC, which was derived from rat fibroblasts rather than derivatives of the implanted human eMSC. However, this new collagen around the eMSC/PA + G mesh filaments showed physiological crimping by scanning electron microscopy, while more scar-like collagen was deposited around the PA + G mesh without eMSC^[89]. This deposition of physiological collagen likely contributed to the improved biomechanical properties of the mesh/tissue complexes harvested at 90 d, where a longer toe region and lower stiffness was observed in the stress strain curves of the cell-seeded PA + G mesh compared with PA + G alone (Figure 7)^[57]. The improved tissue organisation around the mesh filaments shown by histological staining suggests that eMSC promoted tissue regeneration and improved the biocompatibility of the synthetic PA + G mesh^[57,89]. In this xenogeneic model, the eMSC survived a maximum of 14 d indicating that they exerted a paracrine effect in promoting vascularisation and reducing fibrosis similar to MSC effects on many other tissues^[13]. However the percentage of SUSD2⁺ cells in the single sample of passage 6 cells used for the entire study was only 10%. It will be of interest to determine whether more than a paracrine effect will be observed if > 90% of the cells are SUSD2⁺, now a possibility by culturing them in A83-01-containing medium^[87].

Despite the significant biological differences between human females and rodents, mouse models have proven invaluable for the investigation of the underlying biochemical mechanisms involved in the development of POP. The use of genetically modified mice has allowed exploration of the genetic underpinnings of POP, such as lysyl oxidase like-1 (LOXL1), an enzyme involved in elastin biosynthesis within vaginal tissue walls, and fibulin 5 (FBLN5) which regulates expression of collagen and elastin. Depletion of either LOXL1 or FBLN5 has been associated with POP^[20,90]. The LOXL1 deficient mouse creates a POP-like condition where the mice develop an obvious bulge in the perineal region. It would be of interest to determine if an injectable MSC-based cell therapy alleviates the prolapse symptoms of LOXL-1 deficient mice. While extremely useful for investigating genetic contribution, transgenic mice are limited in their utility as models for exploring tissue engineering based treatments for POP due to the small size of their vagina.

Large animal preclinical models

Of the large animal models available for assessing cell-based therapies for POP, the domestic sheep is the most promising candidate due to their ready availability and physiological similarity to the human female pelvis in size and structure. Ewes also have a similar oestrus cycle of 17 d, a long labour and deliver a foetus with a large head to body ratio that is closer to humans than rodents^[91,92]. Like humans, ewes undergo spontaneous POP with similar frequency and predisposing factors, such as parity, age and breeds with a large rump^[91,93].

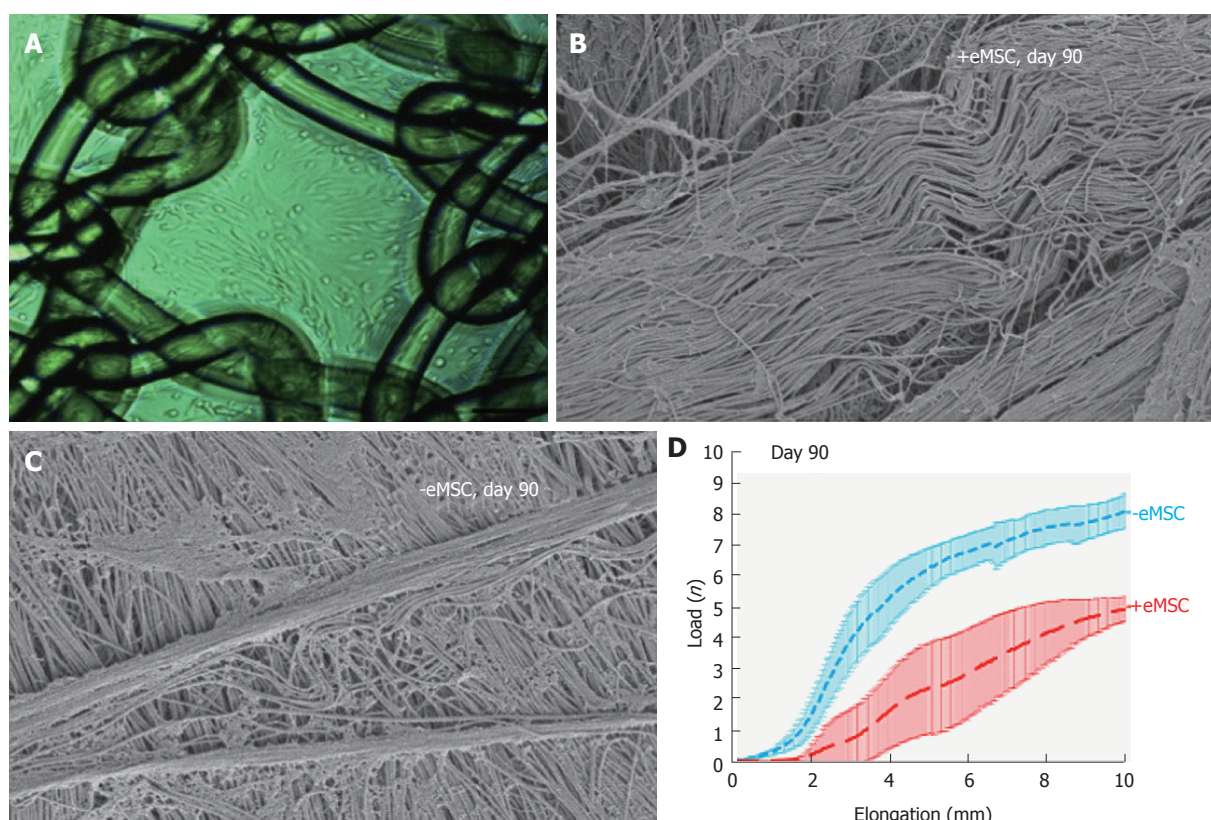


Figure 7 Human e-mesenchymal stem cells improves the biocompatibility of polyamide/gelatin (PA + G) mesh in a fascial wound defect in nude rats. PA + G mesh seeded with 100000 eMSC/cm² and cultured for 48 h, prior to implantation (A); Physiological crimped collagen deposited around eMSC/PA + G mesh (B); Scar-like collagen in PA + G mesh alone as observed by SEM (C); Load-elongation curves of explanted meshes with (red) and without (blue) eMSC showing less stiffness (slope) and longer toe region for mesh seeded with eMSC, indicating improved biomechanical properties (reproduced from ref.[57,86,89] with permission) (D). MSC: Mesenchymal stem cells; PA : Polyamide; G: Gelatin.

Although the ovine species are quadrupeds with a horizontal rather than vertical pelvic floor subject to differing forces, the overall arrangement of the pelvic organs and the similar vaginal dimensions to women make them a useful model for assessing new mesh and tissue engineering constructs^[56]. Additionally, the ovine vagina has a similar histological structure, biochemical and biomechanical properties to that of women. Finally, the most common form of prolapse in sheep involves the bladder (cystocele) as it is for women. Sheep have already been vaginally implanted with various POP mesh materials for evaluation of their efficacy and adverse effects in female pelvic reconstructive surgery^[16,92,94,95]. The biochemical and biomechanical properties of ovine vaginal tissue has already been examined by quantitative histological imaging, biochemical collagen/GAG/elastin assays and biomechanical analyses, providing a platform for the evaluation of next generation eMSC-seeded mesh in the ovine vaginal repair model^[96,97]. It is now possible to evaluate autologous eMSC since methods have been developed for obtaining MSC from the ovine bone marrow^[79] and endometrium (Figure 5)^[81].

Additional large animal models for assessing cell-based therapies for POP surgery include cows, pigs and non-human primates. Cows develop prolapse with similar predisposition and frequency to humans and sheep^[98], however their purchase, handling and agistment costs

make them a less practical model. Pigs are a common preclinical model for various clinical conditions but their foetuses do not have the large head-to body-ratio responsible for inducing spontaneous POP in the ovine model, reducing their utility^[99]. Non-human primates such as Rhesus macaques and squirrel monkeys offer useful animal models due to a similar pelvic anatomy to humans and their more upright posture^[100,101]. Furthermore, the Rhesus species develop spontaneous POP. Non-human primates have been used for assessing new POP meshes and for investigating the mechanism of action of their deleterious effects^[27,102]. However ethical limitations, prohibitive cost of handling and necessary specialist expertise limit their availability for many investigators. Despite these limitations, assessment of tissue engineering constructs in the macaque model, particularly in retired breeders with evidence of POP, might provide the ultimate model of postmenopausal women with POP in which to assess a cell-based therapy. However it would be necessary to develop methods for obtaining MSC populations from the macaque species, for both autologous and allogeneic use.

CONCLUSION

POP is a common hidden disease burden for large numbers of women. Compounding this burden is the

inadequacies of current surgical treatments with or without mesh. To overcome this clinical challenge, recent advances in cellular phenotyping and gene profiling suggest endometrial MSC as a possible compliment to mesh-based POP treatment. The eMSC capacity for regenerating tissue is exemplified during a woman's reproductive life, where they regenerate at least one centimetre of endometrial lining each menstrual cycle for over 400 menstrual cycles. By seeding eMSC onto polyamide/gelatin composite mesh and implanting into vaginal walls, it may be possible to favourably modulate the innate immune response and accelerate organised tissue rehabilitation. That the first attempt at combining eMSC and mesh to treat a fascial defect has been successful using rodent models is encouraging, suggesting that further development of this approach using the ovine model is warranted.

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WJSC covers topics concerning all aspects of stem cells: embryonic, neural, hematopoietic, mesenchymal, tissue-specific, and cancer stem cells; the stem cell niche, stem cell genomics and proteomics, and stem cell techniques and their application in clinical trials.

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Aneuploidy in stem cells

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Abstract

Stem cells hold enormous promise for regenerative

medicine as well as for engineering of model systems to study diseases and develop new drugs. The discovery of protocols that allow for generating induced pluripotent stem cells (iPSCs) from somatic cells has brought this promise steps closer to reality. However, as somatic cells might have accumulated various chromosomal abnormalities, including aneuploidies throughout their lives, the resulting iPSCs might no longer carry the perfect blueprint for the tissue to be generated, or worse, become at risk of adopting a malignant fate. In this review, we discuss the contribution of aneuploidy to healthy tissues and how aneuploidy can lead to disease. Furthermore, we review the differences between how somatic cells and stem cells respond to aneuploidy.

Key words: Chromosomal instability; Aneuploidy; Embryonic stem cells; Induced pluripotent stem cells; Mesenchymal stem cells

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Core tip: Stem cells hold great therapeutic promise for regenerative medicine, especially with new protocols that can create induced pluripotent stem cells from terminally differentiated cells. However, somatic cells and stem cells cope differently with genomic instability. Therefore, it will be of the utmost importance to assess genomic integrity when preparing stem cell cultures for future therapy.

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INTRODUCTION

Chromosomal instability (CIN) is a process that leads to cells with unbalanced genomes, containing structural

abnormalities (*i.e.*, structural CIN leading to amplification, deletions, translocations), numerical abnormalities (*i.e.*, numerical CIN), or both. Numerical CIN leads to aneuploidy, a state in which cells have abnormal numbers of whole chromosomes. While the majority of all cancers are aneuploid^[1], aneuploidy itself appears to act anti-proliferative in non-transformed cells, suggesting that cancer cells somehow have adjusted to the adverse effects of aneuploidy^[2,3]. Emerging evidence is indicating that aneuploidy in somatic cells increases with age and might even contribute to natural ageing^[4-8]. In fact, we are only beginning to understand how our somatic cells and stem cells cope with loss of genomic integrity. As future regenerative medicine-based therapies will likely make use of induced pluripotent stem cells (iPSCs) derived from somatic (aged) patient's cells, aneuploidy will become an important parameter to test for when assessing the quality of the transplanted cells. Furthermore, much is still unknown regarding the effects aneuploidy on the functionality of both somatic stem cells and iPSCs. In this review, we will discuss the impact of aneuploidy on healthy stem cells, how aneuploidy can lead to disease, and how stem cells cope with genomic instability.

ANEUPLOIDY IN HEALTHY TISSUES

Even though systemic aneuploidies or high CIN rates are poorly tolerated during development, various lines of evidence indicate that some of our tissues can cope remarkably well with aneuploidy^[9] and even show aneuploidy under "normal" conditions^[4]. Two tissues that have been associated with increased aneuploidy frequencies are brain and liver.

Aneuploidy in the brain

Aneuploidy in mature brain can affect both the neuronal and non-neuronal populations of the cortex^[10,11], and is thought to originate in ventricular zone progenitors that encounter a variety of cell division defects during embryogenesis^[12,13]. While the most common aneuploid event in progenitors is the loss of a single chromosome, progenitors with up to 5 chromosome losses have been reported. Based on this and other work, cumulatively, 10% of neurons in a healthy brain are estimated to be aneuploid^[14], but the role and fate of these cells remains unclear. For more extensive review see Bushman *et al.*^[15].

The precise contribution of aneuploidy to the healthy human brain remains unclear. One possible explanation is that aneuploidy actually serves a function and is part of a "normal" process during brain development resulting in heterogeneous neuronal and glial populations that contribute to the wide functions that neurons can have^[16]. An alternative explanation however is that aneuploidy rates have been overestimated due to technical limitations of the protocols used to quantify aneuploidy in neurons (in most cases interphase FISH)^[17], which was reinforced by recent studies that used single cell next generation sequencing to determine chromosome

copy numbers in adult brains^[14,18-20]. Aneuploidy in the brain has furthermore been linked to various pathologies such as Alzheimer's disease (AD) (increased trisomy of chromosome 21), schizophrenia, and autism^[21,22]. Further quantification of aneuploidy in the (diseased) human brain is needed to resolve these conflicting observations.

Aneuploidy in the liver

Roughly half of the hepatocytes in an adult liver are polyploid or aneuploid^[23]. Interestingly, liver regeneration has been attributed to proliferation of mature hepatocytes, but not liver stem cells^[24]. This is in line with other observations that stem cells tolerate aneuploidy poorly^[4,9]. The aneuploidization and polyploidization of hepatocytes has been suggested to contribute to a great variety of hepatocyte genotypes that could help the liver adapt to different insults and chronic stressors^[23]. While single cell next generation sequencing failed to detect aneuploidy in adult neurons, it did confirm the polyploidy and aneuploidy rates previously measured in hepatocytes^[18], further emphasizing the importance of increased efforts to quantify aneuploidy in various tissues using several techniques.

ANEUPLOIDY IN HUMAN PATHOLOGIES

Stem cells and ageing

Functional exhaustion of adult stem cells is an important contributing factor to natural ageing, as stem cells are essential for tissue maintenance, especially in tissues that have a high turnover rate, such as the intestinal wall and the skin^[25]. Indeed, ageing coincides with a functional decline of stem cell function in various organs, even though this is not always accompanied by reduced stem cell numbers. For example, in humans and some mouse strains the number of hematopoietic stem cells even increases during ageing^[26-28], but their potential to differentiate decreases^[28]. However, whether increased aneuploidy rates as observed in ageing tissues^[8,29] contribute to stem cells exhaustion needs further testing. There are several syndromes that are caused by systemic aneuploidy that exhibit also premature ageing features, such as Down's syndrome (DS) or Edward's syndrome. However, how the systemic aneuploidy in these patients impacts stem cell integrity and stem cell numbers requires further investigation.

Stable systemic aneuploidy: DS

The most well known condition linked to systemic aneuploidy is DS. This syndrome, caused by a systemic gain of chromosome 21 (trisomy 21), is associated with developmental and cognitive defects and affects about 1 in 700 individuals^[30]. In line with a role of aneuploidy in natural ageing^[8], DS is associated with an earlier onset of aging-related pathologies such as AD, and an increased incidence of cancer^[31]. One proposed driver of the accelerated ageing phenotype in DS is the observed overexpression of *CDKN2A*, as a result of

epigenetic remodulation in various cell lineages including hematopoietic stem cells, mammary epithelium, fibroblasts and neural progenitors cells^[32]. Enhanced *CDKN2A* expression is associated with senescence and stem cell self-renewal defects, further emphasizing the link between aneuploidy and ageing.

STEM CELLS AND CIN

Stem cell biology is a rapidly developing field recently revolutionized through the discovery for protocols to revert terminally differentiated cells back into a pluripotent state (iPSCs)^[33]. Stem cells hold great therapeutic promise for the treatment of a large number of diseases and are defined as cell lineages that have 3 cardinal features: (1) self-renewal through asymmetric cell division yielding one stem cell and one differentiated cell or symmetric division yielding two stem cells; (2) the capacity to produce multiple cell lineages; and (3) the potential to proliferate extensively^[34,35]. We are only beginning to understand how to isolate and maintain stem cells in tissue culture and how to differentiate them into specific tissues, all of which are crucially important to exploit stem cells in future therapies^[36].

Stem cell potency

Stem cells can be subdivided in two subtypes that differ in their differentiation potential or potency: (1) Pluripotent stem cells: Embryonic stem cells (ESCs) are pluripotent, which means that they can form any embryonic tissue, embryonic therefore can form a complete and viable organism when injected into a blastocyst. Mouse ESC culture has revolutionized biology as it allowed for making transgenic and knockout mice^[37,38]; Pluripotent stem cells can form any of the three germ layers of an embryo. The recent discovery of protocols to induce pluripotency in differentiated cells, yielding iPSCs^[33] has propelled the stem cell field as a whole and made this stem cell subtype the current central tool in stem cell research, as, in theory, iPSC protocols would allow us to make any cell type from any patient's somatic cell, overcoming graft versus host disease and omitting the ethical concerns of human ESCs; and (2) The second subtype of stem cells are multipotent, somatic or adult stem cells. These stem cells that can form a number of lineages, typically within one tissue type. Examples of somatic stem cells are neural stem cells, mammary stem cells or hematopoietic stem cells. In many cases somatic stem cells produce unipotent proliferative cells or transit amplifying cells, *e.g.*, through asymmetric stem cell division. These cells can still replicate, but only form one cell lineage before cells terminally differentiate and exit the cell cycle^[39] and are therefore not considered to be genuine stem cells.

HOW CAN STEM CELLS RESPOND TO ANEUPLOIDY?

Because of their important role in development and

tissue maintenance, stem cells have to safeguard their genome integrity. Any genetic alteration that may occur in a dividing stem cell will be inherited by the entire lineage emerging from this single stem cell and thus give rise to severe developmental defects or pathologies. Genomic integrity maintenance in stem cells is not only important for proper embryonic development and adult tissue homeostasis, but also an essential requirement for the use of stem cells in regenerative medicine and research. This is particularly true for mesenchymal stem cells (MSCs), that are already used in therapy. Importantly, both embryonic and somatic stem cells have developed ways to prevent the negative effects of mutations and aneuploidies resulting in an increased DNA damage response in stem cells as compared to somatic cells, thus preventing structural CIN^[40].

DNA damage repair systems in stem cells

One way stem cells accomplish an increased DNA damage response is by increasing the expression of genes involved in DNA repair resulting in increased efficiency in repairing DNA lesions, when compared to differentiated cells^[40]. The most dangerous form of DNA damage is the formation of DNA double strand breaks (DSBs). These can arise from replication stress, reactive oxygen species (ROS), mutagens and other DNA damaging events. To repair DSBs, cells employ two main DNA repair systems: Non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is an error-prone form of repair taking place in the G1 phase of the cell cycle in which the sequence flanking the break point is resected followed by blunt end ligation of the DNA ends, which by definition results in small deletions in the DNA sequence. In contrast, HR utilizes the sister chromatid as a repair template to fully repair the DSB, and can therefore only occur when sister chromatids are available, in the G2 phase of the cell cycle. Another way by which stem cells maintain genomic fidelity is by their predominant use of HR to repair DSBs, while differentiated (non-proliferating) cells can only use NHEJ, as the latter have exited the cell cycle and therefore have no "access" to duplicated sister chromatids^[41,42]. Therefore, the preferred use of HR to repair DNA DSBs combined with more sensitive DNA damage signaling helps stem cells to maintain genomic integrity.

The safe way out: Apoptosis and differentiation

An alternative method to prevent daughter cells from inheriting genomic aberrations is by eliminating the aberrant stem cells from the stem cell pool. This can be done through apoptosis, removing the cell altogether, or by differentiating the compromised stem cell in order to avoid further cell divisions. Indeed, when encountering DNA damage, stem cells activate the p53 pathway to mobilize the DNA repair machinery. When damage remains unrepaired, p53 activity promotes apoptosis. While apoptosis is the main response to DNA damage in stem cells, induction of p53 in human stem cells can

Table 1 Reported chromosomal aberrations in different stem cell types

Species	Stem cell type	Chromosomal aberration
Human	Cultured embryonic stem cells	+1, +12, +14, +17, +20
	Mesenchymal stem cells	4N, -13
Mouse	Cultured embryonic stem cells	+4, +5, +10, +X
		+8

also result in spontaneous differentiation^[43], however, which factors determine the switch between these two choices are so far unclear.

This is exemplified further by the ways by which different stem cell types respond to DNA damage. For example, hematopoietic stem cells (HSCs) enhance DNA repair and stem cell maintenance, and prevent apoptosis to avoid depletion of the HSC pool. Intestinal stem cells on the other hand favor increased cell death and melanocytes respond by terminal differentiation^[44]. Therefore, different responses to DNA damage in different stem cell populations will yield different effects on the integrity of the stem cell pool and their downstream potencies.

STEM CELLS COPE POORLY WITH NUMERICAL CIN

Numerical CIN and the resulting aneuploidy is detrimental for embryonic development, evidenced by early embryonic death observed in mouse models in which high grade numerical CIN was provoked systemically, see for extensive review^[45-47]. While these observations led to the prevailing view that high grade numerical CIN is never tolerated, more recent observations are nuancing this view. For instance, when numerical CIN was provoked in a tissue specific fashion, in mouse epidermis, epidermal hair follicle stem cells were rapidly depleted, while the more committed transit amplifying (unipotent) cells tolerated the resulting high grade aneuploidy remarkably well^[9]. Furthermore, while aneuploid cells seem to accumulate in various somatic cell types in the ageing mouse, aneuploidy in stem cell lineages in the same mice remains rare, further indicating that stem cells are well protected against (or ultra sensitive to) numerical chromosome abnormalities^[4]. However, even though these observations are suggestive of an aneuploidy checkpoint in stem cells, more work is needed to reveal if this checkpoint does exist and if so, how this checkpoint operates.

DO CULTURE CONDITIONS IMPACT STEM CELL GENOMIC INTEGRITY?

As stem cells are typically isolated in small quantities and iPSC protocols are still quite inefficient, stem cells are exposed to a period of tissue culture stress that can yield (further) CIN. While the majority of such alterations will be negatively selected and therefore disappear within a few passages, some genetic alterations could result in

proliferative advantages and outcompete the “normal” stem cells, a process known as “culture adaptation”^[48] resulting in late passage cultures that grow better and show better plating efficiencies^[49].

Frequently reported chromosome aberrations in stem cells

Various cytogenetic abnormalities have been reported in cultured ESCs, with varying frequencies in different cell strains. The most common abnormality reported in murine ESCs is trisomy of chromosome 8 (Table 1). Trisomies of chromosome 12, 14 and 17 are most frequently observed in human ESCs^[50], but not necessarily within the same ES cell line. Further studies that investigated the genomic integrity of > 150 human ES cell lines and 220 iPSC lines found that, while the majority of the stem cell lines were euploid, approximately 12%-13% of human ES cell lines and similar fractions of iPSC lines showed whole chromosome abnormalities^[49,51].

Notably in ESC culture, chromosomal abnormalities increased with prolonged culturing as late passage cultures had approximately twice as many aberrations, which typically involved gain of chromosomes^[49]. In line with culture adaptation, chromosome aberrations were non-random with 60% of the aneuploid stem cell cultures showing abnormalities for chromosomes 1, 12, 17 and/or 20 (Table 1)^[49]. Chromosome 12 gain has been observed by others as well^[52,53] and although selection for chromosome 12 is likely to be driven by multiple genes, it is tempting to speculate that *NANOG* is a key driver for this commonly observed trisomy in stem cell cultures, possibly together with the pluripotency related *DPPA3* and *GDF3*, and the cell cycle regulator *CCND2*. Furthermore, the oncogene *KRAS* is also located on chromosome 12^[49,54]. Importantly, culture conditions appear to also have an important impact on genomic integrity of stem cell cultures, for instance the use of fetal bovine serum as growth supplement results in increased chromosomal abnormalities^[55]. Last, but certainly not least important, iPSC cells are typically derived from somatic (differentiated cells) that appear to tolerate aneuploidy much better than previously anticipated^[9] and therefore the founder cells could already have been aneuploid to start out with. Aneuploidies in these founder cells might lower iPSC protocol's efficiency if the induced stem cells do not tolerate the aneuploidies. Even worse, when aneuploidies in the founder cells are subtle, the aneuploidies could be maintained in the resulting iPSC clones.

As MSCs are frequently used in therapy, their genomic stability is routinely assessed. The most commonly reported type of aberration in MSCs several passages after cell expansion is tetraploidization, observed in 6 out of 21 patient-derived cell lines^[56]. Tetraploidy was observed only in a minority of examined metaphases (approximately 1 in 20-30 per cell line), and whether the contribution of these tetraploid cells will have significant consequences *in vivo* needs further investigation. Other reported aberrations include a preferential loss of chromosome 13 in later passages of the human MSC line UE6E7T-3 (Table 1)^[57],

and the emergence of clonal trisomies for chromosomes 4, 5, 10, and X in a male patient-derived MSC^[58]. Therefore, while cytogenetic aberrations are widely reported for MSCs, their ultimate effect on MSC proliferation and potency *in vivo* are less well understood. This also holds true for other stem cell populations *in vivo*. Novel karyotyping methods (for extensive review see Bakker *et al.*^[17]) that circumvent the limitations of existing techniques will be instrumental in resolving both the incidence and effects of aneuploidy in adult stem cells in various different tissues.

CONCLUSION

Stem cells are the origin of tissue homeostasis and therefore crucially rely on genomic integrity. Fortunately, stem cells appear to be much more sensitive to DNA damage and aneuploidy than their differentiated counterparts^[9,40-42]. However, as iPSC protocols force terminally differentiated cells back into the cell cycle, cells might accumulate mutations in the process of becoming pluripotent, or even harbor chromosomal abnormalities before dedifferentiation starts. Therefore, as iPSC protocols are becoming the new standard in regenerative medicine, it will be of the utmost importance to develop sequencing pipelines that can ensure chromosomal fidelity of the engineered iPSCs or iPSC-derived tissues.

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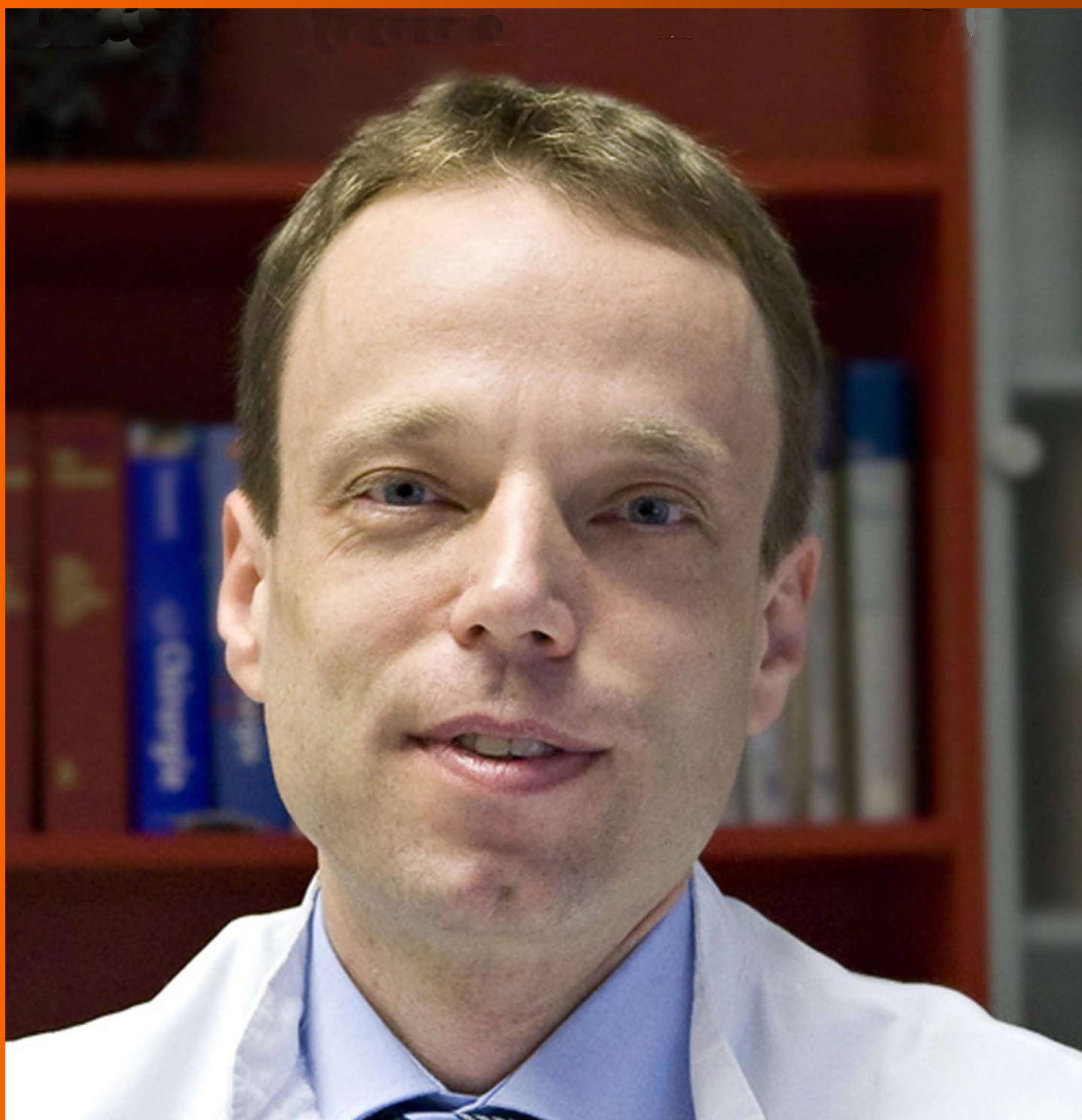
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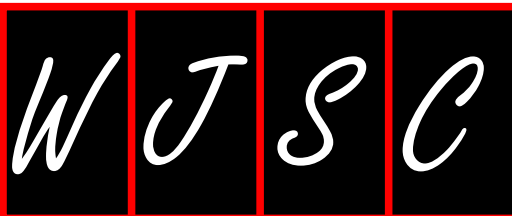
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Clinical Trials Study

Therapeutic potential of human embryonic stem cells in type 2 diabetes mellitus

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Author contributions: Shroff G conceptualized the study, participated in its design and coordination and drafted the manuscript; Shroff G read and approved the final manuscript; Shroff G contributed to study design and data analysis of the manuscript.

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Abstract

AIM: To evaluate the safety and efficacy of human embryonic stem cells (hESCs) for the management of type 2 diabetes mellitus (T2DM).

METHODS: Patients with a previous history of diabetes and its associated complications were enrolled and injected with hESC lines as per the defined protocol. The patients were assessed using Nutech functional score (NFS), a numeric scoring scale to evaluate the patients for 11 diagnostic parameters. Patients were evaluated at baseline and at the end of treatment period 1 (T1). All the parameters were graded on the NFS scale from 1 to 5. Highest possible grade (HPG) of 5 was considered as the grade of best improvement.

RESULTS: Overall, 94.8% of the patients showed improvement by at least one grade of NFS at the end of T1. For all the 11 parameters evaluated, 54% of patients achieved HPG after treatment. The four essential parameters (improvement in glycated hemoglobin (HbA1c) and insulin level, and fall in number of other oral hypoglycemic drugs with and without insulin) are presented in detail. For HbA1c, 72.6% of patients at the end of T1 met the World Health Organization cut off value, i.e., 6.5% of HbA1c. For insulin level, 65.9% of patients at the end of T1 were able to achieve HPG. After treatment, the improvement was seen in 16.3% of patients who required no more than two medications along with insulin. Similarly, 21.5% of patients were improved as their dosage regimen for using oral drugs was reduced to 1-2 from 5.

CONCLUSION: hESC therapy is beneficial in patients with diabetes and helps in reducing their dependence on insulin and other medicines.

Key words: Type 2 diabetes mellitus; Human embryonic stem cells; Insulin; Glycated hemoglobin; Nutech functional scoring scale

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Core tip: We began research on human embryonic stem cell (hESC) therapy in 1999. Today, we have used it in > 1400 patients, and have got patent for our technology in 65 countries including the United States. This study focused on the safety and efficacy of hESCs in patients who were chronically affected with type 2 diabetes mellitus (T2DM). Patients with a previous history of diabetes and its associated complications were enrolled and injected with hESC lines as per the defined protocol. The patients were assessed using Nutech functional score (NFS) (another invention of Nutech Mediworld), a numeric scoring scale to evaluate the patients for 11 diagnostic parameters. All the parameters were graded on the NFS scale from 1 to 5. Highest possible grade (HPG) of 5 was considered as the grade of best improvement. Patients were evaluated at baseline and at the end of treatment period 1 (T1). Overall, 94.8% of the patients showed improvement by at least one grade of NFS at the end of T1. For all the 11 parameters evaluated, 54% of patients achieved HPG after treatment. Important parameters like glycated hemoglobin, insulin and use of oral hypoglycemic drugs with and without use of insulin were measured and their results have been presented in detail. It has been concluded from the study that hESC therapy is beneficial in patients with diabetes and helps in reducing their dependence on insulin and other conventional medicines. Their remarkable properties of reducing risk of immune mediated rejections and absence of hypoglycemic episodes while treating T2DM patients favor their use in management of T2DM.

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INTRODUCTION

Diabetes mellitus (DM) is an endocrine disorder caused by absolute (type 1) or relative (type 2) insulin deficiency leading to hyperglycemia^[1]. Individuals with type 2 diabetes mellitus (T2DM), which is also known as non-insulin dependent diabetes mellitus (NIDDM), present with a combination of varying degrees of insulin resistance and relative insulin deficiency. Insulin resistance caused by impaired β cell functioning is usually associated with abnormal insulin secretion or action. Thus, even after

insulin administration, the cells can no longer use it to keep blood sugar levels in control and insulin deficiency occurs due to loss of insulin producing cells (IPCs)^[2].

The pathogenesis of insulin resistance is complex. Various cellular and molecular mechanisms have been established to explain the cause of insulin resistance. Hyperglycemia itself can impair pancreatic β cell function and lead to insulin resistance, causing a worsening metabolic state. Besides β cell impairment, immune cells like lymphocytes and myeloid cells (monocyte/macrophages) also play important roles in the pathogenesis of DM. Myeloid cells secrete cytokines and regulate the adipose tissue remodeling which accompanies hyper-nutrition, thus are critical players in metabolic homeostasis. Any type of alterations such as pro-inflammatory changes in lymphocyte function causes insulin resistance in T2DM patients^[3-5].

At present, there is no cure for diabetes and β cell failure is progressive; once there is an onset of disease, the impairment can never be fully restored. Patients with T2DM need to control their hyperglycemic condition through various means, including diet and exercise, oral anti-hyperglycemic (blood glucose-lowering) drugs, and/or daily insulin shots. Most people who live with T2DM for a period of time eventually require insulin to survive. However, administering insulin alone *via* conventional techniques does not prevent the long-term complications of the disease, as the optimal insulin dosage is difficult to adjust and also simply re-growing the missing IPCs which could secrete or produce insulin is not enough to solve the problem^[4,5]. Studies for pancreatic transplantation and islet cell replacement at the global level are ongoing; however, they were found to be often associated with complications of donor availability, graft survival, transplant rejection and other complications with long-term use of immunosuppressants^[6-8].

Clinical management of T2DM is complex and is planned as per the severity of the condition. As per the United Kingdom Prospective Diabetes Study (UKPDS) and Action to Control Cardiovascular Risk in Diabetes (ACCORD), the management for diabetes is decided with an objective for intensive glycemic control where it is tried to maintain blood glucose concentrations close to the normal range using either insulin alone or in combination with few oral hypoglycemic drugs followed by conventional diet plans. The dosage regimen is designed in such a manner, so as to overcome the associated risks and complications of the disease. These all have shown successful results in achieving normal glucose levels but have the most common limitation of influencing long-term glycemic control like increased body weight, higher risk of hypoglycemic episodes and insulin resistance^[9-11].

While diabetes can be managed, at present it cannot be cured. Recent advances in stem cell therapy, where it is possible to replace the IPCs of the pancreas that are destroyed by a patient's own immune system, have given a new hope for the management of DM^[12]. Various studies in animal models using stem cells for managing

diabetes have been conducted and ongoing^[5,13-16]. Hayek *et al*^[17] have reported that grafts of human fetal islet like cell clusters when transplanted to immune deficient and streptozocin induced diabetic mice, successfully matured into glucose-responsive IPCs (β cells).

Human embryonic stem cells (hESCs) are useful as they can produce an unlimited number of pancreatic islet cells. The cells are easy to be transplanted and when they were directly tissue-cultured to the endoderm, they differentiated into pancreatic progenitor cells which further lead to the formation of mature pancreatic endocrine cells *in vivo*^[15,18]. We have earlier reported the safe and effective use of hESCs in diabetes in one of our published case studies of patients with diabetes. A reduction in secondary complications associated with high blood sugar such as affection of the heart, kidneys, vision and polyneuropathy, was observed without any adverse events (AEs) or teratoma formation^[19].

Potential of hESCs prepared at our facility has been reported in patients with various terminal/incurable conditions^[20] and disorders like chronic obstructive pulmonary disease (COPD)^[21], cerebral palsy^[22], cortico-visual impairment^[23] and Friedrich ataxia^[19].

Besides appropriate treatment, it is also very important to correctly diagnose the condition. Diagnostic methods available for diabetes do not provide the complete evaluation of patients with diabetes^[24]. Commonly used diagnostic methods are less reliable and have been found to possess higher sensitivity but low specificity^[25]. Currently, there is no scoring system for assessing patients with DM. In the present study, we evaluated the safety and efficacy of hESC therapy in patients with T2DM using an 11-point numeric, Nutech functional scoring scale (NFS). It is composed of 11 diagnostic tests or parameters that are further categorized into five grades. These five ordinal grades (1, 2, 3, 4, and 5) run in the direction 1 \rightarrow 5, *i.e.*, BAD \rightarrow GOOD which represent bad, not so bad, medium, not so good and good, respectively, with reference to the World Health Organization (WHO) cut off point^[26].

MATERIALS AND METHODS

Cell culture and differentiation

hESCs were prepared from a single, spare, expendable, pre-implantation stage fertilized ovum taken during natural *in vitro* fertilization (IVF) process with due consent of the donor. The cells thus obtained are very small (50 nm-2.5 μ m), procured 24 h after fertilization. The cells were cultured and maintained as per our patented technology (United States Granted Patent No. US 8592, 208, 52) in a good manufacturing practice (GMP), good laboratory practice (GLP), and good tissue practice (GTP) compliant laboratory. The cell lines obtained are free of animal product and are chromosomally stable. They harbor all the properties of hESCs and blastocysts and express pluripotent stem cells markers like octamer-binding transcription factor 4, sex determining region Y-box 2, Nanog, stage-specific embryonic antigen-4, trophoectoderm marker, keratin 18, β -human chorionic

gonadotropin (negative), immune-regulatory marker, human leukocyte antigen G (negative), gene activating marker 5-methylcytosin, and other markers like telomerase and α fetoprotein. The detailed procedure of cell culture and differentiation was elaborated previously^[21]. The safety and efficacy of these cells have been established in patients with incurable conditions^[27].

Study population

Patients diagnosed with T2DM who were on conventional therapies of insulin and other commonly used medications were included in the study. We also included those patients who visited our facility and were diagnosed with the disease at the institute by regular diagnostic procedures. A verbal, written and video consent form was obtained from all the patients included in the study. The doctors and the rehabilitation team performed a detailed examination of the patients before, during and after each treatment cycle.

Study design

The study protocol was approved by an Institutional Independent Ethics Committee (IEC) for stem cell research and therapy of our institute. The treatment regimen followed a defined protocol. An initial dose of 0.25 mL (\leq 4 million cells) of hESCs was administered *via* an intramuscular (*i.m.*) route twice daily so as to induce immune tolerance against hESCs, subsequently another dose of 1 mL hESCs (\leq 16 million cells) was administered twice weekly *via* an intravenous (*i.v*) route to inoculate the required area and 5 mL of hESCs were also administered intravenously thereafter every 7 d. This gap was kept in order to allow the injected hESCs to develop into mature cells and regenerate the affected part.

All the patients enrolled were evaluated at baseline and at the end of treatment period 1 (T1) for 11 different diagnostic parameters by NFS. The findings were recorded for those who scored as highest possible grade (HPG), *i.e.*, grade 5 of NFS scale and who showed change in condition by at least one grade of NFS at the end of T1. The results from essential parameters, *i.e.*, glycated hemoglobin (HbA1c) level, insulin level, number of oral hypoglycemics used without insulin, number of oral hypoglycemics used with insulin were reported for each grade of NFS scale.

RESULTS

Patients

Overall 95 patients including 36 (37.9%) women and 59 (62.1%) men were enrolled. The mean age of the patients was 57.1 years.

Table 1 shows the number and percentage of patients evaluated for all the 11 parameters who scored less than the HPG at baseline and reached HPG at the end of T1. Table 2 represents patients who showed improvement by at least one grade of NFS at baseline to the end of T1.

Changes in HbA1c levels from baseline to the end of T1

Figure 1 shows the data of patients being evaluated for HbA1c level at the time of admission, and at the end of

Table 1 Number and percentage of patients who scored less than the highest possible grade at baseline and reached highest possible grade at the end of T1 (*n* = 95)

Parameter	< HPG (<i>n</i>) at baseline	HPG <i>n</i> (%) at T1
FBS	95	61 (64.2)
HbA1c	93	44 (47.3)
Insulin level	91	60 (65.9)
Insulin with medication	92	24 (26.1)
Medication level	93	25 (26.9)
Post-prandial blood insulin 60 min	95	58 (61.1)
Post-prandial blood sugar	95	58 (61.1)
Pre-dinner	95	55 (57.9)
Secondary complication	84	27 (32.1)
Serum insulin	94	74 (78.7)
Serum peptide	90	65 (72.2)

HbA1c: Glycated hemoglobin; FBS: Fasting blood sugar; < HPG: Less than the highest possible grade.

Table 2 Number and percentage of patients who showed improvement by at least one grade of Nutech functional score at baseline to the end of T1 (*n* = 95)

Parameter	Patients (<i>n</i>) affected at baseline	Improved <i>n</i> (%)
FBS	95	93 (97.9)
HbA1c	93	90 (96.8)
Insulin level	91	88 (96.7)
Insulin with medication	92	88 (95.7)
Medication level	93	82 (88.2)
Post-prandial blood insulin 60 min	95	92 (96.8)
Post-prandial blood sugar	95	92 (96.8)
Pre-dinner	95	93 (97.9)
Secondary complication	84	79 (94.0)
Serum insulin	94	80 (85.1)
Serum peptide	90	87 (96.7)

HbA1c: Glycated hemoglobin; FBS: Fasting blood sugar.

T1. Of the 95 patients included, 67 (70.5%) had HbA1c levels \leq 6.5% (normal level as per WHO criteria) at the end of T1.

Percentage of patients who scored HPG and showed improvement by at least one grade of NFS at the end of T1

Before starting the treatment, 93 patients had a score that was less than HPG or had HbA1c levels < 6.0%. After the hESC therapy, 44 (47.3%) patients scored HPG. Overall, 96.8% of patients showed an improvement by at least one grade of NFS at the end of T1.

Changes in insulin level from baseline to the end of T1

Figure 2 shows the data of patients being studied for insulin level at the time of admission, and at the end of T1. As per WHO, the cut-off range for fasting insulin level is \geq 20 μ U/mL or 0-20 IU. Considering grade 5 of NFS as HPG, of 95 patients enrolled, 60 (65.9%) at the

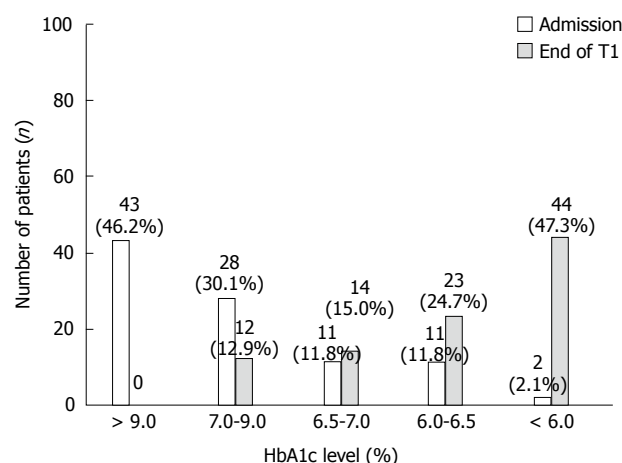


Figure 1 Number of patients assessed for glycated hemoglobin: Before and after the human embryonic stem cell treatment. NFS grades - Grade 1: > 9.0%; Grade 2: 7.0%-9.0%; Grade 3: 6.5%-7.0%; Grade 4: 6.0%-6.5%; Grade 5 (HPG): < 6.0%. HbA1c: Glycated hemoglobin; NFS: Nutech functional score; HPG: Highest possible grade.

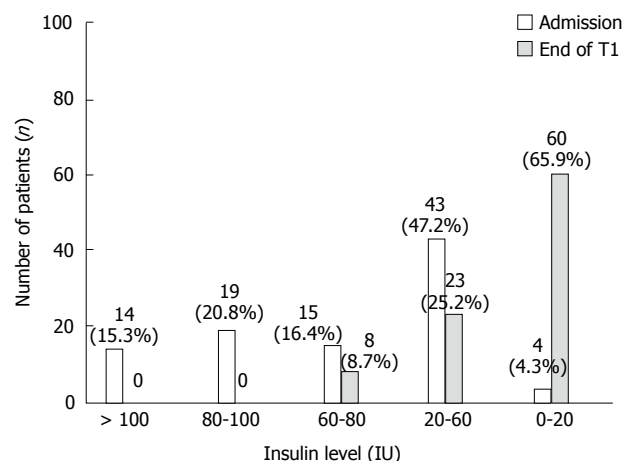


Figure 2 Number of patients assessed for insulin: Before and after the human embryonic stem cell treatment. NFS grades - Grade 1: > 100 IU; Grade 2: 80-100 IU; Grade 3: 60-80 IU; Grade 4: 20-60 IU; Grade 5 (HPG): 0-20 IU. NFS: Nutech functional score; HPG: Highest possible grade.

end of T1 were able to achieve HPG.

Percentage of patients who showed improvement by at least one grade of NFS at the end of T1

Before starting the treatment, 91 (95.7%) patients were qualifying the positive test of fasting insulin for diabetes. After getting treatment, the improvement was seen among 96.7% of patients who scored differently by at least one grade of NFS scale at the end of T1.

Change in number of drugs used from baseline to the end of T1

Of the 95 patients, more than 50% were prescribed to take at least 5 or more than 5 oral hypoglycemics at the time of admission. After hESC therapy, the patients showed remarkable improvement, i.e., the dosage regimen was reduced to 1-2 medicines (grade 4). There

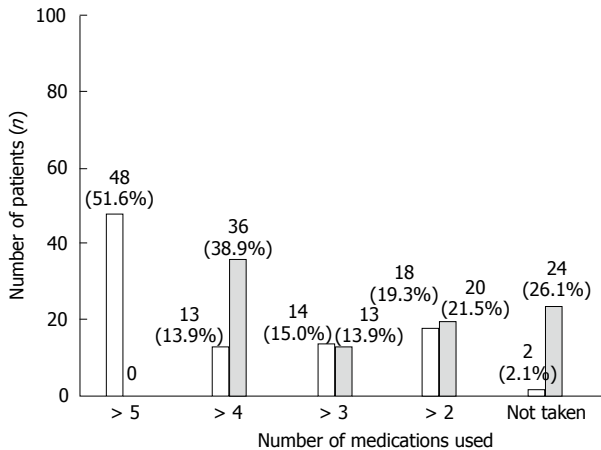


Figure 3 Number of patients assessed for number of medications used without insulin: Before and after the human embryonic stem cell treatment. NFS grades - Grade 1: > 5; Grade 2: < 4; Grade 3: < 3; Grade 4: > 2; Grade 5 (HPG): No medications taken. NFS: Nutech functional score; HPG: Highest possible grade.

were 20 (21.5%) patients at this grade at the end of T1.

Percentage of patients who achieved HPG and showed improvement by at least one grade of NFS at the end of T1

HPG grade (grade 5) where no intake of medicine is required was achieved in 24 (26.1%) patients at the end of T1. Overall, 88.2% of patients showed an improvement by at least one grade of NFS scale at the end of T1 (Figure 3).

Change in number of drugs used along with insulin from baseline to the end of T1

Before the treatment, 92 (97%) patients were taking both insulin and oral therapy to manage their glycemic levels. Depending on the severity of their condition, their dosing regimen was varying with the number of medications being combined with insulin therapy. After hESC therapy, 15 (16.3%) patients were at grade 4 where they required only 2 medications along with insulin (Figure 4).

Percentage of patients who achieved HPG and showed improvement by at least one grade of NFS at the end of T1

Overall, HPG was achieved in 25 (26.9%) patients and 88 (95.7%) patients showed an improvement by at least one grade of NFS scale at the end of T1.

DISCUSSION

T2DM is a complex disorder characterized by hyperglycemia, insulin resistance, and variable degrees of insulin deficiency. To manage T2DM, there are four major factors to be considered which can monitor drug's glycemic and non-glycemic effects: Insulin resistance,

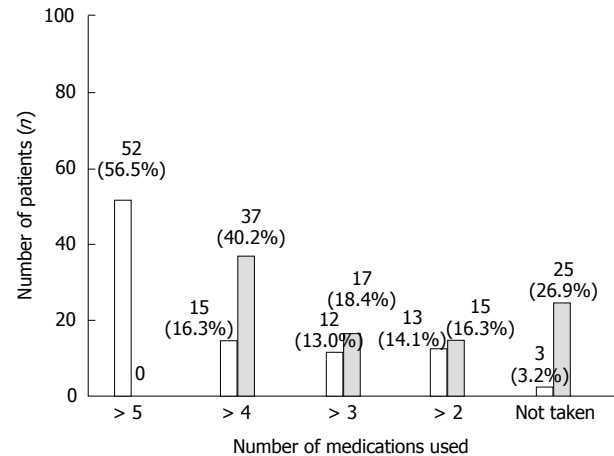


Figure 4 Number of patients assessed for number of medications used with insulin: Before and after the human embryonic stem cell treatment. NFS grades - Grade 1: Insulin with > 4 medicines; Grade 2: Insulin with 4 medicines; Grade 3: Insulin with 3 medicines; Grade 4: Insulin with 2 medicines; Grade 5 (HPG): No medications required. NFS: Nutech functional score; HPG: Highest possible grade.

decreased insulin secretion, increased hepatic glucose production, and reduced glucagon-like peptide-1 levels^[3]. The majority of treatments available focus on intensive glucose control by giving intensive insulin therapy. Intensive glucose control should be done in a stepwise procedure considering the severity of disease and the risk factors experienced with earlier treatment^[28]. American Diabetes Association (ADA) stated that "it is reasonable to recommend tight glucose control in diabetic patients" after the publication of Diabetes Control and Complications Trial (DCCT) results. However, ADA also pointed out that intensive insulin therapy may result in weight gain and unfavorable changes in cardiovascular risk factors among obese and insulin-resistant patients^[29]. The UKPDS (1998) study also concluded that all intensive treatments (either sulphonylureas or insulin) increase the cardiovascular risks in diabetes. It also shows that prolonged use of insulin treatment for T2DM might increase the risk of weight gain and hypoglycaemia and may lead to any cerebrovascular event like stroke due to low glycemic level^[10,11]. It has been observed that insulin injection does not precisely mimic the dynamic regulation of β cells on glucose homeostasis, which increase the risks of renal failure or blindness. It also causes diabetic foot syndrome, the severity of which may force the patient to undergo limb amputation^[1].

The ADA and the European Association for the Study of Diabetes (EASD) have recommended various research based guidelines for the first line and second line management of T2DM which includes use of other drugs like sulphonylureas, thiazolidines, DPP-IV inhibitors either alone or in combination of 2-3 drugs or along with insulin therapy depending on the severity of disease. However, all have other associated risks like cardiovascular events, resistance or hypoglycemia with their long term use^[28].

Stem cell therapy is an emerging area of research for diabetes. Sources for stem cell therapies in DM are multiple, including embryonic stem cells (ESCs), cord blood stem cells, induced pluripotent stem cells (iPSCs), and mesenchymal stem cells which have shown their benefits in the long term treatment of diabetes. hESCs are pluripotent and derived from human fertilized eggs, therefore they have a much lower risk of tumorigenesis than iPSCs^[16,30,31]. Li *et al*^[32] have also reported that hESCs have unique immune-privileged characteristics and therefore there are lesser chances of immune-mediated rejection in their transplantation. A similar study conducted by Drukker *et al*^[33] also favors that hESCs and their differentiated derivatives are less susceptible to immune rejection than adult cells.

Hypoglycemia is also one of the major drawbacks accompanied with management of T2DM. Few more recent studies favor that most of the treatment approaches for diabetes focus on intensive glycemic control, which might increase the risk of hypoglycemia^[34]. hESC therapy overcomes the limitation of hypoglycemic episodes which is usually associated with other conventional regimens to manage T2DM^[35,36].

Preclinical studies conducted by Soria *et al*^[37] have shown that ES-derived insulin-containing cells are able to normalize blood glucose in streptozotocin-induced diabetic mice. The studies reported that glycemic control was found to be not only normal but stabilized as well^[37].

Stem cells are able to differentiate into islet-like cells and have immunomodulatory abilities; they cause paracrine secretion that involves production of growth factors and cytokines. These secreted factors promote differentiation of progenitor cells into endogenous progenitor cells (EPCs) and mobilization and homing of EPCs, resulting in angiogenesis and tissue regeneration in diabetic wounds^[1,4,38]. Khorsandi *et al*^[41] in their studies using advanced genetic techniques showed that cultured adipose-derived mesenchymal stem cells can be differentiated into IPCs. The induced IPCs were morphologically similar to pancreatic islet like cells and were able to produce as well as secrete insulin in response to different concentrations of glucose stimulation in a regulated manner^[41].

Stem cells have the capability to migrate to the damaged cells and repair/regenerate the impaired cells, which also helps in reducing the autoimmune process by producing a new functional immune system and hence shows faster recovery^[39,40]. Various clinical trials registered on ct.gov using stem cells for T2DM also have the same hypothesis that stem cell transplantation in human pancreas results in increased angiogenesis, secretion of various cytokines and up-regulation of pancreatic transcription factors and vascular endothelial growth factor, which creates a microenvironment to support β cell/resident stem cell activation and survival^[41,42].

hESCs used in our study might have shown their therapeutic effect by following the same mechanism. These cells might "home in" to the damaged cells and

repair/regenerate the cells and might reduce the auto-immune process. They are pluripotent in nature and their competency to that with other drug therapies is well proven by the findings obtained; the patients enrolled in the study had chronic diabetic condition and were all kept on the similar pattern of diet and exercises although they had different intake of medicines and insulin therapy as per the severity of disease before starting hESC treatment. After the therapy, most of the patients benefitted as they showed a reduction in medicines and insulin intake to manage hyperglycemia. We did not observe any incidence of hypoglycemia in our patients during hESC treatment. The patients also showed a reduction in HbA1c levels. None of the patients experienced severe AEs or SAEs during the study as a result of hESC therapy. The embryonic stem cell transplantation also reduces the risk of other secondary complications of eyes, heart, kidney and nerves associated with diabetes^[1,2,40,43].

Besides management of diabetes with new therapies, an important area of concern is the precise diagnosis of severity of disease using reliable biochemical parameters so that an appropriate therapeutic strategy can be applied. Factors such as extreme variability in patient populations (*i.e.*, pregnancy, elderly and non-Hispanic Blacks, Mexican white people), lifestyle changes and insulin resistance affect the diagnosis and management of DM^[44-46]. These factors create hindrance for most of the commonly used diagnostic parameters like fasting blood sugar, oral glucose tolerance test, HbA1c, serological examination and post-prandial blood sugar in producing the accurate results^[46], which raises the question on their reliability. No single test can be considered as a perfect method to diagnose diabetes precisely. Many studies in the past few decades have been conducted where combination of diagnostic tests was carried out, but most of the studies could not completely explore the severity of disease accurately. False positive and negative results were also reported during the study^[25,47]. To overcome these limitations of variability, we developed a novel numeric scoring system, NFS which comprises a battery of tests. It has 11 different diagnostic parameters of diabetes on a single scale. The cutoff values of these parameters are as per the WHO criteria.

After hESC therapy, the majority of the patients with diabetes showed an improvement for all the 11 parameters evaluated in NFS. Overall, 94.8% of patients showed improvement by at least one grade of NFS scale at the end of T1. Important parameters like HbA1c and insulin level showed improvement and use of medicines alone and use of medicines with insulin at different levels of treatment phases were reduced after the therapy, suggesting that the hESC therapy helped the patients in being independent of intensive insulin therapy and other conventional drugs.

hESCs might have a good therapeutic potential in the treatment of patients with diabetes. However, well designed studies are needed to prove the long term efficacy and safety of hESCs in the treatment of patients

with diabetes.

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COMMENTS

Background

Type 2 diabetes mellitus (T2DM) is the most common lifestyle disorder nowadays. At present, there is no cure for diabetes. Human embryonic stem cells (hESCs) therapy is a new approach to treat it. hESCs can produce unlimited number of pancreatic islet cells and are easy to be transplanted. When they were directly tissue-cultured to the endoderm, they differentiated into pancreatic progenitor cells which further lead to the formation of mature pancreatic endocrine cells *in vivo*. They have less chances of immune mediated rejection.

Research frontiers

This study focuses on treating the patients who were chronically affected by T2DM with latest stem cell therapy. hESC cell lines produced in their lab are safe, effective and are easily transplantable. The *in vitro* fertilization technique used to prepare hESC is unique. The improvement in diabetic patients is measured by a novel scale, Nutech functional score, which is able to measure 11 parameters simultaneously. Hence the diagnosis is more reliable.

Innovations and breakthroughs

Undoubtedly hESCs have better potential than other treatments. Being pluripotent and derived from human fertilized eggs, they have a much lower risk of tumorigenesis than induced pluripotent stem cells. They are easily transplantable.

Applications

The patients were treated with hESC therapy have shown remarkable improvement. This innovative treatment approach has overcome the life long insulin dependence of patients and have also reduced the intake of oral hypoglycemic and other conventional drugs.

Peer-review

This research article is excellently written. This work has been performed as an advance step for the previous case studies by Dr. Shroff G (*J Diabetes Mellitus* 2015; 5: 313-318). The current report will make a great contribution to the promotion of the hESC-based regenerative medicine in the field of diabetes.

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Therapy-related myeloid neoplasms - what have we learned so far?

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Abstract

Therapy-related myeloid neoplasms are neoplastic processes arising as a result of chemotherapy, radiation therapy, or a combination of these modalities given for a primary condition. The disease biology varies based on the etiology and treatment modalities patients receive for their primary condition. Topoisomerase II inhibitor therapy results in balanced translocations. Alkylating agents, characteristically, give rise to more complex karyotypes and mutations in p53. Other etiologies include radiation therapy, high-dose chemotherapy with autologous stem cell transplantation and telomere dysfunction. Poor-risk cytogenetic abnormalities are more prevalent than they are in *de novo* leukemias and the prognosis of these patients is uniformly dismal. Outcome varies according to cytogenetic risk group. Treatment recommendations should be based on performance status and karyotype. An in-depth understanding of risk factors that lead to the development of therapy-related myeloid neoplasms would help developing risk-adapted treatment protocols and monitoring patients after treatment for the primary condition, translating into reduced incidence, early detection and timely treatment.

Key words: Therapy-related acute myeloid leukemia; Therapy-related myelodysplastic syndromes; Ionizing radiation; Alkylating agents; Allogeneic hematopoietic stem cell transplantation; Topoisomerase II inhibitors; Therapy-related myeloid neoplasms

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Core tip: Therapy-related myeloid neoplasms are becoming

ing an increasing problem as the survival of cancer patients lengthens. The etiology has an important influence on the biological characteristics, time to onset and prognosis of the resultant disease. Although treatment of therapy-related myeloid neoplasms represents a substantial challenge due to prior treatment and comorbidities, cure is possible, especially with allogeneic stem cell transplantation, particularly in those with good-risk karyotype. Ultimately, individual assessment of risk factors may lead to developing risk-adapted therapies to reduce the incidence of this serious complication without affecting therapy for the underlying disorders.

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INTRODUCTION AND EPIDEMIOLOGY

Therapy-related myeloid neoplasms, which include both therapy-related myelodysplastic syndromes (t-MDS) and therapy-related acute myeloid leukemia (t-AML), are well-known sequelae of conventional anticancer chemotherapy and radiotherapy for solid tumors, such as ovarian cancer^[1], breast cancer^[2], testicular cancer^[3] and various sarcomas^[4], as well as hematologic malignancies^[5-7]. Therapy-related myeloid neoplasms constitute approximately 10%-20% of all cases of AML and MDS^[8], with incidence varying depending upon the underlying malignancy, type of cytotoxic agents and/or radiotherapy, and timing of administration and dosage of treatment modalities^[9]. Therapy-related myeloid neoplasms can present at any age, but the median age at diagnosis is reported to be approximately 61 years in adults^[10,11].

After conventional-dose anticancer chemoradiotherapy, the incidence of t-MDS/AML has been reported between 0.8%-6.3% at 20 years post-treatment, with a median time of 3-5 years from treatment to development of t-MDS/AML^[12]. In contrast, the incidence of t-MDS/AML after high-dose chemotherapy and autologous hematopoietic stem cell transplant (auto-HSCT) ranges from 1.1%-24.3% at 5 years post-transplant with a median time to development of only 1-2 years post-transplant^[12-16]. Use of etoposide (a topoisomerase II inhibitor) priming for stem-cell mobilization and total-body-irradiation (TBI) based conditioning regimens are particularly associated with t-MDS/AML after auto-HSCT^[16,17].

According to the World Health Organization classification, therapy-related myeloid neoplasms are broadly categorized into two subtypes: (1) an alkylating agent/radiotherapy-related type; and (2) a topoisomerase II inhibitor-related type^[18]. The development of t-MDS/AML after alkylating agents/radiotherapy usually occurs after a median latency of 4-7 years, with two-thirds of

patients presenting with MDS and one-third presenting with AML^[12,19]. There is prominence of peripheral cytopenias and dysplasia of multiple myeloid lineages with frequently observed abnormalities of chromosome 5 [-5/del(5q)] and chromosome 7 [-7/del(7q)]^[19,20]. Conversely, topoisomerase II inhibitor-related t-MDS/AML has a relatively shorter latency between exposure to drugs and onset (median of 2-3 years)^[21]. Patients with this subtype often present with overt AML without features of preceding MDS. AML in this subtype shows monocytic predominance^[21,22] with a high incidence of balanced translocations involving chromosomal segments 11q23, 17q21 and/or 21q22^[21]. While the risk of developing t-MDS/AML after alkylating agents/radiotherapy rises with increasing age, the risk of the same after topoisomerase II inhibitors appears to remain constant across all age groups^[18,23].

LEUKEMOGENESIS

Therapy-related myeloid neoplasms are clonal hematopoietic stem cell disorders that arise due to iatrogenic somatic mutations after treatment with cytotoxic chemotherapy/radiotherapy. These somatic mutations impart increased proliferative capacity and survival advantage in the affected hematopoietic progenitors^[12].

Alkylating agents have established significant clinical applications in virtually all cancer types and were the first chemotherapeutic drugs to be associated with therapy-related myeloid neoplasms^[24]. These drugs work by transferring alkyl groups to oxygen and nitrogen atoms on DNA bases, resulting in the formation of highly mutagenic DNA base lesions (such as O6-methylguanine and N3-methylcytosine) and inducing DNA damage^[25]. Alkylated DNA-based lesions, specifically O6-methylguanine, cause mispairing during DNA replication, and while this replication error is efficiently repaired by mismatch-repair enzymes, alkylated bases cannot be cleaved by mismatch-repair enzymes, leading to mutagenicity, secondary DNA double-stranded breaks and eventual cytotoxicity^[26,27]. Mono-functional alkylating agents, such as nitrosoureas, dacarbazine and temozolomide, have one active moiety and are able to induce such lesions. In contrast, bi-functional alkylators, such as cyclophosphamide, melphalan and chlorambucil, have two active moieties and are able to form crosslinks within and between DNA strands in addition to forming alkylated base lesions^[28]. Inter-strand DNA crosslinks halt replication forks during DNA replication, resulting in the formation of double-stranded DNA breaks. These breaks can give rise to chromosomal translocations, insertions, inversions and loss-of-heterozygosity involving several vital cellular genes^[29,30].

Drugs targeting DNA topoisomerases are also well-known to cause t-MDS/AML^[31]. DNA topoisomerase enzymes mediate the unknotting and relaxing of DNA supercoils, thereby allowing DNA replication to occur. These enzymes accomplish this by creating transient single-stranded (DNA topoisomerase I) and double-

stranded (DNA topoisomerase II) DNA breaks. The release of topoisomerases from the DNA strands is followed by the re-ligating of these transient DNA breaks^[32]. Topoisomerase II inhibitors, such as epipodophyllotoxins (etoposide and teniposide) and anthracyclines (daunorubicin, doxorubicin, *etc.*) prevent the release of topoisomerase II from cleaved DNA, preventing the re-ligation of strands and persistence of double-stranded breaks^[26]. These DNA breaks are highly mutagenic and frequently result in translocations involving the genes *MLL* at 11q23, *RUNX1* at 21q22 and *RARA* at 17q21^[33-35].

The substantial incidence of various leukemias and myeloid disorders in the survivors of the Hiroshima and Nagasaki nuclear attacks has established a firm causal relationship between ionizing radiation and hematologic malignancies^[36-38]. Epidemiological data from several studies involving individuals receiving therapeutic radiation has corroborated its leukemogenicity^[3,39-41]. Cellular exposure to ionizing radiations has multiple mechanisms of causing DNA damage and mutations. Energy in each individual photon of radiation is able to disrupt the sugar-phosphate backbone of the DNA molecule, leading to single- and double-strand breaks^[28]. In addition to this direct effect, cellular exposure to ionizing radiations results in radiolysis of water molecules leading to the formation of reactive oxygen species (most notably hydrogen peroxide, superoxide and hydroxyl radicals)^[42]. These highly reactive molecules are capable of oxidizing and deaminating DNA bases and disruption of the sugar-phosphate backbone. As discussed with alkylating agents and topoisomerase II inhibitors earlier in this section, double-stranded breaks are highly mutagenic and contribute to leukemogenesis in therapy-related myeloid neoplasms.

In the context of auto-HSCT, DNA damage is multifactorial, arising as a result of treatment with cytotoxic agents used in induction therapy prior to auto-HSCT, possibly from the transplant process itself (stem cell mobilization, stem cell collection and storage) and from the stress of engraftment and hematopoietic recovery during the post-transplant period^[43-46], apart from the chemotherapy agents and TBI used in the conditioning regimen. It is probable that some progenitor cells persist within the patients despite pre-transplant conditioning and acquire mutations overtime, for example from injury caused by the conditioning regimen, leading to t-MDS/AML after auto-HSCT^[16]. To scientifically ascertain this hypothesis, future studies may focus on genetically marking the autograft and performing assays of t-MDS/AML clones in patients who develop this complication post-transplant to ascertain whether progenitor cells persisting in the patient after pre-transplant conditioning give rise to t-MDS/AML or is it the rescuing hematopoietic progenitors that give rise to t-MDS/AML. Currently, the ongoing Center for International Blood and Marrow Transplant Research study LE14-01 is the largest retrospective study to date (to the best of our knowledge) on t-MDS/AML after auto-HSCT^[47]. The results of this study may provide deeper insight into t-MDS/AML in patients

receiving auto-HSCT.

The *p53* gene plays a crucial role in DNA damage response pathways, DNA repair mechanisms, cell cycle control and apoptosis. Abnormalities affecting *p53* hinder the cell's ability to repair damaged DNA and results in genomic instability and accumulation of various genetic lesions that contribute to leukemogenesis^[12]. It is noteworthy that less than 10% of patients with *de novo* MDS and AML harbor *p53* mutations, whereas 27%-50% of patients with t-MDS/AML demonstrate *p53* mutations^[48-50]. These are non-germline mutations that are often seen as a late adverse effect of therapy with alkylating agents and often occur simultaneously with chromosome 5 [-5/del(5q)] and chromosome 7 [-7/del(7q)] losses^[12,50].

Telomeres are repeat sequences of non-coding DNA that flank the 3' ends of linear chromosomes, permitting the replication of 3' chromosomal ends and are vital for preventing dicentric fusion and chromosomal abnormalities^[51]. Each mitotic division results in fractional loss of telomeric DNA, with cumulative telomeric loss leading to cellular senescence, a process by which normal cells lose their ability to divide after a specific number of cell divisions. In addition, loss of telomeric DNA also leads to genomic instability and somatic mutations^[52,53]. Exposure to chemotherapeutic agents places proliferative stress on the bone marrow to allow for hematopoietic recovery after/in between cycles of chemotherapy^[54]. The increased proliferative rates accelerate the loss of telomeric DNA, which would otherwise be conserved by the telomerase enzyme under physiologic conditions^[52]. It is evident that telomere shortening is associated with the development of myeloid malignancies, such as MDS and AML, in both *de novo*^[55] and therapy-related settings^[43,56,57]. The nested case-control study by Chakraborty *et al.*^[57] showed that after auto-HSCT, those patients who developed t-MDS/AML showed a substantial increase in the rate of telomeric shortening after day +100 in comparison to the control group who did not develop t-MDS/AML. Other studies^[43,56] also demonstrated similar observations. These findings corroborate that increased telomeric loss and telomere dysfunction contributes to leukemogenesis and likely precedes the development of t-MDS/AML in premalignant cells.

TREATMENT AND OUTCOMES

Conventional chemotherapy

Intensive chemotherapy is one of the established therapeutic approaches to t-MDS/AML and its role has been investigated in earlier studies. In a retrospective study of 122 patients with t-MDS/AML at the MD Anderson Cancer Center, intensive chemotherapy with cytarabine yielded a complete remission (CR) rate of 37%^[58]. In the same study, pooled data of 496 patients from 13 different studies revealed a cumulative CR rate of 27%^[58]. No doubt, CRs have been achieved in this and other early studies on t-MDS/AML, but these rates are lower and short-lived in comparison to *de novo* MDS/

AML^[11,59,60]. The fatal course of t-MDS/AML is due to profound and persistent cytopenias due to ineffective hematopoiesis regardless of the fraction of immature blasts accumulating in the bone marrow^[61]. In contrast, a subsequent study reported a surprisingly high CR rate of 82% for t-MDS/AML treated with high-dose cytarabine + mitoxantrone^[62].

For therapy-related acute promyelocytic leukemia (t-APL) and t-AML with good-risk cytogenetics, specifically inv(16) and t(8;21), induction chemotherapy is recommended, similar to the treatment guidelines for their *de novo* counterparts^[28]. For t-APL, outcomes are encouraging with regimens containing all-trans retinoic acid, as evidenced by two large European studies^[63,64]. One study reported a CR rate of 87%^[64]. The other study reported a CR rate of 80% with actuarial survival of 59% at 8 years^[63]. Since outcomes with non-transplant strategies are encouraging in t-APL, this allows patients to be spared from the toxicities associated with allogeneic hematopoietic stem cell transplant (allo-HSCT). However, recent evidence does not favor the same recommendations for t-AML with inv(16) and t(8;21) as these patients have shown shorter event-free and overall survival in comparison to patients with *de novo* AML exhibiting inv(16) and t(8;21)^[65-67]. This suggests that these patients may also require allo-HSCT for a durable cure, as is the case with t-MDS/AML with intermediate- and poor-risk cytogenetics^[12,61,68]. The general conclusion drawn from literature on the subject is that outcomes of t-MDS/AML treated with conventional chemotherapy are generally poor, with median survival as low as only 6 mo^[12].

Role of hypomethylating agents in therapy-related myeloid neoplasms

With suboptimal survival rates for t-MDS/AML after allo-HSCT and even lower with conventional chemotherapy, exploration of alternative treatments and novel therapies is highly warranted to improve survival in this subset of patients. Azacitidine has shown promising efficacy in the treatment of high-risk MDS and AML^[69,70] with a limited side effect profile and impressive tolerability, especially in patients with poor performance status and comorbidities^[71]. Several recent retrospective studies suggested notable activity of azacitidine against t-MDS/AML, with overall response rates ranging from 39%-43% and median overall survival from 14.5-21 mo^[72-74]. Azacitidine yielded the most benefit and better overall survival when used as first-line therapy^[74] and detailed analysis of these studies showed similar outcomes between patients with *de novo* MDS/AML and those with t-MDS/AML^[72,73]. A recent retrospective account of patients treated with azacitidine at the Memorial Sloan-Kettering Cancer Center and patients treated with decitabine in two industry-sponsored clinical trials (D0007^[75] and DACO-020^[76]) was published by Klimek *et al*^[77]. In a cohort of 42 patients with t-MDS, this account reported an overall response rate (CR + marrow CR + hematologic response) of 38%^[77]. However, a multi-

center retrospective case series published in 2015 reported relatively inferior outcomes compared to the aforementioned studies (overall survival: 9.6 mo; overall response rate: 35.7%)^[78].

Prebet *et al*^[79] recently reported results of the E1905 study, a phase II randomized trial comparing the effects of combination therapy with azacitidine and the histone deacetylase inhibitor, entinostat, against monotherapy with azacitidine. The results showed lower hematologic normalization rates (17% vs 46% in the monotherapy arm), shorter overall survival (6 mo vs 13 mo in the monotherapy arm) and increased toxicity in the combination arm, recommending against the use of the azacitidine + entinostat combination for t-MDS/AML^[79]. A predecessor of the same study demonstrated pharmacologic antagonism of entinostat when added to azacitidine^[80]. However, the same study showed that prolonged administration of azacitidine alone increased the rate of hematologic responses when compared to standard dosing, representing an area of future research interest^[80].

Allogeneic hematopoietic stem cell transplant

The standard approach for most patients with t-MDS/AML is allo-HSCT, which has consistently been shown to be a potential curative option for t-MDS/AML^[12,61,68]. Outcomes of patients with t-MDS/AML after allo-HSCT, albeit limited and mostly based on retrospective studies, are still uniformly poor due to the high-intensity and transplant-related complications associated with the procedure and the refractory nature of the disease. For example, an account of 13 patients receiving allo-HSCT for t-MDS/AML after auto-HSCT reported that all patients died of either transplant-related complications (11 patients) or relapse (2 patients) with a median overall survival of only 1.8 mo^[81]. One study reporting outcomes of 461 patients estimated a 35% overall survival 3 years after allo-HSCT^[82]. Another large study involving 306 patients reported a median survival of only 8-10 mo and a 5 year overall survival of less than 10%^[35]. Other studies have also reported poor outcomes^[68,83-86], with non-relapse mortality ranging between 54%-58%^[86-88]. Since most clinical trials in the AML or MDS arena have usually excluded t-AML/MDS, to our knowledge, prospective phase III randomized data evaluating the role of allo-HSCT in t-MDS/AML is lacking.

Some studies have described notable influences of conditioning regimens on survival rates. In a large study by Witherspoon *et al*^[88], the 5-year disease-free survival for patients receiving conditioning with busulfan (BU) targeted to 600-900 ng/mL steady-state plasma concentration with cyclophosphamide (CY) [(t-BU/CY)] was 30%, the highest in the patient cohort. Survival rates were significantly lower for other regimens (standard BU/CY: 19%; chemotherapy/TBI: 8%) in comparison to t-BU/CY ($P = 0.006$). In the same report, the 5-year cumulative non-relapse mortality was lowest for t-BU/CY (42%) vs that for standard BU/CY and chemotherapy/

TBI regimens (52% and 58%, respectively); ($P = 0.02$)^[88]. Subsequently, an even larger study (including 251 patients) also showed a greater 5-year disease-free survival for patients conditioned with t-BU/CY (BU targeted to 800-900 ng/mL steady-state plasma concentration) of 43% vs that for standard BU/CY, fludarabine (Flu)/BU, Flu/TBI and high-dose TBI/CY (28%, 24%, 23%, 18%, respectively); ($P = 0.001$)^[87]. This study also showed the lowest 5-year cumulative non-relapse mortality for the t-BU/CY regimen (28%) vs high-dose TBI/CY, Flu/TBI and standard BU/CY (53%, 54% and 61%, respectively); ($P < 0.001$)^[87].

Factors affecting outcomes

The dismal outlook of these patients is likely multifactorial, resulting from relapse-related and/or non-relapse-related mortality. The likelihood of relapse significantly correlates with disease stage. For example, a report from the Fred Hutchinson Cancer Research Center showed varying rates of relapse among their patient cohort (no relapses in the refractory anemia/refractory anemia with ringed sideroblasts group; 22% relapse in the refractory anemia with excess blasts group; and 36% relapse in the refractory anemia with excess blasts in transformation/AML group)^[85]. Another study reported similar findings^[88]. Likewise, disease karyotype also correlates with relapse rate. The impact of karyotype on outcomes in both *de novo* and t-MDS/AML were compared in large prospective studies which showed disease karyotype to be an independent prognostic factor in both groups, with poor-risk cytogenetic abnormalities more common in the t-MDS/AML group^[84,89]. An optimized, 3-group cytogenetic classification proposed by Armand *et al.*^[90] was found to be the strongest predictor of overall survival in t-MDS/AML by its impact on relapse risk after allo-HSCT. Through this classification, cytogenetic abnormalities in these patients were divided into good-risk [normal, -5, (del)20q or -Y], poor-risk (chromosome 7 abnormalities, complex karyotype) and intermediate-risk (all others)^[90]. Also, relapses are less likely with unrelated donor transplants, likely due to a more potent graft vs leukemia effect^[12,91] and lower peripheral blood blast count (correlating with early-stage disease and low disease burden)^[92].

Other outcome parameters after allo-HSCT have been scrutinized. Patient performance status strongly influences survival^[79]. Treatment for the primary malignancy causes injury to various organ systems and depletion of normal hematopoietic progenitors, diminishing the patients' ability to withstand the intensive nature and toxicities associated with allo-HSCT. In addition, damage to bone marrow stromal elements from prior therapy (especially radiotherapy) alters the bone marrow microenvironment, making hematopoietic regeneration more difficult^[61]. Younger patients (children, adolescents, young adults) have a better bone marrow reserve and better ability to withstand the toxicities associated with multiple treatments (both for the primary disease and allo-HSCT)^[4], hence it would be expected that survival is

better in this group in contrast to elderly. Since therapy-related myeloid neoplasms are relatively uncommon in young age groups^[8,9], there is paucity of literature concerning the prognostic factors and survival in younger patients. This is a potential area of research interest. Future studies are warranted to ascertain if different prognostic factors confer survival advantage in younger patients with therapy-related myeloid neoplasms, or if the dismal outcomes in elderly are just a result of sheer fact of age.

Patients are also immunocompromised from prior treatment regimens and hence often acquire life-threatening infections, a well-known and feared cause of mortality after allo-HSCT. Additionally, relapse of the primary malignancy, especially metastatic cancer or disseminated lymphoma, carries its own risks of morbidity and mortality^[61]. Also, the timing of allo-HSCT affects the outlook of patients, as a recent study demonstrated that those who received allo-HSCT later than 6 mo after diagnosis have inferior survival rates^[93]. Thus it is imperative to refer a newly diagnosed case of t-MDS/AML to a transplant center early.

In addition to disease stage and karyotype, somatic mutations of specific genes may also have implications on prognostication. For example, frame-shift mutations of the nucleophosmin gene, internal tandem duplications of the *fms*-like tyrosine kinase 3 gene and double mutations in the *CEBPA* gene are now routinely assessed in the workup of AML patients and incorporated into therapeutic algorithms^[94]. They have also been observed in t-MDS/AML^[95,96]. While these (and perhaps other specific gene mutations) may have impact on t-MDS/AML prognosis, these mutations usually occur and have prognostic value in cases with normal cytogenetics^[94], a karyotype which is relatively rare in t-MDS/AML, making their prognostic utility uncertain in cases of t-MDS/AML.

When taking only t-MDS into account, the International Prognostic Scoring System, a cornerstone in the prognostication of patients with MDS, has shown unsatisfactory ability to predict the outcome of patients after treatment^[81]. Instead, an alternative prediction model utilizes the following four factors to gauge survival for patients with t-MDS and t-AML after allo-HSCT: (1) age greater than 35 years; (2) poor-risk cytogenetics; (3) advanced-stage t-MDS or t-AML not in CR after allo-HSCT; and (4) donor other than an HLA-identical sibling or a matched or partially-matched unrelated donor^[68]. Five-year overall survival varies with the number of these factors present: None (50%), 1 (26%), 2 (21%), 3 (10%) and 4 (4%)^[68]. Male sex has also been indicative of poor outcomes^[86]. A proposed algorithmic approach to patients with therapy-related myeloid neoplasms is elaborated in Figure 1.

GAUGING THE RISK OF THERAPY-RELATED MYELOID NEOPLASMS

Keeping in mind the poor outcomes of t-MDS/AML, mea-

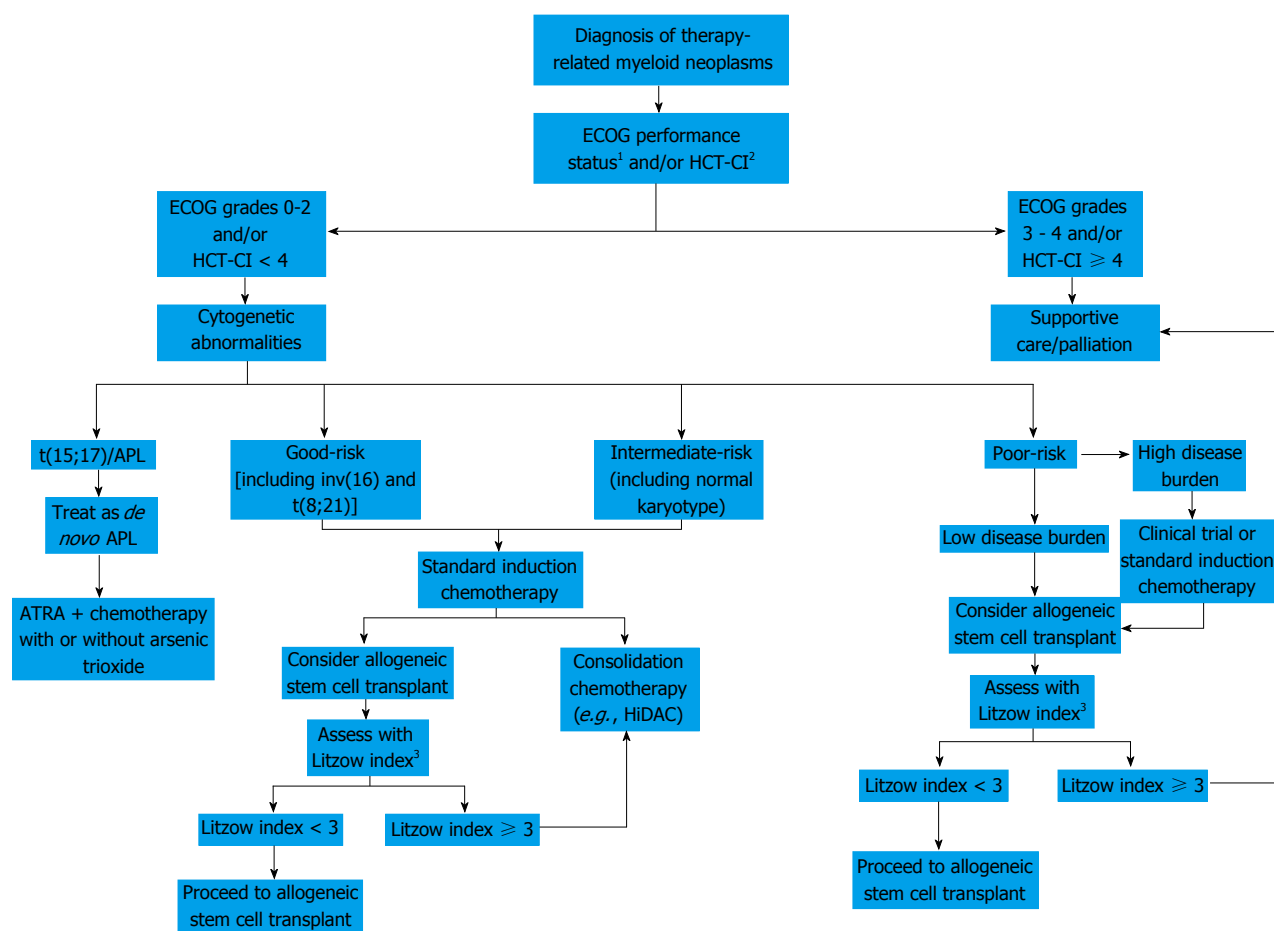


Figure 1 Algorithmic approach to patients with therapy-related myeloid neoplasms. ¹Oken *et al.*^[109]; ²Sorror^[110]; ³Litzow *et al.*^[68]. ECOG: Eastern Cooperative Oncology Group; APL: Acute promyelocytic leukemia; ATRA: All-trans retinoic acid; HiDAC: High-dose cytarabine; HCT-CI: Hematopoietic cell transplant-co-morbidity index.

asures for early detection of this disorder would allow for timely and pre-emptive treatment approaches, such as reduced intensity conditioning allo-HSCT. This approach would yield substantial advantages as opposed to waiting for the development of overt t-MDS/AML, when disease burden is higher and requires more intensive therapy which can have its own risks of morbidity and mortality^[28]. In this section we will outline some methods for prediction and/or early detection of t-MDS/AML in patients at risk.

Metaphase cytogenetics and karyotyping analyze actively dividing cells, though the number of cells analyzed is limited (20-30 cells)^[44]. It is worthy of note that patients developing t-MDS/AML, for example after auto-HSCT, may not show karyotypic abnormalities before the procedure. Conventional cytogenetics may lack sufficient sensitivity and specificity to efficiently recognize patients with increased predisposition to t-MDS/AML^[16,44].

Interphase fluorescence *in situ* hybridization (FISH) offers several advantages over conventional cytogenetics, mainly the lack of need for cells to be actively dividing and the ability to analyze a greater number of cells (several hundreds)^[44]. FISH is also able to detect abnormal clones prior to auto-HSCT. For example, in one report, FISH was

able to detect clonal abnormalities in 9 out of 12 patients (75%) who later developed t-MDS/AML after auto-HSCT^[97]. In another study, FISH identified abnormal cell clones in 20 out of 20 patients who went on to develop t-MDS/AML^[98]. Identification of clonal abnormalities in a high percentage of cells may indicate proliferative and survival advantages and foreshadows development of t-MDS/AML^[44]. However, the locus specificity of FISH requires prior selection of multiple markers for adequate analysis and its labor- and time-intensive methodology are notable limitations^[44].

Loss of heterozygosity (LOH) employs a polymerase chain reaction (PCR) analysis of a selected sample to detect loss of one allele at a specific locus and large chromosomal deletions. This technique is also labor- and time-intensive and is a population-based assay that requires prior selection of loci to be analyzed. In addition, its sensitivity is poor, unable to detect less than 20% cells for LOH of a selected locus^[44]. Nevertheless, it may have impressive specificity, as a positive result suggests an abnormal cell clone. Thus, LOH may prove to be a viable "rule-in" test in this context and may be followed by more sensitive techniques, such as high-throughput analysis and next-generation sequencing (NGS)^[44,99]. However, prospective studies with large numbers of

patient samples are needed to ascertain its validity as a predictor of t-MDS/AML.

Clonality assay based on X chromosome-inactivation at the human androgen receptor gene is another useful method. This is a PCR-based technique that does not require information about loci prior to analysis and detects abnormal clones with survival/proliferative advantage over normal polyclonal cells^[44]. In a single center study by Mach-Pascual *et al.*^[100], monoclonal hematopoiesis, as indicated by X-inactivation-based clonality at the human androgen receptor locus, prior to auto-HSCT was predictive of the development of t-MDS/AML. Four out of 10 patients (40%) demonstrating monoclonal hematopoiesis before transplant subsequently developed t-MDS/AML vs only 2 out of 53 patients with polyclonal hematopoiesis ($P = 0.004$)^[100]. However, this method is limited by the need for high numbers of monoclonal cells to be present for diagnosis (low sensitivity) and its applicability only to female patients^[44]. Altered gene expression in CD34⁺ progenitors may also be used. A large study by Li *et al.*^[101] showed that a 38-gene panel analyzing gene expression in peripheral blood CD34⁺ progenitors showed remarkable ability to distinguish patients who would eventually develop t-MDS/AML from those who would not develop the complication after auto-HSCT. The implication of this study is that development of t-MDS/AML requires the acquisition of mutations in multiple genes as opposed to just one gene^[44]. Additionally, due to different kinds and combinations of mutations, patients with this disorder show significant heterogeneity with multiple subtypes. Therefore, characterization of single gene mutations may not have a satisfactory predictive value in identifying patients prone to developing t-MDS/AML^[12,28,44].

Significant advances have happened for identification of unique biomarkers associated with leukemias which is mainly driven by gene expression analysis and NGS, which have the potential to significantly improve the diagnostic and prognostic criteria. The utilization of a signature NGS panel for each disease (*e.g.*, AML, ALL, MDS, *etc.*) is increasing worldwide^[102,103]. In t-MDS/AML, the impact of NGS panel on long term outcomes are awaited. What we do know is some of clonal mutations with known association with leukemogenesis, *i.e.*, *TET2*, *DNMT3A*, and *ASXL1*^[104,105], if found in a patient who is at risk of t-MDS/AML may predict a high likelihood of developing t-MDS/AML. Caution must be exercised with such an approach, as some cases of t-MDS/AML may have germline mutations in cancer susceptibility genes^[106], thus a careful family history to discover cancer susceptibility is warranted in at-risk patients.

In summary, when a bone marrow biopsy is being obtained for work up for cytopenias in an at-risk patient (*e.g.*, cancer survivor who received chemotherapy or radiation), obtaining an NGS panel specific for MDS and AML should be considered.

RISK REDUCTION STRATEGIES

Based on our knowledge of the risk factors and patho-

genesis of t-MDS/AML, development of risk reduction strategies is a certain possibility. Standardized screening tests, including but not limited to the ones discussed in the previous section, may help identify patients at substantial risk. Accordingly, alterations of chemotherapeutic regimens and treatment modalities may be made under a risk-adapted model, thereby minimizing the risk of t-MDS/AML while providing adequate treatment to the underlying malignancy^[12].

In the context of high-dose chemotherapy and auto-HSCT, modifications can be made to stem cell mobilization and harvesting and pre-transplant conditioning regimens, circumventing the use of alkylating agents, topoisomerase inhibitors and radiotherapy, to eliminate as many risk factors as possible. Specific FISH loci, such as 5q-, 7q-, +8, -11 and 20q-, may be screened preemptively to predict outcomes when any specific abnormalities in blood work are being worked up^[44]. Alternatively, if the risk of t-MDS/AML is substantial (for example, in the case of hematologic malignancies evidence of cytogenetic or FISH abnormalities prior to transplant and high risk disease), these patients can be offered other therapeutic options, such as pre-emptive work up for allo-HSCT (HLA typing) and non-transplant modalities (emerging novel therapies and targeted agents).

CONCLUSION

There is much needed effort for further exploration and validation of biomarkers specifically for t-MDS/AML to develop a viable risk assessment tool for this subgroup of patients. When it comes to cancer survivorship, we urge the current professional societies, *e.g.*, National Comprehensive Cancer Network, American Society of Clinical Oncology, and European Society for Medical Oncology to consider screening the at-risk population of cancer survivors for t-MDS/AML, at least with a complete blood count with peripheral smear annually, which is a relatively simple and economically feasible option for screening for t-MDS/AML.

Lastly, most of the large randomized studies in the arena of AML and MDS have traditionally excluded t-MDS/AML and thus prospective phase III data for t-MDS/AML with regards to outcomes is absent. It is imperative that prospective clinical trials be conducted specifically for t-MDS/AML to delineate optimum treatment options. The cancer community has accomplished a lot in the past five decades in alleviating the burden of cancer by improvements in both radiation and chemotherapy fields, and current efforts on personalized or individualized medicine are looking very promising for further improvements in decreasing cancer mortality. However, as the cancer survivors are living longer^[107,108], the incidence of t-MDS/AML continues to increase and currently is one of the fastest growing cancers worldwide. Efforts must be made by clinicians and researchers globally for establishment of risk reduction strategies for this fatal cancer.

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Targeting stem cells by radiation: From the biological angle to clinical aspects

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Abstract

Radiotherapy is a cornerstone of anticancer treatment. However in spite of technical evolutions, important rates of failure and of toxicity are still reported. Although numerous pre-clinical data have been published, we address the subject of radiotherapy-stem cells interaction from the clinical efficacy and toxicity perspective. On one side, cancer stem cells (CSCs) have been recently evidenced in most of solid tumor primary locations and are thought to drive radio-resistance phenomena. It is particularly suggested in glioblastoma, where CSCs were showed to be housed in the subventricular zone (SVZ). In recent retrospective studies, the radiation dose to SVZ was identified as an independent factor significantly influencing overall survival. On the other side, healthy tissue stem cells radio-destruction has been recently suggested to cause two of the most quality of life-impacting side effects of radiotherapy, namely memory disorders after brain radiotherapy, and xerostomia after head and neck radiotherapy. Recent publications studying the impact of a radiation dose decrease on healthy brain and salivary stem cells niches suggested significantly reduced long term toxicities. Stem cells comprehension should be a high priority for radiation oncologists, as this particular cell population seems able to widely modulate the efficacy/toxicity ratio of radiotherapy in real life patients.

Key words: Cancer; Neoplastic stem cells; Radiation therapy; Efficacy; Toxicity

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Core tip: Radiotherapy is a cornerstone of anticancer treatments. However, significant levels of toxicity and recurrences are still reported. On the one hand, cancer stem cells have been recently suggested to be the root of radio-resistance, with strong pre-clinical rational. On the other hand, convincing pre-clinical data suggesting the importance of healthy tissue stem cells radiation-induced destruction in long term side effects of radiotherapy surfaced. This article provides an overview of the available literature analyzing from the clinical efficacy and toxicity perspective the interactions between stem cells and radiation. Significant improvement of radiotherapy toxicity/efficacy ratio is suggested.

Vallard A, Espenel S, Guy JB, Diao P, Xia Y, El Meddeb Hamrouni A, Ben Mrad M, Falk AT, Rodriguez-Lafrasse C, Rancoule C, Magné N. Targeting stem cells by radiation: From the biological angle to clinical aspects. *World J Stem Cells* 2016; 8(8): 243-250 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v8/i8/243.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v8.i8.243>

INTRODUCTION

Radiotherapy is a cornerstone of anticancer treatments, since it proved efficacy in various primary tumor location when performed with intent to cure^[1-4]. It also proved to be efficient for palliation of bone^[5] and brain metastases^[6], whatever histologic diagnosis. However, significant rates of failure and of radiation-induced toxicities are still reported in spite of recent technological improvements^[1-6]. Radiation resistance seems mainly caused by biological phenomenon driven by cancer stem cells (CSCs)^[7]. CSCs have been evidenced in the mid-90s in hematological tumors, but their presence has been proved recently in most of solid tumors (glioblastoma, prostate, breast, rectum, colon, and head and neck cancers)^[7]. The CSC is defined by three main characteristics: It can initiate tumorigenesis and endlessly proliferate, it can self-renew, and it can give birth to a high number of progenitor parental cells (Figure 1). Although CSCs account for a very small number of cells considering the whole pool of tumor cells, they are thought to play a leading role in radiation resistance. Pre-clinical data showed that CSCs were able to redirect their cell cycle toward a radiation resistant state (the S-G0 phase), had a considerable capacity of tumor re-population, were not dependant of oxygen, and above it all - possessed hyperactive DNA repair processes^[8]. Besides, CSCs seem highly gifted for invasion and migration^[9] making them the supposed - main responsible for local and metastatic post-radiotherapy recurrences. Targeting CSCs in order to increase the therapeutic index (efficacy/toxicity ratio) of radiotherapy is a very promising way of research^[10]. But from another angle, it might lead to

concurrently kill stem cells located in the surrounding healthy tissues, and induce serious radiation-caused toxicities. Ideally, radiotherapy should simultaneously destroy CSCs and spare normal tissue stem cells. Several research approaches actually tried to reach this goal with recent publications regarding the CSC pharmacological targeting^[10], the CSC dosimetric targeting, and the healthy tissue stem cells sparing. Interesting potential pharmacological targets have been recently suggested: Wnt/ β -caderines pathway inhibitors are currently under clinical investigation^[11], with the strong pre-clinical rational that Wnt/ β -caderines ex-pression is directly related with radiation-resistance^[12], de-differentiation, adhesion, and invasion^[13]. Notch-1 (involved in CSC repopulation^[14], proliferation and radiation-induced apoptosis resistance^[15]), SHH (involved in metastases^[16], CSC proliferation, survival, morphogenesis and radioresistance^[17]), JAK/STAT (involved in CSC de-differentiation, apoptosis resistance, and proliferation^[18]) and PI-3 kinase/Akt (involved in CSC survival after radiation^[19]) are pharmacological targets of interest, with inhibitors that are currently tested in pre-clinical studies. Hypoxia is also a major topic of interest, since CSCs are thought to be located in hypoxic niches. In pre-clinical studies, decreasing CSC hypoxia resulted in reduced CSCs self-renewing and multiplication^[20,21]. The pharmacological targeting of tumor and vascular stroma (using PDGF inhibitors) seems therefore promising, with the *in vitro* radio-sensitisation of CSCs that were initially radio-resistant^[22]. Contrary to pharmacological targeting, the CSC "dosimetric targeting" (*i.e.*, directly targeting stem cells by radiation) is still at its early stages. However, most of the publications consist in clinical studies with already promising outcomes. The sparing of organs at risk stem cells is also a hot topic, since healthy tissue stem cell death was suggested to be directly related to side effects widely impacting patients' quality of life, occurring after both curative and palliative radiotherapy. The present article's objective is to address the radiotherapy/stem cells topic from the clinical efficacy and perspective.

TARGETING CSC WITH RADIATION: EFFICACY DATA

Clinical outcomes: The glioblastoma model

Glioblastoma is a major model of radioresistance since in spite of a multi modal approach (ideally combining surgery, radiotherapy and chemotherapy), the median overall survival time only reaches 12-15 mo, with most of the recurrences located in the radiation fields. The underlying phenomena leading glioblastoma to radioresistance are still misunderstood but it was suggested in animal pre-clinical models that the genesis of glioblastoma was linked to a loss of tumor suppressor gene in neural stem cells (NSCs)^[23]. NSCs were shown to be physiologically housed in the subventricular zone (SVZ), an area surrounding the lateral ventricles^[24-27]. Therefore, delivering high doses of radiation to niches of "healthy tissue" (*i.e.*, the SVZ) possibly harboring glioblastoma CSCs might allow to

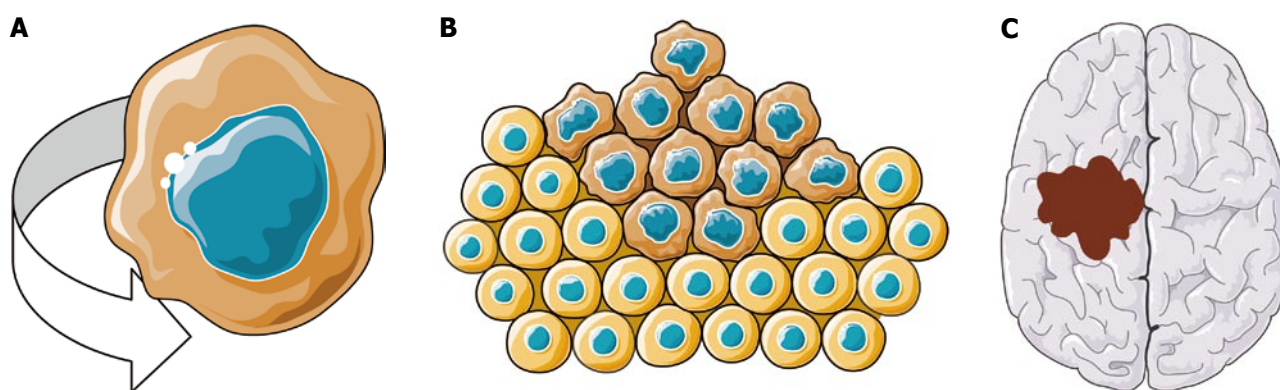


Figure 1 Cancer stem cells main biological characteristics. A: Self-renewal/ endless proliferation; B: Giving birth to a high number of progenitor parental cells; C: Tumorigenesis initiation.

overcome its radioresistance. This hypothesis was tested in 2010 by Evers *et al.*^[28]. Data of 55 patients treated for a glioblastoma between 2003 and 2009 in California, United States, were retrospectively reviewed. Dosimetric data of radiotherapy were analyzed in order to estimate the dose delivered to the supposed CSC niches (*i.e.*, the SVZ), and correlate it with patient global outcomes. Only patients with histopathologically diagnosed anaplastic glioma (grade 3) or glioblastoma (grade 4), with at least 1 mo of follow-up, and who completed the whole planned radiotherapy were included. SVZ was defined based on previous publications, and doses to the volumes of interest could be a posteriori calculated. The authors estimated that the median dose received by the bilateral SVZ was 43 Gy. They then divided the population into a "low dose group" (receiving less than the median dose, $n = 27$) and a "high dose group" (receiving more than the median dose, $n = 28$). The two groups were well balanced on all essential prognosis factors (RPA classification, age, Karnofsky performance scale), but one. Complete resection was less achieved in the "high dose" group ($n = 6$, 21%) than in the "low dose" group ($n = 16$, 59%). The mean dose received by bilateral SVZ was $50 \text{ Gy} \pm 2 \text{ Gy}$ for the "high dose group" and $27 \text{ Gy} \pm 5 \text{ Gy}$ for the "low dose group". The median progression free survival (PFS, defined as the time between radiotherapy completion and glioblastoma recurrence) was 15 mo for the "high dose group" and 7.2 mo for the "low dose group". This difference was statistically significant ($P = 0.03$). Hazard ratio concerning glioblastoma progression was significantly decreased for the "high dose" group (HR = 0.74, 95%CI: 0.567-0.951, $P = 0.0019$). All other statistical analyses comparing important characteristics could not evidence significant differences, particularly regarding the total dose ($P = 0.83$), highlighting the high degree of glioblastoma's radioresistance due to CSCs^[29]. No correlation was shown between the total dose and dose to SVZ, since SVZ was most of the time outside of the clinical target volume. Therefore, doses per fraction on the SVZ were limited (1.36 Gy CI: 1.2-1.5). The fact that low doses of radiation could result in an increased radio-sensitivity

has already been described in glioblastoma^[30,31], but not in CSCs^[32]. The underlying biological phenomenon is hypothesized to be the non-detection of DNA damages in case of small doses per fractions, while the CSC radio-resistance is supposedly linked with the over-expression of DNA damage checkpoints^[33]. However, CSC high sensitivity to low doses must be studied in prospective clinical studies. Interestingly, when statistical analyses were performed regarding the doses received by the ipsilateral periventricular zone only, no significant difference could be evidenced. Linked with the observation that glioblastoma cells can widely migrate within the healthy brain tissue, causing frequent contralateral recurrences^[34], it was hypothesized that ipsilateral CSCs could take shelter in contralateral CSC niches. Targeting radio-resistant CSC might therefore be more efficient if all the possible CSC harbors are damaged, but this hypothesis is still to be demonstrated. In 2012, Gupta *et al.*^[35] published outcomes of 40 glioblastoma patients treated between 2008 and 2010 at the Tata Memorial Centre, India. All patients were treated for histologically proven glioblastoma using standard treatment. Dosimetric data were retrospectively reviewed, and doses to SVZ were a posteriori calculated and linked with global outcomes. Median dose to bilateral SVZ was 56.2 Gy, and patients were divided as previously described into a "high dose group" ($n = 20$, mean dose to ipsilateral, contralateral and bilateral SVZ of 60.1 Gy, 59.9 Gy and 60 Gy respectively) and a "low dose group" ($n = 20$, mean dose to ipsilateral, contralateral and bilateral SVZ of 57.5 Gy, 47.4 and 52.5 Gy respectively). Most of known prognosis factors were unfavorably distributed in the "high dose group" vs "low dose group": Patients were older (55 yo vs 46 yo), with higher RPA class (85% of class IV-V vs 55%), with less frequent extensive resection (50% vs 70%), and with more frequent MGMT methylation (55% vs 40%). At a median follow-up of 15 mo, 25 out of the 40 patients experienced progression, with 21 deaths. Age and RPA class (well known prognosis factors) were significantly linked with survival in univariate analysis, as well as the dose to contralateral SVZ ($P = 0.05$). A Kaplan-meyer

analysis showed significantly increased overall survival ($P = 0.05$) and progression free survival ($P = 0.02$) for patients with the highest doses to contralateral SVZ. In multivariate analysis, RPA class, Karnofsky performance status and dose to ipsilateral SVZ were identified as independent prognosis factors of overall survival (HR = 0.87, 95%CI: 0.77-0.98, $P = 0.025$). These results corroborate the efficacy of targeting CSCs by radiation in glioblastoma. However, the ideal target (ipsilateral or contralateral SVZ) and the dose threshold (43 Gy? 50 Gy?) are still to be clarified. The brain model is certainly one of the most interesting models for the CSC dosimetric targeting: Due to its anatomical conception, CSC niches are distinct from differentiated cells, making the result of a precisely delivered radiotherapy easier to interpret.

Properly imaging CSC through hypoxia: A necessary condition for an efficient radiotherapy?

These two publications also reflect the need for reliable imaging of CSC niches. The recent development of spectroscopy (identifying the specific metabolic profile of glioblastoma CSCs) is certainly a very promising technique that could allow a precise dosimetric targeting of CSCs in the future^[36,37]. Out of the glioblastoma model, the CSC imaging systems are mainly based on hypoxia^[38]. Hypoxia is thought to be a cornerstone of radiation resistance since it was clearly proven that the biological effects of conventional radiotherapy (*i.e.*, the DNA damages caused by chain oxidation) are potentiated by oxygen. In case of hypoxia, the efficacy of radiotherapy is *de facto* significantly reduced. It also seems clear that tumor hypoxic niches harbor CSCs (in glioblastoma but also in other solid tumors^[39]) and therefore represent a target of interest for radiotherapy: The most radioresistant cells are housed in a micro-environment enhancing radioresistance. Imaging the hypoxic niches and targeting them by radiation might be the key to overcome cancers radioresistance since higher doses could induce the destruction and the re-oxygenation of these niches, initiating a virtuous cycle. The challenge of properly imaging hypoxia is still ongoing. Efficient nitroimidazole-based tracers were developed during the past 30 years, based on the fact that hypoxia induces a transformation of nitro-imidazole intermediates into alkylating agents that bind to cell component^[40]. These elements could be then coupled with positron emitting radionuclides (^{18}F , ^{64}Cu , ^{60}Cu) in order to be detected by positron emission tomography (PET) imaging devices. (^{18}F) Fluoromisonidazole and (^{18}F) 1-(5-fluoro-5-deoxy- α -Darabinofuranosyl)-2-nitroimidazole were validated (regarding specificity) by invasive gold standard methods and can be now clinically used. However, sensitivity is still limited due to low tumor-to-plasma ratios and poor spatial resolution of PET imaging systems^[38]. Techniques based on magnetic resonance imaging (MRI) have been developed, resolving the issue of spatial resolution (Blood oxygen dependent MRI imaging, Mapping of

Oxygen by Imaging Lipid Relaxation Enhancement, and Dynamic-Contrast-Enhanced MRI), but sensitivity issues remained^[38]. Moreover, recent data suggested that CSC were not necessarily located in the most hypoxic areas^[41], making multi-modal imaging methods absolutely needed (coupled PET-MRI, or imaging techniques detecting CSC surface marker). In this field, nanoparticles are very promising theragnostic tools, since they can be used both as MRI contrast agents, and as radiotherapy targets^[42,43]. Finally, the ideal solution might be a radiotherapy technique capable of destroying as well CSC as differentiated cancer cells. Hadrontherapy (carbon or proton-based radiotherapy) seems to fulfill these criteriae, showing *in vitro* the ability to kill with the same efficacy CSCs and conventional cancer cells, thanks to the absence of oxygen effect^[44]. However, the high cost of this technique might be a clear drawback to its routine application. Moreover, radio-resistance phenomena have been very recently described *in vitro* and need to be fully investigated to evaluate their possible clinical impact^[45].

SPARING NORMAL STEM CELLS DURING RADIOTHERAPY: TOXICITY DATA

Clinical outcomes: The whole brain radiotherapy model

Memory disorders are a well known long term side effect of whole brain radiotherapy (WBRT), performed in case of multiple brain metastases. Radio-damaged neural stem cells (NSCs) located in the subgranular zone of the hippocampal dentate gyrus^[46] have been hypothesized to cause the reported cognitive decline following WBRT^[47]. Thanks to the development of the intensity modulated radiotherapy (IMRT), Gondi *et al.*^[48] showed the feasibility of a WBRT avoiding (*i.e.*, reducing the delivered dose of $\geq 80\%$ to) the hippocampal NSC niches, without impairing the quality of coverage of the remaining brain. IMRT offers the possibility to spare areas that could not be spared with conventional radiotherapy indeed, thanks to highly conformal dose painting (Figure 2). Gondi *et al.*^[49] published in 2014 the outcomes of an international single-arm phase II trial, comparing the results of a WBRT sparing hippocampal NSCs with the results of a 2003 phase III trial using conventional WBRT for brain metastasis. Patients treated using WBRT for solid tumor brain metastasis were assessed for standardized cognitive assessments [Hopkins Verbal Learning Test-Revised Delayed Recall (HVLTR-DR)] at baseline, 2-, 4- and 6-mo follow-up, with a primary endpoint being the HVLTR-DR at 4 mo. At 4 mo, the mean relative decline in HVLTR-DR score from baseline was of 30% in the 2003 control trial. In the experimental trial, hippocampal NSC niches definition was standardized and based on MRI fusion with planning computed tomography-scan. Standard (and similar to the control trial) fractionation scheme was delivered, with 30 Gy in 10 fractions. Doses were limited to 9 Gy to the entire hippocampus, with a maximum focal dose of 16 Gy. Between 2011 and 2012, 113 patients were included, with 42 patients being

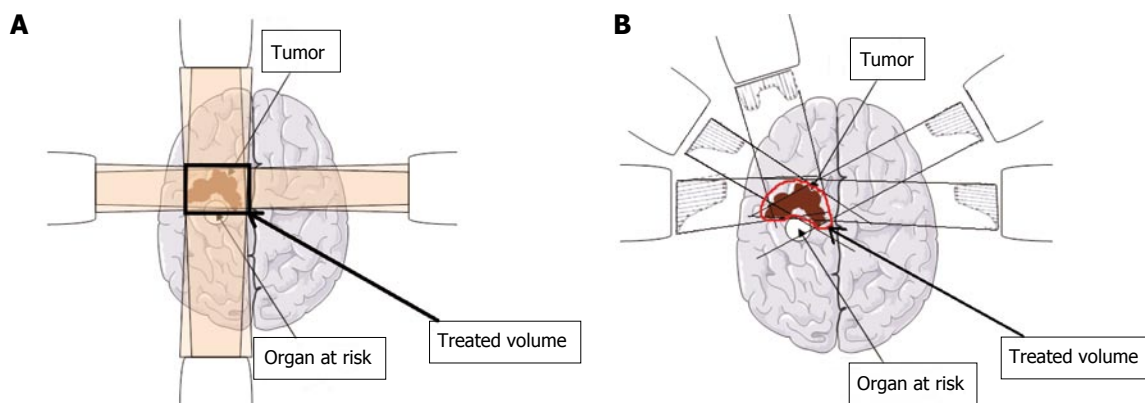


Figure 2 Three dimensional conventional radiotherapy vs intensity modulated radiotherapy. A: Three dimensional conventional radiotherapy; B: Intensity modulated radiotherapy.

analyzable for the primary endpoint. At 4 mo, the mean relative HVL-R DR impairment was significantly lower in the population who experienced the hippocampal NSC protection compared to the population who did not (7% vs 30%, $P < 0.001$). Interestingly, if most of patients experienced intracranial progression, with a mean overall survival of 6.8 mo, only 4.5% of patients developed intra-hippocampal progression. The authors concluded that the avoidance of hippocampal NSC was significantly related to memory preservation, bringing a direct clinical evidence that hippocampal NSC niche was implicated in the pathophysiology of radiotherapy-induced memory decline. Of course, the main limitation of this article is the absence of direct control group, but phase III trials have been approved and will clarify the place and efficacy of NSC avoidance during WBRT.

Clinical outcomes: The head and neck radiotherapy model

Xerostomia is one of the most quality of life-impacting late side effects of head and neck radiotherapy. Oral dryness frequently ruins patients' everyday life inducing ulcerations, speech, taste and swallowing difficulties. Even with modern radiotherapy techniques minimizing mean dose to salivary glands, important rates of mucosal complications (15% to 40% of treated patients) are still reported^[50,51]. It was clearly demonstrated that the xerostomia was linked with the irradiation of salivary glands, because of the high radiosensitivity of stem cells niches located in the salivary glands^[50,52,53]. Xerostomia seemed to be proportionally linked with the dose delivered to salivary gland stem cells niches, determining the quantity of post radiotherapy viable salivary stem cells^[52,54]. However, the clinically relevant threshold dose of radiotherapy damaging stem cells is still undetermined and only techniques delivering doses as low as reasonably achievable to parotid stem cells-rich regions were tested. Moreover, the exact location of these areas is still debated, the strongest hypothesis being they could be located in the larger excretory ducts^[55]. Based on animal models, van Luijk *et al.*^[56] suggested that the centre of the parotid (containing the major ducts) was certainly

rich in stem cells, since its restricted irradiation led to long term saliva production collapse. This hypothesis was recently tested in humans^[55]. Salivary and dosimetric data of 74 patients treated for a head and neck cancer without salivary gland involvement were retrospectively reviewed. Spatial dose distribution inside the parotid could be correlated to salivary flows 1 year after radiotherapy completion (with a dose-dependent effect relationship), defining a stem cell region located near the dorsal edge of the mandible, at the occurrence of the first branching of Stensen's duct, in concordance with animal stem cells locations. Doses delivered to this area were more predictive of salivary flow than (routinely used) parotid mean dose. Moreover, after radiotherapy, only cells provided by biopsies of these zones could be grown *in vitro*. A feasibility study was performed in 22 patients, showing that the preservation of the parotid stem cell niche seemed feasible with IMRT, even in case of impossible avoidance of the whole parotid. Other areas of parotid have been suggested to house stem cells capable of salivary long-term regeneration. It was suggested in one retrospective cohort derived from an important phase III study that sparing the superficial lobe of the two parotid glands could induce a better salivary preservation than complete contralateral parotid gland sparing^[57]. These data need to be validated in larger patient cohorts, but might be a significant progress in order to limit radiation-induced xerostomia. The main limitation of these articles (out of their retrospective nature) is that the link between salivary flow and xerostomia is still unclear: The major salivary glands (parotid glands, submandibular glands and sublingual glands) produce 90% of saliva, but minor salivary glands (thousands of small glands located in the oral cavity) secrete the major quantity of mucin, the saliva lubricating agent. Mucin is also secreted for a small account by submandibular glands and sublingual glands. Therefore, only shielding parotids stem cells might insufficient to guarantee the restoration of good quality saliva after radiotherapy. Pre-clinical and clinical data are certainly needed concerning the radio-sensitivity and the location of stem cells in the submandibular and minor salivary glands. Currently, no reliable biological or

imaging markers have been validated to precisely locate salivary stem cells, making progresses difficult to be made.

CONCLUSION

If the interaction between radiotherapy and CSCs is an en vogue topic^[58], targeting CSC by radiation is at its early stage of development. Combining radiotherapy with biological drugs targeting CSC could be an efficient mean to overcome local and metastatic recurrences, with various agents that are currently tested based on solid pre-clinical rationales^[59]. But directly targeting CSC using radiation is also a promising anticancer therapy with already interesting clinical results. The evolution of modern techniques of radiotherapy might widely depend of the imaging progresses in term of sensitivity. In order to increase the therapeutic index of radiotherapy, sparing stem cells of healthy tissue is also a major topic of interest since significant improvements regarding quality of life-impacting side effects following radiotherapy can be achieved. More than ever, prospective trials with solid methodologies are needed to confirm or infirm the suggested trends. Finally, both cancer and normal tissue stem cells seem to be central elements modulating the toxicity and the efficacy of radiotherapy. A better comprehension of stem cells location and their intrinsic radio-sensitivity is crucial, and permanent return trips between pre-clinical and clinical data are mandatory.

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Reprogramming of germ cells into pluripotency

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Abstract

Primordial germ cells (PGCs) are precursors of all gametes, and represent the founder cells of the germ-line. Although developmental potency is restricted to germ-lineage cells, PGCs can be reprogrammed into a pluripotent state. Specifically, PGCs give rise to germ cell tumors, such as testicular teratomas, *in vivo*, and to pluripotent stem cells known as embryonic germ cells *in vitro*. In this review, we highlight the current knowledge on signaling pathways, transcriptional controls, and post-transcriptional controls that govern germ cell differentiation and de-differentiation. These regulatory processes are common in the reprogramming of germ cells and somatic cells, and play a role in the pathogenesis of human germ cell tumors.

Key words: Primordial germ cell; Embryonic germ cell; Germ cell tumor; Reprogramming; Induced pluripotent stem cell; Small molecule compound; Gene; Signal; Transcription factor

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Core tip: Primordial germ cells can be reprogrammed into pluripotent stem cells called as embryonic germ cells *in vitro* and into pluripotent germ cell tumors *in vivo*. Germ cell reprogramming can be regulated by signaling pathways, including PI3K/Akt signaling, mitogen-activated protein kinase signaling, transforming growth factor- β signaling, RA signaling. These mechanisms are also involved in somatic cell reprogramming, indicating that there exist common regulatory networks regulating germ and somatic cell reprogramming. On the other hand, regulators for germ cell development prevent germ cell dedifferentiation in unique manners.

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INTRODUCTION

The germ lineage is a privileged cell lineage that transmits genetic and epigenetic information from generation to generation^[1]. Primordial germ cells (PGCs) are embryonic germ cell (EGC) precursors that eventually differentiate into sperm or oocytes^[2,3]. In mice, a population of proximal epiblast cells in egg cylinder-stage embryos is committed to PGC precursors at embryonic day 6.25 (E6.25). During gastrulation, PGC precursors migrate out of embryos into the extraembryonic region, where a small number of nascent PGCs emerge at E7.0. PGCs return to embryos at E7.75, migrate through the hindgut and dorsal mesentery, and finally colonize the genital ridges until E11.5. PGCs actively proliferate and increase in number from E7.0 to E13.5, being transiently arrested in the G2/M phase at E8.5. In the gonads, PGCs undergo sex-dependent differentiation under the influence of somatic cells. Male germ cells enter into mitotic arrest after E13.5 and retain mitotic quiescence during embryogenesis. After birth, male germline stem cells (GSCs) called spermatogonia resume proliferation and produce sperm *via* meiosis and sperm morphogenesis (spermiogenesis). In contrast, female germ cells enter into meiosis at E13.5, and oocytes mature and are ovulated after birth.

Although totipotency is restored after fertilization, germ-lineage cells differentiate into only sperm or oocytes, but never into somatic cell types, during normal development. However, PGCs can be reprogrammed into pluripotency or can de-differentiate under experimental and pathological conditions as described below. In this review, we present an overview of the molecular mechanisms underlying germ cell preprogramming and germ cell tumor pathology, and discuss the features shared by germ cell and somatic cell reprogramming.

DIFFERENTIATION AND DE-DIFFERENTIATION OF PGCS

PGC differentiation

A number of events take place during PGC specification^[2,3]. These include transcriptional activation of germ cell-specific genes [Stella and Deadend-1 (*Dnd1*)], reactivation of pluripotency-related genes (*Sox2* and *Nanog*), and repression of the somatic cell differentiation program. Epigenetic reprogramming occurs concomitantly. DNA methylation is globally erased through two waves by passive and active demethylation mechanisms, and unique genome-wide histone modification patterns are established (acquisition of H3K27me3 and loss of H3K9me2).

Three transcription factors, Blimp1 (*Prdm1*), *Prdm14*, and *Tfap2c* (*AP2γ*), play central roles in the specification of PGCs from the epiblast. Blimp1 expression commences in PGC precursors, the most proximal layer of the

epiblast, at E6.25^[4]. Expression of *Prdm14* follows soon after the onset of *Blimp1* expression in the precursors^[5]. *Tfap2c* may be a downstream target of Blimp1^[6]. In mice lacking these transcription factors, PGC precursors and nascent PGCs have abnormal gene expression patterns and epigenetic status. Gene expression analysis has revealed that Blimp1 represses somatic cell gene expression and *Prdm14* activates germline and pluripotency genes^[5,7]. Additionally, forced expression of these three transcription factors sufficiently promotes the differentiation of PGC-like cells from embryonic stem cells (ESCs) in culture^[8,9].

PGC specification is regulated by interactions with surrounding somatic-lineage cells. Bone morphogenetic protein 4 (BMP4) is secreted from extraembryonic ectoderm, and is critical for the induction of PGC precursors and mesodermal cells from the epiblast *in vivo*^[10]. Furthermore, treatment of epiblast explants with BMP4 activates the expression of *Blimp1* and *Prdm14* and induces the formation of PGC-like cells in culture^[11], which suggests that BMP4 is an upstream regulator of *Blimp1* and *Prdm14*. Other BMP family proteins, BMP8b and BMP2 (which are secreted from extraembryonic ectoderm and visceral endoderm, respectively), may support PGC specification along with BMP4^[11-14]. Wnt3a is also essential for the specification of PGCs and mesodermal cells. Since epiblast explants isolated from *Wnt3a*-deficient mice do not generate PGC-like cells in response to BMP4^[11], Wnt3 seems to enable epiblast to respond to BMP4. Finally, the suppression of mitogen-activated protein kinase (MAPK) signaling is critical for the induction of PGC-like cells in the lineage choice between germ and mesodermal cells^[15].

Testicular teratomas

Germ cell tumors are classified into two groups: Germi-nomas (seminomas) and non-germinomatous tumors^[16,17]. Testicular teratomas belong to the latter group, and contain a variety of differentiated cells and tissue structures, which belong to the ectoderm, endoderm, and mesoderm lineages. Undifferentiated cells called embryonal carcinoma cells (ECCs) are also found in testicular teratomas^[18]. ECC lines can be established from teratomas and maintained indefinitely in culture. However, these cell lines are usually multipotent rather than pluripotent because the cells differentiate into a limited number of cell types *in vitro* and *in vivo*. Teratomas often occur outside of the testis. Non-germinomatous germ cell tumors include yolk sac tumors and choriocarcinomas.

The etiology of testicular teratomas has been extensively studied using the 129/Sv inbred mouse strain, which frequently develops juvenile testicular teratomas^[18]. Early teratomatous foci can be detected in E15.5 testes. Seminiferous tubule structures are disorganized, and teratomatous cells are found outside of the tubules thereafter. The foci contain a number of mitotically active cells, suggesting that these cells have failed to enter into mitotic arrest.

Teratoma onset is considered to be at around E12.5

in 129/Sv mice based on two lines of evidence. First, investigation of the sizes of the spontaneous tumors at various embryonic ages has indicated that tumor onset occurs at E12.5^[18]. Secondly, when E12.5 gonads of 129/Sv mice were transplanted into the testes of adult 129/Sv mice, about 80% of the grafts developed into teratomas; conversely, the incidence of experimental teratomas was dramatically lower when E13.5 gonads were transplanted^[19]. It is noteworthy that testicular teratomas do not develop in other inbred mouse strains both spontaneously and experimentally, suggesting that the genetic background affects the occurrence of teratomas.

The homozygous steel (*Sl*) mutant mouse has been used to show that testicular teratomas originate from germ cells in the gonads^[20]. The *Sl* locus encodes a growth factor Kit ligand (KITLG, also known as stem cell factor), which activates the receptor tyrosine kinase c-Kit. c-Kit is expressed in migratory and gonadal PGCs, and its signaling is required for their proliferation and survival *in vivo*. When E12.5 gonads of 129/Sv mice carrying the homozygous *Sl/Sl* mutation were transplanted, no grafts developed into experimental teratomas, clearly demonstrating that teratomas are derived from PGCs.

EGCs

Studies that searched for PGC growth factors uncovered methods for reprogramming PGCs into pluripotent EGCs *in vitro*^[21,22]. Treatment of PGCs with individual growth factors, such as KITLG, leukemia inhibitory factor (LIF), or basic fibroblast growth factor (bFGF), can promote the proliferation and survival of PGCs in culture. PGCs are responsive to these growth factors for only a few days, and eventually die *via* apoptosis. However, when LIF, KITLG, and bFGF are simultaneously added in culture, PGCs actively proliferate to form ESC-like, dome-shaped colonies (EGC colonies) within 5–7 d. In contrast, PGCs cultured in the presence of KITLG and LIF generate scattered colonies of cells with elongated morphology and do not lead to EGC formation.

After secondary cultures, EGCs can be propagated indefinitely in the presence of LIF, but without KITLG and bFGF^[21]. When transplanted into blastocysts, EGCs can be incorporated into development and contribute to the three germ layers and germline in chimeric mice, indicating that EGCs have pluripotency equivalent to ESCs. However, when PGCs are transplanted into blastocysts immediately after isolation without culture, they never contribute to chimeric mice^[23]. Thus, stimulation with KITLG, LIF, and bFGF can reprogram germline-committed PGCs into pluripotent EGCs. bFGF can be replaced by retinoic acid (RA) or forskolin^[24,25], which increases the intracellular cyclic AMP (cAMP) concentration and leads to the activation of protein kinase A (PKA).

EGC derivation efficiency gradually decreases as germ cell differentiation proceeds. Efficiency is highest in E8.5 migratory PGCs, and sharply declines in E13.5 PGCs^[21]. No EGCs can be derived from germ cells after E15.5^[26]. In contrast to testicular teratomas, EGCs can be

derived not only from 129/Sv mice but also from various other mouse strains. This indicates that PGCs intrinsically have the potential to be reprogrammed, regardless of genetic background, although genetic background has a strong influence on the pathogenesis of testicular teratomas *in vivo*.

PI3K/AKT SIGNALING

PI3K/Akt signaling in germ cell reprogramming

As stimulation with KITLG, LIF, and bFGF is required for the derivation of EGCs, signaling pathways downstream of these growth factors are likely critical for PGC reprogramming. Phosphoinositide-3 kinase (PI3K) is a lipid kinase activated by these growth factors. PI3K produces phosphatidylinositol 3,4,5-triphosphate (PIP3) from phosphatidylinositol 4,5-bisphosphate (PIP2) and transmits signals *via* downstream effector proteins, such as the serine/threonine kinase Akt and the small GTPases Rac1 and Cdc42^[27]. Akt promotes physiological and pathological processes, such as proliferation, survival, metabolism, and tumorigenesis, through the phosphorylation of various target proteins^[28]. On the other hand, the tumor-suppressor gene product phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a lipid phosphatase that converts PIP3 to PIP2 and antagonizes PI3/Akt signaling.

PGC-specific *Pten*-deficient mice develop juvenile testicular teratomas with a high frequency despite their mixed genetic background^[29]. In mutant mice, PGC differentiation appears normal until E13.5, because the expression of germ cell-specific genes such as mouse vasa homolog (Mvh) is activated in mutant PGCs as well as in control PGCs. However, mutant PGCs do not enter into mitotic arrest and a number of PGCs undergo apoptosis after E14.5. Teratomatous foci, which are weakly positive or negative for Mvh, are detected in the E15.5 testes of mutant mice. Additionally, EGC derivation efficiency is much higher in E11.5 PGCs isolated from *Pten* mutant mice than in those from control mice. These findings show that *Pten* is essential for the establishment of the male germ lineage, and suggest that hyperactivation of PI3K reprograms PGCs into pluripotent cells *in vivo* and *in vitro*.

The effects of downstream Akt signaling have been examined using transgenic mice expressing the Akt-Mer fusion protein, which is composed of the myristoylated active form of Akt and mutated ligand-binding domain of estrogen receptor (Mer)^[26,30]. The kinase activity of Akt-Mer can be turned on or off by the addition or withdrawal, respectively, of the Mer ligand, 4-hydroxytamoxifen (4OHT). When E11.5 PGCs from transgenic mice are cultured in the presence of KITLG, LIF, and bFGF, EGC derivation efficiency is greatly enhanced by 4OHT treatment. Furthermore, whereas bFGF is essential for EGC derivation, EGCs can be efficiently derived from transgenic PGCs cultured with 4OHT, KITLG, and LIF but without bFGF, showing that Akt hyperactivation can replace bFGF. Thus, the PI3K/Akt signaling axis plays

pivotal roles in PGC reprogramming.

Male GSCs in the testes of postnatal mice also reportedly de-differentiate into pluripotent cells in culture, albeit much less frequently than do PGCs. For example, it has been shown that GSCs, which are established from neonatal mouse testis, spontaneously generate ESC-like colonies during long-term culture^[31]. These cells are called multipotential GSCs (mGSCs), and show pluripotency equivalent to ESCs and EGCs. Although both PGCs and GSCs are germ-lineage cells, Akt activation does not enhance the emergence of mGSCs from GSCs^[32].

Cellular processes and target molecules in the reprogramming of germ and somatic cells

Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by the introduction of the transcription factors Oct4, Sox2, Klf4, and c-Myc (OSKM)^[33,34]. E-Ras is an ESC-specific small GTPase that activates PI3K. Overexpression of E-Ras and downstream active Akt enhance OSKM-induced iPSC derivation efficiency^[35,36]. In this section, we discuss the cellular processes and target molecules downstream of PI3K/Akt signaling by comparing the germ cell and somatic cell reprogramming systems.

The tumor suppressor Trp53 is a gatekeeper that checks the balance between proliferation and apoptosis^[37]. The amount and activity of Trp53 are regulated transcriptionally and post-transcriptionally by intrinsic and external stimuli that cause DNA damage and oncogenic activation. Mice lacking *Trp53* frequently develop testicular teratomas against the 129/Sv genetic background^[38]. Akt activation in cultured PGCs inhibits nuclear accumulation of Trp53 and the phosphorylation required for maximal transcriptional activation of Trp53^[26], suggesting that Akt inhibits Trp53 activity in PGCs during reprogramming. Furthermore, deletion of *Trp53* not only enhances the derivation efficiency of EGCs in the presence of KITLG, LIF, and bFGF, but also can replace bFGF^[26]. This shows that Trp53 inhibition is a critical event downstream of Akt signaling.

Deletion or knockdown of *Trp53* also greatly enhances iPSC induction^[39]. Whereas OSKM introduction and/or culture conditions induce cell cycle arrest in somatic cells during reprogramming, inhibition of Trp53 suppresses cell cycle arrest, promotes cell proliferation, and eventually leads to a high frequency of iPSC production. Moreover, the cell proliferation rate is well-correlated with reprogramming efficiency in iPSC production^[40], suggesting the existence of proliferation-dependent reprogramming processes. Likewise, PGC reprogramming also seems to be proliferation-dependent as failure of mitotic arrest in both 129/sv mice and *Pten*-deficient mice *in vivo* leads frequent incidence of PGC dedifferentiation^[19,29]. Akt activation enhances proliferation but suppresses apoptosis in cultured PGCs *in vitro*^[26,30]. In addition to inhibiting Trp53, Akt is known to promote proliferation through many other target proteins, such as cyclin D and cyclin-dependent protein kinase inhibitors (CDKIs), p21Cip1, and p27Kip1^[28,41]. In fact,

mutation in *INK4* CDKI promotes incidence of spontaneous testicular teratomas in the absence of *Trp53*^[42]. Cell cycle arrest represents a roadblock for reprogramming that can be overridden by higher proliferative activity both in somatic and germ cells.

Metabolic reprogramming, shifting from oxidative phosphorylation to glycolysis, is required for somatic cell reprogramming toward iPSCs^[43]. Akt signaling promotes glycolysis by phosphorylation of the Foxo family transcription factors^[28,41]. Foxo1 regulates the expression of genes involved in glycogenesis and gluconeogenesis, as well as in proliferation and apoptosis^[44]. Akt inhibits the transcriptional activity of Foxo1 through its exclusion from the nuclei, leading to enhanced glycolysis. In fact, forced expression of the dominant-negative form of Foxo1 enhances the derivation efficiency of iPSCs^[36]. The mechanistic target of rapamycin complex 1 (mTORC1) is another target of Akt that regulates metabolism^[45]. As activation of mTORC1 by Akt inhibits mitophagy, Akt can promote oxidative phosphorylation in mitochondria and thereby antagonize metabolic reprogramming^[46,47]. On the other hand, little is known about the metabolic status of PGCs or metabolic changes during germ cell reprogramming.

It has been suggested that only a fraction of cells are randomly selected for reprogramming because of the stochastic nature of the epigenetic reprogramming processes^[40]. A number of repressive epigenetic modifications, such as DNA methylation, H3K9me3, and H3K79me2, and their regulators, have been identified as barriers to somatic cell reprogramming^[48]. In addition, inhibition of histone deacetylase complex enhances iPSC induction^[49,50]. Mbd3 is a component of the nucleosome remodeling deacetylase (NuRD) complex, which is involved in heterochromatin formation. It has been reported that the majority of cells are reprogrammed into iPSCs by knockdown of *Mbd3* in the secondary iPSC induction system^[51], showing that the NuRD complex is one of the most important epigenetic roadblocks. In addition, the deletion of *Mbd3* also enhances the efficiency of EGC derivation from PGCs^[51]. Gene expression analysis during PGC reprogramming shows that a great number of *Mbd3* target genes are affected by Akt activation^[52]. Additionally, Akt activation decreases expression of *Mbd3* during somatic cell reprogramming. Collectively, the evidence suggests that PI3K/Akt signaling may promote germ and somatic cell reprogramming through multiple pathways, including proliferation, survival, metabolic change, and epigenetic regulation.

PI3K/Akt signaling in human germ cell tumors

Mutants and variants of *KIT* and *KITLG* have been identified as risk factors for human germ cell tumors^[17]. A strong association between a variant of *KITLG* and the occurrence of testicular teratomas has been reported. *KIT* mutations, which activate kinase activity in a ligand-independent manner, are found frequently in testicular seminomas but not in testicular teratomas or yolk sac tumors^[53,54]. *CBL* mutations have been found in

teratomas, yolk sac tumors, and mixed-type tumors composed of germinomas and non-germinomatous tumors, all of which occur intracranially^[54]. Because *CBL* encodes ubiquitin ligase for receptor tyrosine kinases, including KIT, mutations may lead to KIT overexpression.

The PI3K/Akt and MAPK signaling pathways are associated with the occurrence of germ cell tumors. *KRAS* and *NRAS* mutations, which activate both PI3K/Akt and MAPK signaling, are frequently detected in seminomas and teratomas^[54]. Single nucleotide polymorphisms (SNPs) of *PTEN* have been identified as risk factors for testicular teratomas^[55]. In addition, mutations in *MTOR* and *TRP53* and copy number gains in *AKT1* are frequently observed in intracranial teratomas and yolk sac tumors^[54,56]. On the other hand, variants of sprouty-4, encoding a negative regulator for MAPK signaling, are associated with testicular teratomas^[53]. Thus, the KIT, PI3K/AKT, and MAPK signaling pathways could be promising therapeutic targets for human germ cell cancers, including testicular teratomas.

REPROGRAMMING BY SMALL MOLECULE COMPOUNDS

In somatic cell reprogramming, reprogramming-inducing transcription factors can be replaced by chemical compounds. For example, the effects of Sox2 and Klf4 can be reproduced by transforming growth factor- β receptor inhibitor (TGF β Ri, SB431542 and A83-01)^[57-59] or Kempaullone^[60], respectively. Kempaullone is an inhibitor of kinases, including glycogen synthase kinase-3 (GSK3) and cyclin-dependent protein kinases. Oct4 can be substituted by forskolin, 2-methyl-5-hydroxytryptamine, and D4476^[61]. As forskolin substitutes for bFGF in PGC reprogramming^[24], the cAMP/PKA axis mediates cellular reprogramming in both somatic and germ cells.

PGCs are never converted to EGCs when cultured on mouse embryonic fibroblast (MEF) feeder layers with LIF, which is a standard culture condition for ESCs. When post-migratory PGCs at E11.5 are treated with TGF β Ri under ESC culture conditions, EGCs can be derived without KITLG and bFGF, showing that TGF β Ri can reproduce the effects of KITLG and bFGF^[62]. Although Kempaullone alone does not induce EGCs from E11.5 PGCs, simultaneous treatment with TGF β Ri and Kempaullone synergistically enhances EGC induction efficiency. In contrast, when E13.5 PGCs are cultured under ESC culture conditions, Kempaullone efficiently induces EGCs, while TGF β Ri merely promotes EGC derivation. In addition, the effects of Kempaullone are inhibited completely by TGF β Ri in E13.5 PGCs. It remains to be elucidated how PGCs respond differentially to these compounds in a differentiation stage-dependent manner.

ESCs are derived from the epiblast in blastocysts before implantation, whereas epiblast stem cells (EpiSCs) are established from the epiblast in post-implantation stage embryos^[63,64]. While mouse ESCs can be propagated in the presence of LIF and form multi-layered

colonies, mouse EpiSCs can be expanded and form mono-layered colonies in the presence of bFGF and TGF- β family member activin. These differences may reflect the distinct developmental stages of epiblast. On the other hand, primate ESCs resemble mouse EpiSCs in terms of colony morphology and growth factor requirements. While the pluripotent states of mouse ESCs are called naïve pluripotency, those of mouse EpiSCs and primate ESCs are called primed pluripotency.

Mouse ESCs can be maintained in a more undifferentiated state, so-called "ground-state" pluripotency, when cultured with LIF and two inhibitors (2i), namely inhibitors of MAPK/ERK kinase and GSK3 (PD0325901 and CHIR99021, respectively)^[14]. The efficiency of iPSC production is enhanced by treatment with 2i^[65,66]. Furthermore, EGCs are derived from migratory PGCs at E8.5 by 2i without KITLG and bFGF^[67]. Treatment with 2i also increases EGC derivation efficiency in post-migratory PGCs at E11.5, and the effect is further enhanced by TGF β Ri treatment^[68].

It has recently been reported that iPSCs can be derived from MEFs by sequential treatment with chemical compounds alone^[61,69]. These compounds include TGF β Ri (616452), GSK3i (CHIR99021), a cAMP/PKA agonist (forskolin), an RA agonist (AM580), a histone deacetylase complex inhibitor [valproic acid (VPA)], an inhibitor of H3K4 demethylase LSD1 (tranylcypromine), inhibitors of H3K79 methyltransferase DOT1L (EPZ004777 and SGC0946), and a DNA methyltransferase (Dnmt) inhibitor (5-aza-dC). Despite their positive effects on somatic cell reprogramming, VPA and 5-aza-dC inhibit EGC derivation from E11.5 PGCs, indicating differences in epigenetic status between somatic and germ cells^[68].

REGULATORS OF GERM CELL DEVELOPMENT

A homozygous Teratoma (*Ter*) mutation dramatically increases the occurrence of testicular teratomas against the 129/Sv genetic background^[70,71]. Although germ cells in *Ter/Ter* mutant mice appear normal until E13.5, the cells do not enter into mitotic arrest after E14.5, undergo massive apoptosis, and generate early teratomatous foci after E15.5, which are essentially the same phenotype as those of *Pten*-deficient mice. However, the *Ter/Ter* mutant mice, against other genetic backgrounds such as C57/BL6, do not develop testicular teratomas but exhibit germ cell deficiency. A homozygous *Ter* mutation causes germ cell death during embryonic development regardless of the genetic background. There exist genetic and epigenetic modifiers required for teratoma formation in the 129/sv genome.

Dnd1 is a gene responsible for *Ter* mutation phenotype^[72]. *Dnd1* is an evolutionarily conserved RNA-binding protein that counteracts micro RNA (miRNA)-mediated translational inhibition of target mRNAs in zebrafish and mammals^[73-75]. The miRNA targets include mRNAs for negative cell cycle regulators (*p27*, *Lats*,

Trp53), pluripotency and germ cell-related genes (*Oct4*, *Sox2*, *Nanos1*) and anti-apoptotic factors (*Bax*, *Bclx*). As translation of these target mRNAs is de-repressed by *Dnd1*, *Ter* mutation brings about decreased levels of these proteins, which can lead to germ cell deficiency and uncontrolled cell proliferation and survival. *Dnd1* is a binding partner of the RNA-binding protein *Nanos2*, which interacts with the CCR4-NOT deadenylase complex and regulates the stability of mRNAs for germline genes such as *Sycp3*, *Dazl*, *Nanog*, and *Strab*^[76]. Dereglulation of RNA metabolism may also be implicated in tumorigenesis in *Ter* mutant germ cells.

Doublesex-related transcription factor (*Dmrt1*) promotes male differentiation in germ and somatic cells in fetal and neonatal testes. In the absence of *Dmrt1*, testicular germ cells prematurely enter into meiosis and Sertoli cells transdifferentiate into female somatic cells^[77-79]. Like *Ter/Ter* mutant mice, over 90% of *Dmrt1*-deficient mice develop testicular teratomas against the 129/Sv genetic background, but not other genetic backgrounds. Conditional knockout mice demonstrate that the loss of *Dmrt1* in PGCs, but not in Sertoli cells, leads to teratoma formation^[80]. Pluripotency-related genes and Nodal pathway genes are upregulated, whereas the glia-cell derived neurotrophic factor (GDNF) receptor genes including *Ret* and *Gfra1* are downregulated in mutant fetal testes^[81]. As deletion of *Gfra1* in 129/Sv mice modestly increases the incidence of testicular teratomas^[81], the effects of *Dmrt1* deletion are at least partly mediated by downregulation of GDNF signal. Alternatively, enhanced RA signaling in germ cells lacking *Dmrt1* may drive dedifferentiation, as RA treatment induces PGC reprogramming *in vitro*^[25,77,79]. In addition to these effects on fetal germ cells, depletion of *Dmrt1*, together with *Trp53* depletion, increases the efficiency of mGSC derivation from GSCs^[82]. It has been reported that SNPs near *DMRT1* are associated with testicular germ cell cancer in humans^[83].

The transcription factors *Blimp1*, *Prdm14*, and *Tfap2c* are critical for the specification and differentiation of PGCs. While forced expression of *Blimp1* in ESCs reduces the expression of pluripotency genes, deletion of *Blimp1* in PGCs promotes the derivation of EGCs even in the absence of bFGF^[52]. In addition, heterozygous *Tfap2c* mutant mice develop testicular teratomas against the 129/Sv background^[84]. *In vitro*, PGC-like cells induced from homozygous *Tfap2c* mutant ESCs show upregulation of cell cycle regulators (*Cdk6*) and pluripotency genes (*Eras*, *Klf4*), but downregulation of germline genes (*Dmrt1*, *Nanos3*)^[84]. Furthermore, the susceptibility locus for human testicular germ cell cancer has been found near *PRDM14*^[85]. Collectively, these germline genes also function as gatekeepers of PGC dedifferentiation.

CONCLUSION AND PERSPECTIVES

Reprogramming of germ cells and somatic cells is controlled by common signaling pathways, which are activated

by PI3K/Akt, MAPK, GSK3, TGF β , RA, and cAMP/PKA. Therefore, it is critical to understand which downstream effectors are important for reprogramming, and which cellular processes are modulated by these signaling pathways during reprogramming. In contrast, the roles of epigenetic regulators on reprogramming seem to differ to some extent between germ and somatic cells. Furthermore, certain regulators of germ cell differentiation, which are essential for the establishment of the male germline, play critical roles in the prevention of germ cell dedifferentiation.

129/Sv mice frequently develop testicular teratomas. Additionally, mutations in *Dnd1*, *Dmrt1*, and *Tfap2c* lead to testicular teratomas in only the 129/Sv mouse strain. Therefore, it has been suggested that 10-15 susceptibility genes are present in the 129/Sv genome^[86,87]. These modifiers include *Ter*, *Trp53*, testicular germ cell tumor 1, and primordial germ cell tumor 1^[86-88]. *Ter* mutation increases the incidence of teratomas along with mutations in the genes encoding *Trp53*, *KITLG*, the translational regulator *Eif2s2* (*A'* mutation), and the cytidine deaminase *Apobec1*^[89,90]. Furthermore, the introduction of chromosome 19 from MOLF mice into the 129/Sv background greatly increases the tumor incidence^[86]. Investigating the genetic network among susceptibility genes will be necessary to understand the development of germ cell tumors.

Genome-wide association studies have revealed a number of candidate genes for human germ cell tumors. Variants have been found near genes involved in male germ cell development (*DAZL*, *HPGDS*, *SMARCA1*, *SEPT4*, *TEX14*, *RAD51C*, *PPM1E*, and *TRIM37*), chromosomal segregation (*MAD1L1*, *TEX14*, and *SKA2*), the DNA damage response (*SMARCA1*, *RFWD3*, and *RAD51C*), and epigenetic regulation (*JMJD1C/KDM3A* and *KDM2A*)^[83,85,91]. Mouse models would help to evaluate the roles of these genes in the tumorigenesis of germ cells.

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Epithelial plasticity in urothelial carcinoma: Current advancements and future challenges

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Abstract

Urothelial carcinoma (UC) of the bladder is characterized by high recurrence rate where a subset of these cells undergoes transition to deadly muscle invasive disease and later metastasizes. Urothelial cancer stem cells (UroCSCs), a tumor subpopulation derived from trans-

formation of urothelial stem cells, are responsible for heterogeneous tumor formation and resistance to systemic treatment in UC of the bladder. Although the precise reason for pathophysiologic spread of tumor is not clear, transcriptome analysis of microdissected cancer cells expressing multiple progenitor/stem cell markers validates the upregulation of genes that derive epithelial-to-mesenchymal transition. Experimental studies on human bladder cancer xenografts describe the mechanistic functions and regulation of epithelial plasticity for its cancer-restraining effects. It has been further examined to be associated with the recruitment of a pool of UroCSCs into cell division in response to damages induced by adjuvant therapies. This paper also discusses the various probable therapeutic approaches to attenuate the progressive manifestation of chemoresistance by co-administration of inhibitors of epithelial plasticity and chemotherapeutic drugs by abrogating the early tumor repopulation as well as killing differentiated cancer cells.

Key words: Cancer stem cells; Clinical management; Cytotoxic effects; Epithelial plasticity; Therapeutic resistance; Urothelial carcinoma; Urothelial stem cells

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Core tip: A subset of bladder cancer cells, known as urothelial cancer stem cells, have abilities to self-renew, generate tumor heterogeneity *via* differentiation, and are actually responsible for tumor relapse and metastasis formation. Delineating the mechanistic complexity between epithelial plasticity and cancer stemness in malignant transformation of urothelial carcinoma provides the basis for designing rational therapies. Differentiation and elimination therapies targeting the potential biomarkers could prove to be clinically beneficial by suppressing the cancer stemness and inhibiting epithelial-to-mesenchymal transition phenotype and would provide novel opportunities for targeted therapeutic approaches in the clinical management of patients.

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INTRODUCTION

Urothelial carcinoma (UC) of the bladder, also known as transitional cell carcinoma of the bladder, is the sixth most common cause of cancer-related deaths worldwide^[1]. It is the second most frequent cancer of the genitourinary tract where men are at four times greater risk than women. It is caused by the accumulation of genetic or epigenetic changes in the urothelium due to its exposure to multiple risk factors including tobacco and occupational/environmental carcinogens (polycyclic aromatic hydrocarbons). People working in leather, dye, rubber industries, painters, pesticide applicators or those having chronic urinary tract infections are more prone to develop urothelial carcinoma.

UC of the bladder is a heterogeneous disease, which can arise through two different pathways - non-invasive papillary pathway and invasive pathway. It represents a spectrum of neoplasms, including non-muscle invasive bladder cancer (NMIBC), muscle invasive bladder cancer (MIBC) and metastatic lesions. Tumor staging and grading (Tumor Node and Metastasis classification by World Health Organization/International Society of Urology Pathologists, 2004) are the gold standard prognosticators for defining the various entities of UC of the bladder (Figure 1)^[2]. Despite the successful treatment of NMIBC through transurethral resection of bladder tumor (TURBT), 70% to 80% of them have a tendency to recur. Hence, there is a need for regular cystoscopy and examination of cytologic and molecular markers in urine, blood or tumor tissues in bladder cancer patients. This intense surveillance after treatment makes this cancer, one of the most costliest cancers to manage. Although in the majority of the cases, these papillary bladder tumors are not lethal, however, 20%-30% of them can progress to more aggressive, invasive and metastatic bladder tumors with an overall survival rate of 5% (Figure 2).

Characterization of molecular and biological mechanisms responsible for distinct bladder tumor phenotypes would facilitate personalization of more effective treatment decisions. Multiple genetic and epigenetic abnormalities are known to be associated with diverse types of urological malignancies. Cancer stem cell theory sheds further light on understanding the biology of the origin of distinct oncological pathways and heterogeneous nature of this disease.

This paper discusses the current concepts on the aberrant activation of epithelial-to-mesenchymal transition (EMT), also known as epithelial plasticity, as one of the primary causes of transformation of urothelial

stem cells (UroSCs). Further, recent advancements on the functions of urothelial cancer stem cells (UroCSCs), a tumor subpopulation derived from transformation of UroSCs, in the pathophysiology and its clinical implications in the treatment of UC of the bladder are reviewed.

UROTHELIAL STEM CELLS AND UROTHELIAL CANCER STEM CELLS

The stratified epithelial lining of the urinary bladder wall, also known as urothelium, consists of unilayered polygonal basal cells which are in direct contact with the basement membrane, intermediate cells and umbrella cells. Many recent studies report the existence of a self-renewing unipotent population of slow cycling, label-retaining cells with long life span and high integrin subunit beta 4 expression, also known as urothelial stem cells, as clonal patches among basal cell layer. High nuclear-cytoplasmic ratio and expression of CD44, laminin receptor, cyto-keratins (CK-5/14, CK17), $\beta 1$ and $\beta 4$ integrins are some of the characteristic features of UroSCs^[3]. These cells confer increased regenerative and proliferative potential, lower apoptosis rate and multilineage differentiation at the edge of the basement membrane as compared to other cell types. These cells undergo cellular differentiation to give rise to transit-amplifying cells of intermediate cell layers and later umbrella cells. However, an alternative hypothesis suggests that adult stem cells can give rise to two cell lineages and hence, umbrella cells are formed separately from intermediate/basal cells (Figure 3). Lineage tracing experiments in the murine model of carcinogenesis provide a cellular and genetic basis for the diversity in bladder cancer lesions which could be responsible for their clinical and morphological differences. According to the experimental results of this study, the low grade, non-invasive papillary lesions arise from intermediate cells whereas Keratin 5 expressing basal cells are likely the progenitors of flat carcinoma *in situ*, a flat aggressive lesion, as well as of muscle-invasive lesions depending on the genetic background^[1]. A study by Dancik *et al*^[4] screened 874 bladder cancer patients in five cohorts for the identification of UroCSCs in muscle invasive tumors and validated the hypothesis of differential origin of non-muscle invasive and muscle invasive tumors from distinct progenitor cells. These results provide a paradigm shift in better understanding the biology of urothelial carcinoma for significant diagnostic and therapeutic implications.

Mutational insults in adult UroSCs and differentiated progenies, help them in acquiring tumorigenic properties and result in the origin of a subpopulation of high tumor-initiating potential cells called UroCSCs. Characterization studies on these cells describe their self-renew ability, clonogenic and proliferative potential. In addition, their capability to conserve cellular heterogeneity *via* differentiation can be explained by the research studies on the regrowth of heterogeneous tumor after *in vivo*

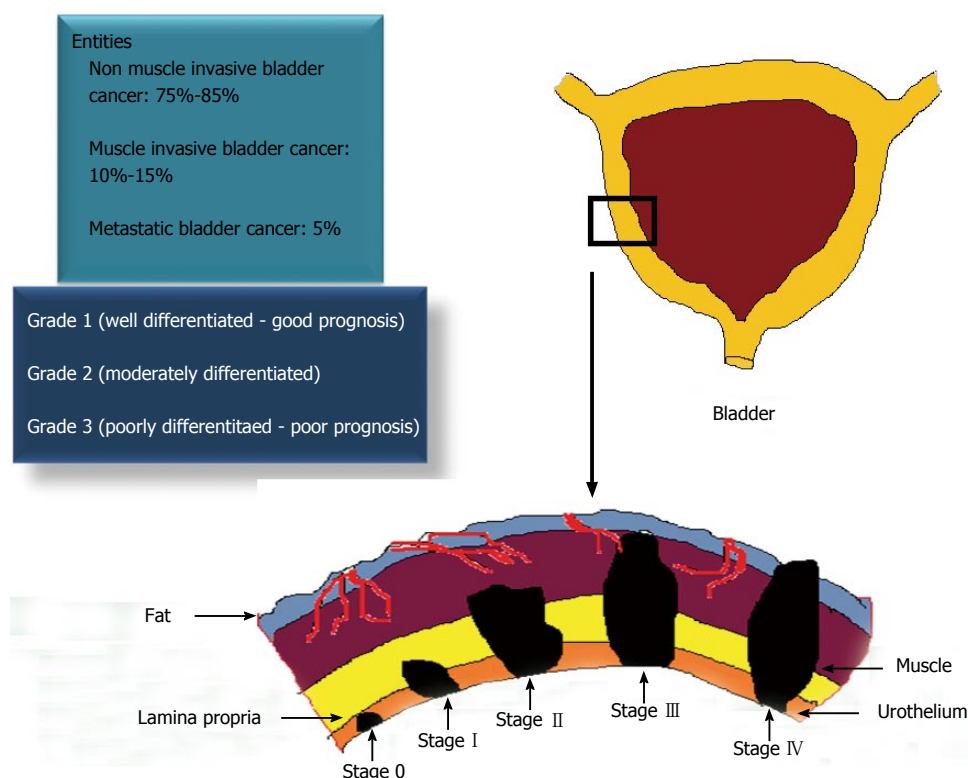


Figure 1 Staging, grading and prognosis of urothelial carcinoma of the bladder.

xenotransplantation of a small number of UroCSCs in immunodeficient mice (Figure 3). These characteristic features of UroCSCs document their large amount of functional resemblance with the normal adult stem cells.

UroCSCs have been examined for the upregulation of various oncogenes which help them to acquire self-renewability. Beta-catenin (β -catenin), signal transducer and activator of transcription 3, glioma associated oncogene 1, B lymphoma Mo-MLV insertion region 1 homolog (BMI1), POU domain, class 5, transcription factor 1/octamer-binding transcription factor 4 (POU5F1/Oct4), sex determining region Y-box 2 (SOX2), Kruppel-like factor 4, v-myc myelocytomatosis viral oncogene homolog (avian) (MYC, formerly C-MYC) and NANOG are the oncogenes/transcription factors that have been observed to be responsible for maintaining the pluripotent properties of stem cells and aggressiveness of tumor invasion^[5-7].

Studies on the identification of co-expression of keratin 5 and CD44 markers on UroCSCs distinguish them from differentiated tumor cells and support their basal-like phenotype. Binding of CD47, a marker of tumor-initiating cells, to signal-regulatory protein alpha on macrophages and subsequent inhibition of phagocytosis of tumor cells make it a suitable drug target^[8]. Increased expression of POU5F1, an embryonic stem cell marker, and high aldehyde dehydrogenase activity in a fraction of CD44⁺ tumors correlate with increased clonogenic capacity of UroCSCs, and poor prognosis in UCs^[9]. Identification of an extracellular marker, prominin 1 (PROM1⁺) (CD133⁺) and intracellular markers POU5F1⁺, and nestin (NES⁺) on putative UroCSCs confer them self-renewal ability and

proliferative advantages in clonogenic assays. However, in due course of time, they allow these UroCSCs to lose stem cell phenotype as well as proliferative capacity and initiate the process of differentiation^[10]. Differentially expressed cancer stem cell markers CD24/CD44/CD47 in the urothelial cancer cells of bladder cancer patients undergoing radical cystectomy could be of therapeutic value as their presence influenced cancer-specific survival of patients^[11]. Many cell surface markers, intracellular proteins and their activities are examined to identify and characterize the putative UroCSCs, however, due to the lack of consensus on these markers, functional assays have been studied to confirm the stem cell phenotype of these tumor cells.

Pumping of DNA-binding dyes, Hoechst 33342 and DyeCycle violet, out of the cells due to overexpression of ABC (ATP-binding cassette) transporters/multidrug resistance (MDR) pumps are considered important features of a side population of urothelial cancer cells, enriched for CSCs. Co-localization of ABC transporters, ABCG2 and ABCB1 (MDR1) and other stem cell markers including POU5F1 and BMI1 further validates their identity and existence^[12]. Initiation of tumor formation upon subcutaneous injection of a small number of SP of urothelial cancer cells into immunocompromised mice has been examined by clonogenic assays, and these cells showed rapid cell growth, chemo and radioresistance.

Accumulating evidence suggests that UroCSCs/progenitor cells exhibiting epithelial plasticity are quiescent, show increased DNA damage response, pump drugs out of the cells, reside in difficult-to-reach CSC protective

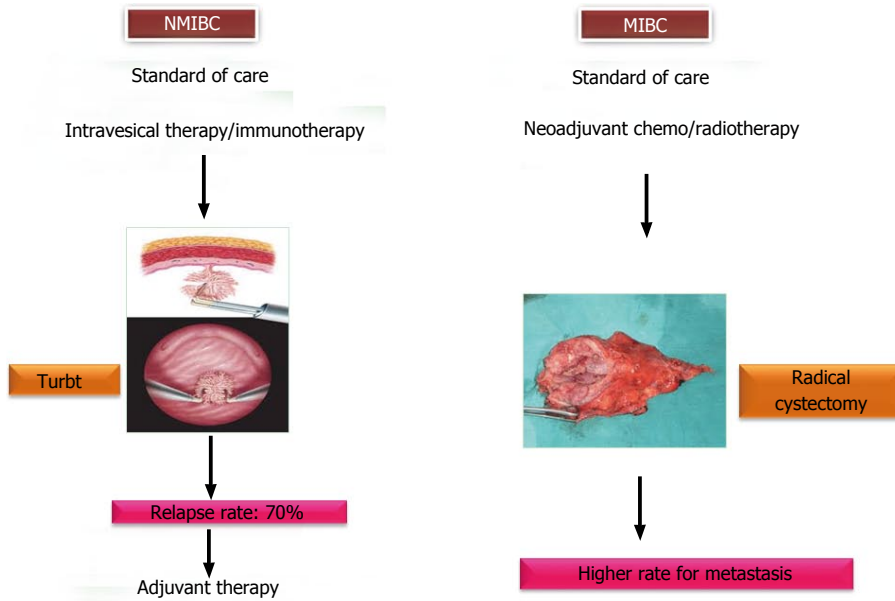


Figure 2 Multimodality approaches for urothelial carcinoma of the bladder. NMIBC: Non-muscle invasive bladder cancer; MIBC: Muscle invasive bladder cancer.

niches and are less affected by antiproliferative therapies.

UROTHELIAL CANCER STEM CELLS AND EPITHELIAL PLASTICITY

During the analysis of transcriptome of microdissected muscle invasive urothelial carcinoma of bladder/MIBC, the cancer cells expressing multiple progenitor/stem cell markers were found to be enriched with elevated levels of genes that derive and regulate EMT^[13]. The process of EMT is characterized by the loss of cell polarity and cell-cell adhesion by sessile, epithelial cells and their transition to motile, mesenchymal stem cells with increased migratory and invasive potential. Cells acquire phenotypic or epithelial plasticity when they gain the ability to dynamically switch over between different phenotypic states^[14]. EMT helps to establish metastasis by allowing the motile cells to invade the surrounding tissues, intravasate, move to distant sites through bloodstream, extravasate and colonize the target organs. Re-establishment of cancer cells with more epithelial phenotype at metastatic sites can be induced through mesenchymal-to-epithelial transition (MET) (Figure 4).

A study by Franzen *et al*^[15] demonstrates the increased expression of several mesenchymal markers, including α -smooth muscle actin, S100A4 and snail, in urothelial cells treated with muscle invasive bladder cancer exosomes (small secreted vesicles that contain proteins, mRNAs and miRNAs and can potentially modulate signaling cascades in recipient cells) as compared with phosphate-buffered saline-treated cells. Moreover, these treated urothelial cells showed loss of epithelial markers, E-cadherin and β -catenin in association with increased migratory and invasive properties.

Loss of E-cadherin, a tumor suppressor gene, and abnormal expression of N and P-cadherin (cadherin switch)

ching) have been shown to be key mediators in invasive and malignant phenotype of cancer. In addition, activation of WNT signaling cascade by tumor cells owing to decreased E-cadherin levels, loss of β -catenin expression, its nuclear translocation and increased transcriptional activity have been examined to be associated with epithelial plasticity of tumor cells, disease aggression and metastasis formation. One of the serious implications of cadherin switching include the development of cancer stem cell phenotype and this makes the cadherin cell adhesion molecules and associated pathways, the probable target candidates for inhibition of cancer progression^[16].

Tumor stroma/microenvironment has been shown to regulate tumor behavior by maintaining UroCSC population, its properties and EMT. Although the exact mechanism is not known, secretion of stroma-modulating growth factors including basic fibroblast growth factor 2, vascular endothelial growth factor, platelet-derived growth factor, epidermal growth factor receptor (EGFR) ligands, colony stimulating factors, and transforming growth factor-beta; extracellular matrix-degrading proteins, such as matrix metalloproteinases; and chemoattractants result in activation of fibroblasts, inflammatory cells, mesenchymal stem cells, smooth muscle cells, and adipocytes^[17,18]. This contributes to angiogenesis, tumor growth, invasion and metastasis formation.

THERAPEUTIC IMPLICATIONS AND CHALLENGES

Intravesical instillations of drugs or adjuvant therapies following TURBT are the standard of care for non-muscle invasive cancer. Similarly neoadjuvant therapies with radiotherapeutic or chemotherapeutic drugs and in some cases radical cystectomy are the standard treatment

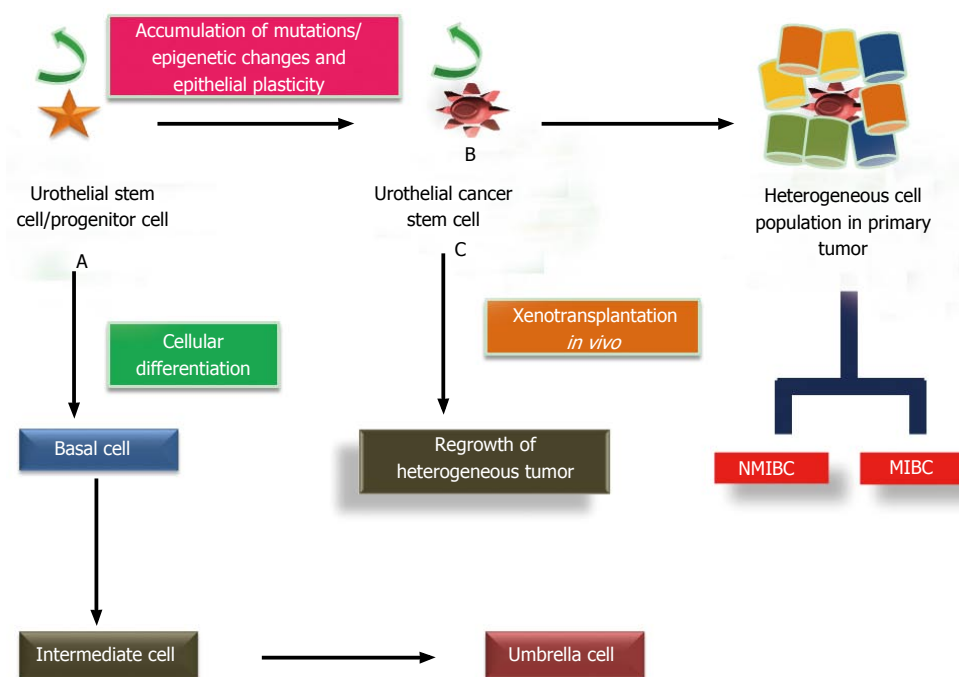


Figure 3 Cellular differentiation and mutational transformation of urothelial stem cells and dual pathways of carcinogenesis. A: Cellular differentiation of UroSCs (exist in the form of clonal patches in basal layer) gives rise to basal cells which further differentiate to intermediate cells and then into single layer of umbrella cells; B: Mutational insults including epithelial plasticity result in malignant transformation of UroSCs into self-renewing UroCSCs which undergo aberrant activation and differentiation to form papillary non muscle invasive and muscle invasive bladder cancer; C: *In vivo* xenotransplantation of a small number of UroCSCs that have the potential for the regrowth of heterogeneous tumor cells. UroCSCs: Urothelial cancer stem cells; NMIBC: Non-muscle invasive bladder cancer; MIBC: Muscle invasive bladder cancer.

options for more aggressive muscle invasive disease^[19].

Cytotoxic effects of these drugs can potentially de-bulk tumor masses initially but tumors progressively develop between or after multiple treatment cycles in due course of time. The SP of tumor cells was found to be enriched for UroCSCs which can possibly contribute to progressive development of therapeutic resistance through enhanced survival. A number of experimental studies on human bladder cancer xenografts provide the probable mechanistic explanation for unexpected proliferative response to repopulate residual tumor cells between chemotherapy cycles. Urothelial carcinoma cell lines were examined for enriching CSCs with CD90 and CK14 expression and the effects of short- and long-term treatment with cisplatin on tumor initiating potential of these separated cells were studied. Substantial phenotypic plasticity as evident by increased expression of EMT markers, an altered pattern of CKs, and WNT-pathway target genes were observed in these sublines and instead of inducing apoptosis, it promoted neighboring CSC repopulation and subsequently the development of clinical resistance to cisplatin^[20]. A strong correlation between the existence of CSC-like cells in the population of cisplatin-resistant bladder cancer cells, levels of Bmi1 and Nanog expression and the degree of malignancy of urothelial carcinoma tissues has been observed. This may play a role in the progression and drug resistance of bladder cancer^[21].

Recruitment of a quiescent pool of UroCSCs into cell division in response to the cytotoxic effects of clinical

drugs, similar to the mobilization of UroSCs during wound repair, reduces the efficacy of existing drugs and dramatically accelerates the pathophysiological spread of more aggressive type of bladder cancer. Combinatorial approaches based on *in vivo* administration of inhibitors of epithelial plasticity could be the probable therapeutic strategy for enhancing chemotherapeutic drug-induced damages by abrogating early tumor repopulation (source of cancer) and killing a bulk of bladder cancer cells, thereby customizing a new method to counter CSC-driven resistance, prevent relapse and improve the survival outcome in the patients with UC of the bladder.

Sox4, a biomarker of UroCSCs and one of the important candidate oncogenes, results in advanced cancer stages and poor survival rate. The results of its knockdown include reduced sphere formation and enriched cell population with high levels of aldehyde dehydrogenase [ALDH (high)]; inhibition of cell migration, colony formation as well as MET; and decreased tumor formation potential of urothelial cancer cells^[22]. The essential role of αv integrins has been shown in migration, EMT and maintenance of ALDH activity, tumor growth and metastasis. Therefore, targeting of αv integrins could be a promising therapeutic approach for prevention of metastatic bladder cancer. Treatment with an αv integrin antagonist and its knockdown in the bladder carcinoma cell lines resulted in reduced expression levels of EMT-inducing transcription factors including SNAI2 and self-renewal genes NANOG and BMI1; low ALDH activity; and decreased CDH1 (E-cadherin)/CDH2 (N-cadherin),

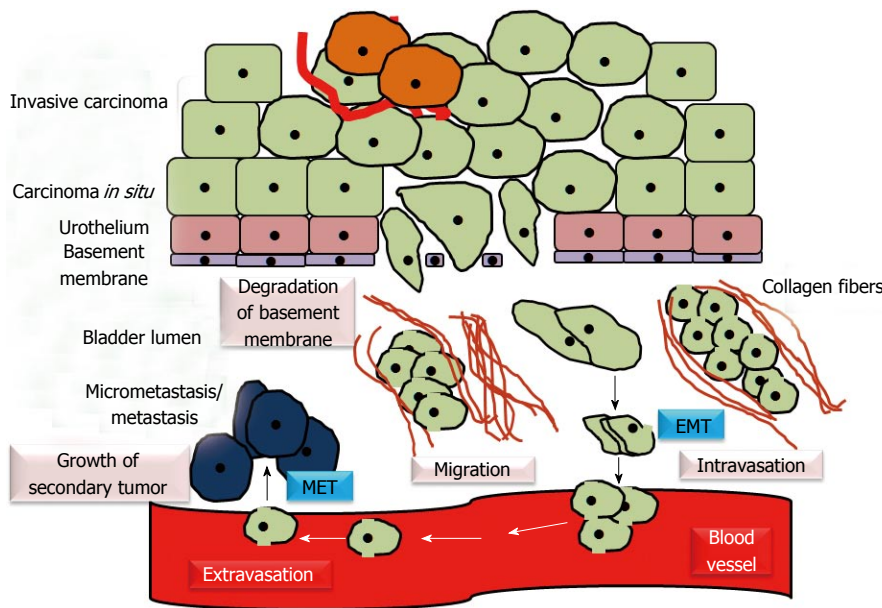


Figure 4 Epithelial mesenchymal transition and mesenchymal epithelial transition in urothelial carcinoma. EMT/epithelial plasticity allows invasive bladder cancer cells to become motile, invade the surrounding tissues and intravasate. Through bloodstream, primary tumor cells move to distant sites, extravasate, colonize the target organs and establish the metastasis. MET induces regrowth and re-establishment of cancer cells with epithelial phenotype at secondary/metastatic sites. EMT: Epithelial mesenchymal transition; MET: Mesenchymal epithelial transition.

indicative of a shift towards epithelial phenotype and decreased proliferative, migratory, clonogenic capacity and metastatic growth^[23]. Overexpression of EGFR has been examined to be associated with poor prognosis in epithelial cancers. Hence, targeting cancer cells with an EGFR inhibitor (anti-EGFR antibody, cetuximab) has been shown to increase the expression of CDH1 and confer cancer cells with epithelial phenotypic property^[24]. Implications of miRNAs (a class of small non-coding RNA molecules of 21-23 nucleotides in length) in the maintenance of epithelial plasticity, cancer stemness and mediating drug sensitivities make it a potential therapeutic system towards eradication of tumor recurrence and metastasis^[25,26]. Forced expression of miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) has been associated with induction of MET in mesenchymal bladder cancer cell lines, which thereby restored EGFR inhibitor sensitivity to attenuate tumor aggressiveness in bladder cancer^[25]. Re-expression of miR-23b may be a beneficial therapeutic strategy for the treatment of human bladder cancer by targeting Zeb1, a crucial regulator of EMT, inhibiting cell proliferation and migration and inducing apoptosis^[27].

Direct suppression of epithelial plasticity with the use of inhibitors or knocking down EMT markers can also potentially reduce migration, invasion, and survival of cancer cells. Inhibitory effects of prostate-derived E-twenty six (Ets) factor (PDEF), an epithelium-specific member of the Ets family of transcription factors, on the proliferation, invasion, and tumorigenesis have been studied. Its ectopic overexpression in bladder carcinoma cells has been examined to modulate EMT by upregulating E-cadherin expression and downregulating the expression of N-cadherin, SNAIL, SLUG, and vimentin,

thereby resulting in lower migration and invasion abilities of cancer cells^[28]. Molecular mechanisms for ERK1/2 inhibitor to exert its antiproliferative effects in bladder cancer have been investigated. Treatment of SV-HUC-1 cells with ERK1/2 inhibitor (U0126) significantly reduced the expression of EMT markers including Snail, β -catenin, Vimentin, and MMP-2^[29].

Besides inhibiting epithelial plasticity which can check dissemination and migration of invasive cells, it is also important to attenuate the reestablishment of cancer cells at distant sites through MET mechanism. In addition, elimination therapies are required to modulate the properties of UroCSCs, hence facilitate their chemosensitivity and apoptosis. This can be achieved by the application of inhibitors to target ABC transporters and drug-detoxifying enzymes. Cracking the difficult-to-reach protective niche of UroCSCs and creating an inhospitable microenvironment for them as well as for heterogeneous cancer cells at primary and distant sites may provide a basis for developing improved and effective therapeutic strategies for selective elimination of tumor cells. One of the recent studies identify the possible role of connexins, gap junction proteins found in the smooth muscles of detrusor muscle, in bladder tumorigenesis. Preliminary assessment detects the upregulation of connexin 43 in human urothelial carcinomas. Its functions in enhancing the adherence of tumor cells to stroma, increased migration potential as well as dissemination of cancer cells make it a promising target for genetic therapeutic approaches^[30].

Long-term follow-up of patients and definite prediction of the biomarkers for patient survival or disease progression are the most important requirements in designing suitable therapies. High-throughput drug

screening for its anticancer effects, reliable methods for detecting the population of UroCSCs, their characterization and validation in appropriate disease models are some of the additional challenges for successful therapies.

Understanding the mechanisms and biology of UroCSCs that can control their proliferation and differentiation allows the possibility of developing effective anti-cancer drugs. Deciphering the connection between epithelial plasticity and cancer stemness paves the way to design rationale therapies for its anti-tumor effects in the clinical management of bladder cancer.

CONCLUSION

Depending upon the genomic integrity and its background, UroCSCs in basal urothelium aggressively colonize a significant region of stratified urothelium to generate histologically different tumor lesions, identical to muscle invasive bladder cancer and carcinoma *in situ*. However, intermediate cells derived from the cellular differentiation of UroSCs can give rise to non-muscle invasive papillary lesions, suggestive of dual pathways of urothelial carcinogenesis. Basal-cell specific markers are examined to be good candidates for enriching UroCSCs in the SP of tumor cells. These cells are characterized by remarkable plasticity, contribute to tumor heterogeneity, relapse, and metastasis, and thereby carry significant information in the clinical management of bladder cancer. Therapeutic applications of EMT inhibitors to reverse the epithelial plasticity may account for inhibitory functions of UroCSCs, reduced migratory and invasive properties of cancer cells and can improve therapeutic planning for better patient management.

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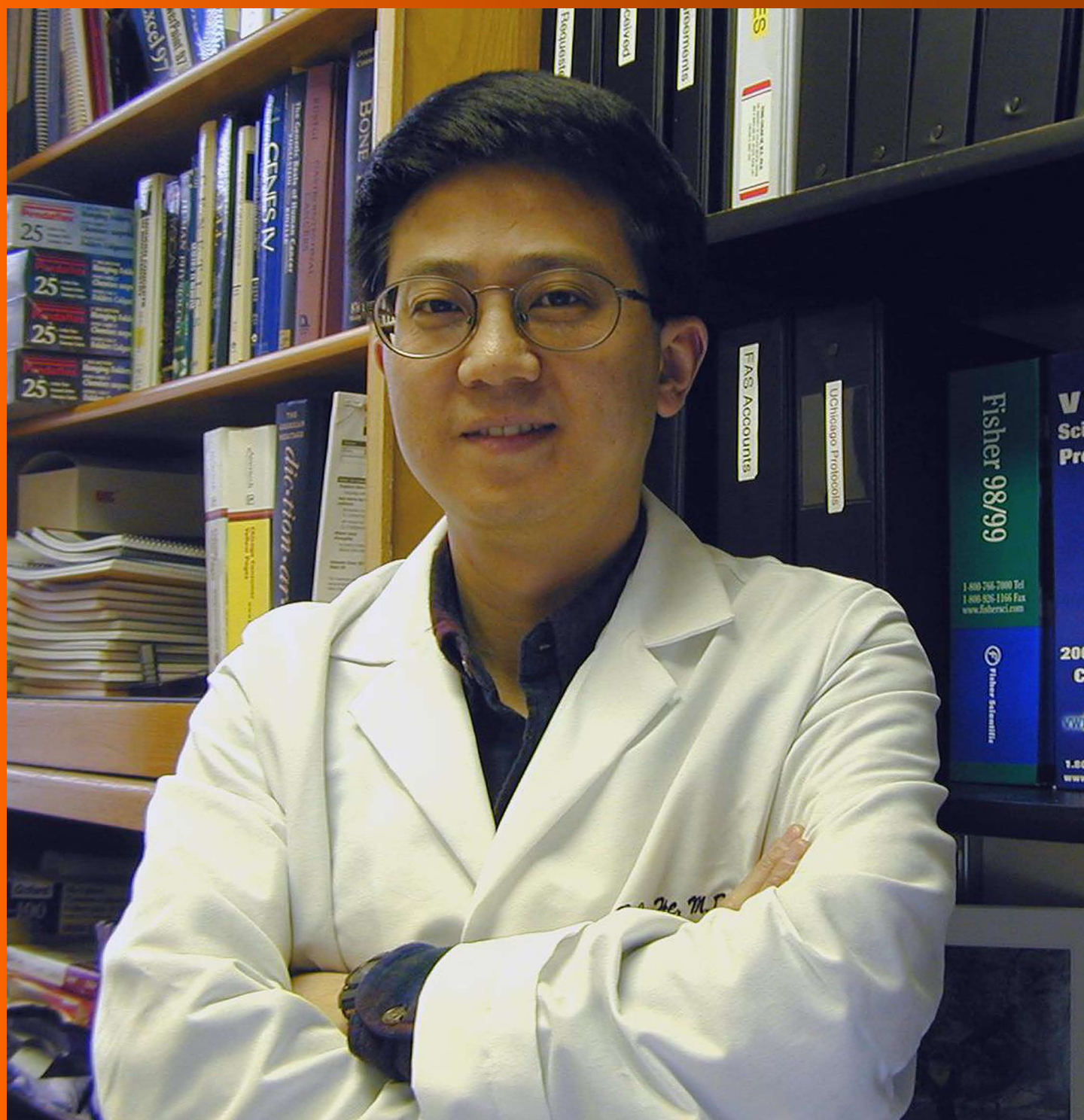
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Immunomodulation by mesenchymal stem cells: Interplay between mesenchymal stem cells and regulatory lymphocytes

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Abstract

Mesenchymal stem cells (MSCs) possess immunomodulatory properties, which confer enormous potential for clinical application. Considerable evidence revealed their efficacy on various animal models of autoimmune diseases, such as multiple sclerosis, systemic lupus erythematosus and uveitis. MSCs elicit their immunomodulatory effects by inhibiting lymphocyte activation and proliferation, forbidding the secretion of proinflammatory cytokines, limiting the function of antigen presenting cells, and inducing regulatory T (T_{reg}) and B (B_{reg}) cells. The induction of T_{reg} and B_{reg} cells is of particular interest since T_{reg} and B_{reg} cells have significant roles in maintaining immune tolerance. Several mechanisms have been proposed regarding to the MSCs-mediated induction of T_{reg} and B_{reg} cells. Accordingly, MSCs induce regulatory lymphocytes through secretion of multiple pleiotropic cytokines, cell-to-cell contact with target cells and modulation of antigen-presenting cells. Here, we summarized how MSCs induce T_{reg} and B_{reg} cells to provoke immunosuppression.

Key words: Mesenchymal stem cells; Regulatory T cells; Regulatory B cells; Immunomodulation; Autoimmunity

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Core tip: In this review, we summarized the mechanisms involved in regulatory T (T_{reg}) and B (B_{reg}) cell induction by mesenchymal stem cells (MSCs). In an inflammatory environment, MSCs secrete various anti-inflammatory cytokines, actively interact with immune cells and modulate them to acquire regulatory properties, thus, generate a tolerogenic environment. Particularly, by

inducing T_{reg} and B_{reg} cells, the immunomodulation of MSCs is amplified. Therefore, genetic engineered MSCs to enhance their ability to induce T_{reg} and B_{reg} cells may increase their therapeutic efficacy.

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INTRODUCTION

Mesenchymal stem cells (MSCs) are mesodermal progenitor cells that have a wide range of differentiation capacity. They can differentiate into adipocytes, osteocytes, chondrocytes, myocytes, fibroblasts and stromal cells^[1]. In addition, some research studies have shown that MSCs, under certain conditions, can trans-differentiate to cells from ectodermal and endodermal lineage^[2,3]. Among them, the ability of MSCs to develop into neurons is of particular interest. Considering that neural stem cells are limited in number and extremely difficult to be isolated while, comparatively, massive numbers of MSCs can be derived from numerous adult tissues, including, liver, kidney, adipose tissue, bone marrow, dental pulp, peripheral blood and umbilical cord blood. MSCs may serve as a reliable source of neural cells for potential cell replacement therapy or regenerative medicine.

Aside from its diverse differentiation capacity, their immunomodulatory properties also prompt researchers to study profoundly. MSCs are capable of regulating both innate and adaptive immunity. They secrete a large variety of soluble factors, including interleukin (IL)-6, IL-8, transforming growth factor- β 1 (TGF- β 1), indoleamine 2,3-dioxygenase (IDO), human leukocyte antigen-G (HLA-G) and prostaglandin E2 (PGE2)^[4]. These factors allow MSCs to interact with components of the innate and adaptive immunity, subsequently modulate inflammation and immune tolerance. Monocytes, for instance, under the influence of MSCs-secreted IL-6, IDO and PGE2, tend to develop into anti-inflammatory M2 macrophages instead of proinflammatory M1 macrophages^[5-9]. In addition, recent reports showed that human gingiva derived MSCs have converted M1 macrophages to M2^[5]. Natural killer (NK) cells, on the other hands, express CD73 and acquires regulatory phenotype when exposed to MSCs^[10,11]. Similarly, regulatory dendritic cells (DC) induced by MSCs were capable of secreting IL-10, a powerful anti-inflammatory cytokine^[12-14]. Thus, MSCs are able to suppress innate immunity by skewing their differentiation into regulatory subtype (Figure 1).

MSCs can regulate adaptive immune system by suppressing the proliferation, differentiation and activation of T cell and B cell. A number of studies have demonstrated that MSCs can inhibit the proliferation of

Th1 and Th17 cell, decrease the production of interferon (IFN)- γ , IL-2, IL-6 and IL-17, and downregulate the T cell activation markers, CD38 and HLA-DR^[15-19]. When MSCs were co-cultured with B cell and in the presence of different B cell trophic stimuli, B cell proliferation was inhibited and they were arrested in G₀/G₁ phase. Moreover, B cell differentiation was prohibited as indicated by limited production of IgG, IgM and IgA^[20]. In addition, the regulatory-skewing propensity of MSCs observed in innate immune system also applies to T and B lymphocyte. In fact, the ability of MSCs to expand regulatory T (T_{reg}) cells and regulatory B (B_{reg}) cells have been intensively studied. However, the mechanism of how T_{reg} and B_{reg} cells are induced by MSCs has not been fully understood. Some suggest regulatory lymphocytes induction by MSCs requires mediation of other immune cells, while others propose MSCs-released cytokines are sufficient to expand T_{reg} and B_{reg} cell populations, but more and more researchers have come to the consensus that MSCs can use multiple pathways to generate regulatory lymphocytes and which pathways are more favorable is determined by the microenvironment that MSCs encounter^[21]. Altogether, MSCs modulate immune cells to acquire regulatory phenotype, hence, alter the inflammatory milieu into a tolerogenic one (Figure 1).

There is another advantage of using MSCs for cellular therapy. MSCs have low immunogenicity, implying that MSCs can be used for allogeneic transplantation. This property is particularly helpful to the patient whose MSCs are compromised. Thereby, MSCs possess valuable therapeutic potential to treat immune-mediated disorders^[22].

Although MSCs have demonstrated as a promising immunoregulator for clinical use, the immunomodulatory and low-immunogenicity properties of MSCs are not constitutive. The function of MSCs is based on the signals from the vicinity. MSCs, in the absence of tumor necrosis factor (TNF)- α and IFN- γ may adopt pro-inflammatory phenotype, which activate T cells to response. On the contrary, when MSCs are exposed to high level of TNF- α and IFN- γ they will behave as an anti-inflammatory regulator by producing TGF- β 1, IDO, and PGE2^[23]. Likewise, depending on the level of IL-6, MSCs can convert monocyte into M1 or M2 macrophages^[22,24-26]. Thus, before any clinical application, the plasticity of MSCs should be carefully considered. In this review, we summarized current understandings on how MSCs interact with regulatory lymphocytes, T_{reg} and B_{reg} cells particularly, to attenuate autoimmunity, and how this knowledge can contribute to therapeutic development.

T_{reg} LYMPHOCYTE

The notion of "suppressive" T cells has long been proposed in 1970s. Due to technical limitation, their identities and phenotypic characteristics cannot be described until 1995, Sakaguchi *et al.*^[27] isolated a unique CD4⁺ CD25⁺ T cells that can suppress immune responses and maintain immunologic self-tolerance^[28]. Later, this subpopulation

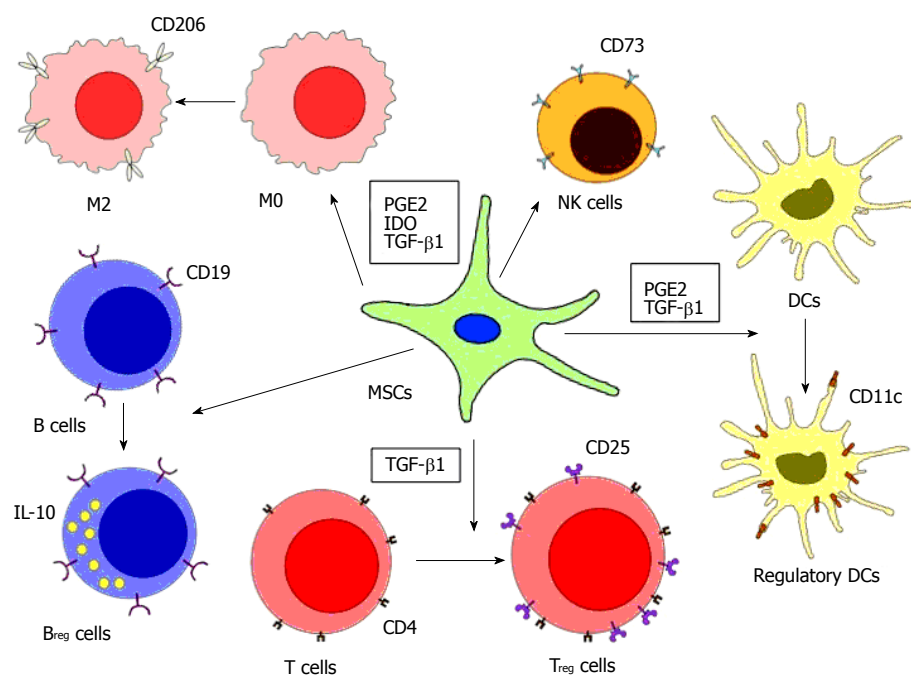


Figure 1 Immunosuppression by mesenchymal stem cells. MSCs suppress innate and adaptive immune responses by enhancing regulatory immune cells with tolerogenic properties. MSCs suppress macrophages by favoring monocyte polarization to anti-inflammatory M2 macrophages, increasing the production of IL-10, and decreasing the production of TNF- α and IL-12. MSCs can also regulate DCs by downregulating the expression of MHC, CD40, CD80, CD83 and CD86, thus, diminishing their antigen presenting ability, while upregulating the expression of IL-10. MSCs can reduce the NK cell cytotoxicity and decrease their production of TNF- α and IFN- γ . T_{reg} and B_{reg} cells can be induced by MSCs, further increase the production of anti-inflammatory cytokines (IL-10 and TGF- β 1). However, the mechanisms of how B_{reg} cells are induced by MSCs are still not clear. MSCs: Mesenchymal stem cells; TNF: Tumor necrosis factor; IL: Interleukin; NK: Natural killer; DCs: Dendritic cells; IFN- γ : Interferon- γ ; T_{reg}: Regulatory T; B_{reg}: Regulatory B; TGF: Transforming growth factor; PGE2: Prostaglandin E2; IDO: Indoleamine 2,3-dioxygenase.

of T cells was named as T_{reg} cells. For those T_{reg} cells that undergo maturation in thymus, are referred to as thymus-derived T_{reg} (tT_{reg}) cells. Three days post-maturation, tT_{reg} cells will relocate from thymus to periphery^[29]. Surprisingly, tT_{reg} cells only comprise 5%-10% of peripheral T cells, but they are the critical regulator of autoimmunity. This is evidenced in mice lacking peripheral T_{reg} cells. They were lethal due to various autoimmunity enhancements^[29,30].

Apart from tT_{reg} cells, T_{reg} cells can also be generated in periphery^[31,32]. Periphery-derived T_{reg} (pT_{reg}) cells are converted from naïve T cells (CD4⁺CD25⁺Foxp3⁺CD45RB^{hi}). Upon activation of naïve T cells and in the presence of particular cytokines, two main types of T_{reg} cells can be differentiated in the periphery and *in vitro*, namely, T helper 3 (Th3) cells and type 1 regulatory T (Tr1) cells. Th3 cell and Tr1 cell differentiation are promoted by TGF- β and IL-10, respectively^[33-35]. Both Th3 and Tr1 cells are suppressive to effector and memory T cells, and they are able to secrete cytokine for self-activation. However, one distinct phenotypical difference is Th3 cells are Foxp3⁺ whereas Tr1 cells are Foxp3⁻.

Forkhead box P3 (Foxp3) is a transcription factor that constitutively express in tT_{reg} cells and some types of pT_{reg} cells. It has been recognized as the master regulator of T_{reg} cells. Scurfy, a Foxp3 gene mutated mouse, is lethal by one month after birth, displays hyperactivation of CD4⁺ T cells and overproduction of proinflammatory cytokines^[36]. In human, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is X-linked recessive disorder caused by mutation in

Foxp3 gene^[37]. T_{reg} cells from the patients with IPEX are either dysfunction or completely vanished. As a result, IPEX patients are afflicted with various autoimmune diseases, allergy and/or inflammatory bowel disease^[38]. The provoked inflammation on IPEX patients indicates the failure of immune tolerance. Foxp3 promotes its regulatory effect by enhancing the expression of IL-2 receptor (CD25), cytotoxic T cell-associated antigen-4 (CTLA-4), and glucocorticoid-induced TNF receptor family-related protein (GITR), meanwhile suppressing the production of IL-2, IL-4 and IFN- γ ^[39]. T_{reg} cells monitor the inflammatory status by the exogenous level of IL-2. Binding of IL-2 to CD25 would enhance the expression of T_{reg}-cell associated genes and regulate the inflammation by suppressing effector T cell proliferation or by altering the function of antigen presenting cells^[40]. Retroviral transfer of Foxp3 to naïve T cells (CD4⁺CD25⁺Foxp3⁻) can upregulate the expression of some T_{reg} cell-associated genes, including CD25, CTLA-4, GITR and CD103, and the Foxp3-transduced T cells were shown to be suppressive^[41]. Altogether, Foxp3 is critical to the function and the development of T_{reg} cells and to a greater extent, the maintenance of immune homeostasis^[42,43].

T_{reg} LYMPHOCYTE INDUCTION BY MSCs

MSCs are able to induce Foxp3⁺ T_{reg} cell population *in vitro* and *in vivo*. So far, several mechanisms have been proposed, including: (1) secretion of soluble mediators; (2) cell-cell interaction; and (3) modulation of antigen

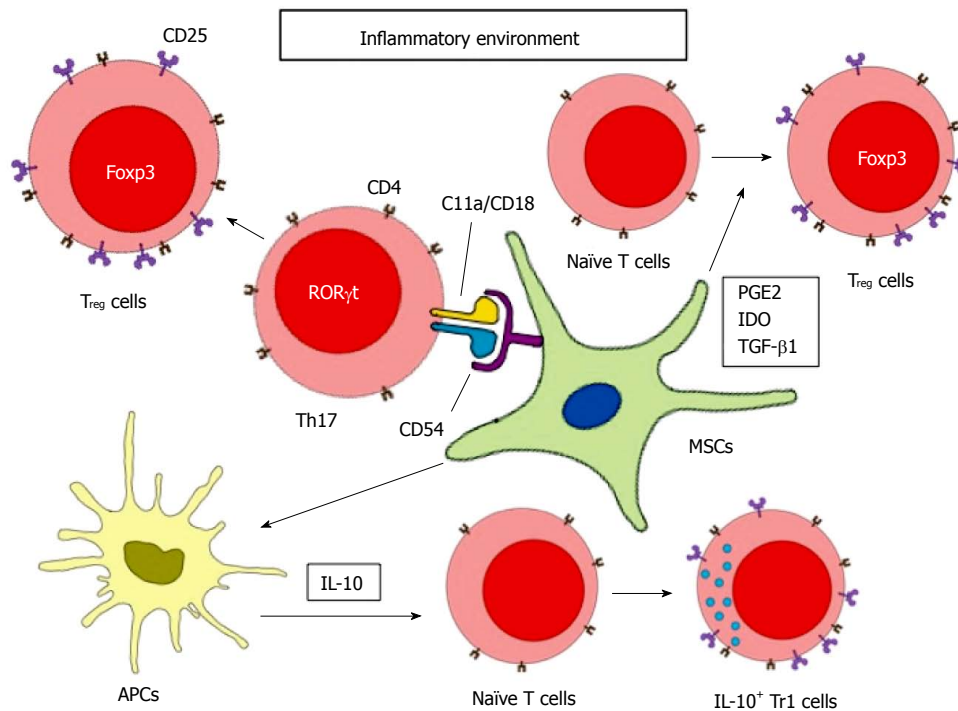


Figure 2 Mesenchymal stem cells-mediated regulatory T cell induction. MSCs induce T_{reg} cells through soluble mediators stimulation, cell-cell interaction, and modulation of antigen-presenting cells. Under inflammatory environment, MSCs secrete TGF- β 1, PGE2 and IDO to facilitate the differentiation of naïve T cells to Foxp3 $^{+}$ T_{reg} cells. MSCs can also interact with Th17 cells by direct contact via CD54 and C11a/CD18. With the presence of PGE2, differentiated Th17 cells can be converted to functional Foxp3 $^{+}$ T_{reg} cells. MSCs can increase the secretion of IL-10 by antigen presenting cells, which will then induce Tr1 cells differentiation. MSCs: Mesenchymal stem cells; IL: Interleukin; T_{reg} : Regulatory T; TGF: Transforming growth factor; PGE2: Prostaglandin E2; IDO: Indoleamine 2,3-dioxygenase.

presenting cells (Figure 2).

Secretion of soluble mediators

TGF- β 1: MSCs can secrete TGF- β 1 to promote T_{reg} cell differentiation, especially when MSCs are placed in an inflammatory environment^[21]. TGF- β 1 is a potent immunosuppressor secreted by every leukocyte lineages, including macrophages, DCs, NK cells, T cells and B cells. Both TGF- β 1 knockout mice and T-cell specific TGF- β receptor II knockout mice develop severe autoimmunity, leading to multiple organs failure and death, suggesting the importance of TGF- β 1 in regulating peripheral tolerance^[44,45]. Generally, TGF- β 1 can suppress the proliferation of T cells, the activation of B cells, the maturation and antigen presentation of DCs, the cytotoxicity of NK cells, and phagocytic effect of macrophages^[46]. Moreover, as mentioned earlier, TGF- β 1 is able to convert naïve T cells to Foxp3 $^{+}$ Th3 cells, although such conversion seems to be concentration-dependent. High concentrations of TGF- β 1 suppresses the expression of IL-23R and shifts the conversion to Foxp3 $^{+}$ Th3 cells, whereas at lower concentrations and in the presence of IL-6 and IL-21, the expression of IL-23R is enhanced and results in ROR γ t $^{+}$ Th17 differentiation^[47]. In addition, neutralizing TGF- β 1 reduced mRNA and protein level of Foxp3 and CD25, further confirms its essential role in promoting T_{reg} cell differentiation^[48]. In conclusion, MSCs-secreted TGF- β 1 not only acts as a suppressor of innate and adaptive immune response, it can also induce development of T_{reg} cells from

naïve T cells, which further enhance the regulatory effects.

PGE2: MSCs can also secrete PGE2 to induce T_{reg} cells. PGE2 plays a major role in suppressing chronic inflammation. PGE2 can reduce IFN- γ production of NK cells, limit the phagocytic ability of macrophages and interfere early activation of B cells^[49-52]. Although PEG2 can suppress early development of DCs, it is surprising that PGE2 also stabilize matured DCs and enhance its antigen presenting capacity^[53-55]. Moreover, despite PGE2 is able to shift the differentiation of naïve T cells from Th1 to Th2 cells, PGE2 also promote proinflammatory Th17 cell development by elevating IL-23 production^[56]. Thereby, PEG2 is not exclusively anti-inflammatory. It also possesses the ability to provoke inflammation. Nevertheless, like TGF- β 1, PGE2 can induce Foxp3 $^{+}$ T_{reg} cell differentiation and it is one of many soluble mediators that produce by MSCs. Diminishing PGE2 signaling when co-culture CD4 $^{+}$ T cells with MSCs by antagonist indomethacin fail to upregulate Foxp3 and CD25 expression. In fact, when inhibiting both TGF- β 1 and PGE2 signaling, the expression of Foxp3 and CD25 further decreased^[48]. Furthermore, after transferring adipose tissue-derived MSCs in asthmatic mice, the number of infiltrated inflammatory cells was significantly reduced and no obvious goblet cell hyperplasia was found in the lung. Meanwhile, the number of T_{reg} cells was elevated. When TGF- β 1 neutralizing antibodies or indomethacin was added to MSCs-treated asthmatic mice, the anti-

inflammatory effects promoted by MSCs as well as the T_{reg} cell expansion. These results demonstrated the necessity of TGF- β 1 and PGE2 for T_{reg} cell induction as well as the anti-inflammatory effect of MSCs^[57].

IDO: IDO is a rate-limiting enzyme that catalyzes the degradation of tryptophan *via* kynurenine pathway. IDO is expressed in various cell types, including macrophages, DC and MSCs. Interestingly, IDO expression can be induced by IFN- γ and other proinflammatory cytokines. Munn *et al*^[58] treated pregnant mice carrying allogeneic or syngeneic fetus with 1-methyltryptophan, an IDO inhibitor. As a result, allogeneic, but not syngeneic, fetuses provoked severe immune rejection^[58]. Also, some studies suggested the association of tryptophan catabolism with inhibition of T cell proliferation, emphasizing its tolerogenic potential^[59,60]. In addition, kynurenines, a tryptophan catabolite, can promote T_{reg} cell induction^[61]. Infusion of MSCs to kidney allograft murine model prevented graft rejection, and the T_{reg} cell population was elevated. In contrast, allograft tolerance and T_{reg} cell expansion diminished when the recipients were treated with IDO-deficient MSCs. These results demonstrated the importance of IDO in MSCs-mediated Treg cell induction and graft tolerance^[62]. Other soluble factors, like human leukocyte antigen-G5 and haem oxygenase 1, are also shown to be involved in MSCs-mediated T_{reg} cell induction^[63,64]. However, the underlying mechanisms are not clear. More studies need to be done in order to further increase the efficacy of MSCs-based therapy and to reveal the potential risk that could cause to the patients.

Cell-cell interaction

Apart from soluble mediators, cell-cell interaction is also important to the modulatory function of MSCs and T_{reg} cell induction. MSCs are known to express adhesion molecules on their surface, although only low level of expression can be detected in normal condition. However, after placing MSCs in inflammatory conditions, adhesion molecules, ICAM-1 and VCAM-1, chemokine ligands of CCR5 and CXCR3 are upregulated. Through these molecules, T cells are attracted and anchored to MSCs. With close proximity, adhesion molecules co-operate with IDO and NO, suppress T cell activity by inducing their apoptosis or cell arrest^[65-68]. It is also worth to note that MSCs can inhibit the expression of ICAM-1, CXCR3 and α -integrin on CD3⁺ T cell, reduced the interaction between T cells and endothelial cells, thus, disrupted T cells from infiltrating into CNS^[69]. On the other hand, MSCs can attach to Th17 cells *via* CCR6 and CD11a/CD18 and facilitate Th17 to adopt regulatory phenotype^[70]. Moreover, when co-culture MSCs with CD4⁺ T cells in transwell system; T_{reg} cells cannot be induced, even in the presence of PGE2 and TGF- β ^[48]. These results further confirmed cell-cell interaction is essential to the overall suppressive effect of MSCs. However, T_{reg} cell induction ability was recovered if MSCs were co-cultured with peripheral blood mononuclear

cells instead of isolated CD4⁺ T cells, suggesting there is an alternative pathway that does not require cell-cell contact, and it is likely, through soluble mediators in peripheral blood mononuclear cells^[48].

Modulation of antigen presenting cells

Increasing evidence has indicated MSCs are able to shift macrophages, DCs and NK cells to a regulatory phenotype and alter their cytokines production. For example, MSCs skew monocyte toward M2 macrophage differentiation. Subsequently, M2 macrophages secrete CCL18 and IL-10 to exert suppressive response and induce T_{reg} cell differentiation^[26]. As discussed above, IL-10 is able to induce naïve T cell to Foxp3⁺ Tr1 cell, which secrete high level of IL-10 and TGF- β to modulate the inflammatory microenvironment. Interestingly, although MSCs express neither IL-10 nor its receptor, MSCs are able to induce NK cells, DCs, macrophages, T cells and B cells to produce IL-10^[5,10-12,17]. In addition, IL-10 is a powerful anti-inflammatory cytokine that suppresses antigen-specific immune responses, reduces pathological immune responses and promotes allograft tolerance.

In conclusion, the mechanisms underlying MSCs-mediated T_{reg} cell development are complicated, which involve synthesis and secretion of multiple mediators, direct interaction with target cells and modulation of certain antigen-presenting cells. Apparently, there is no single pathway that governs the whole induction process, indicating that MSCs possess certain degree of plasticity. Regardless of how T_{reg} cells are enhanced by MSCs, MSCs-activated T_{reg} cells play a significant role on immunoregulation and affect a wide spectrum of immune responses^[43,71,72]. Certainly, T_{reg} cells can massively amplify the immunomodulatory effect of MSCs. However, the mechanism in regard to T_{reg} cell induction is far from elaborate and additional researches are required.

B_{reg} LYMPHOCYTE

In recent decade, B_{reg} cells were being intensively investigated due to its immunosuppressive effect on excessive inflammation. Like T_{reg} cells, B_{reg} cells can produce anti-inflammatory cytokines, like TGF- β and IL-10. Among these, IL-10 is strongly associated with B_{reg} cells since depleting IL-10-producing B cells result in chronic inflammation, outgrowth of proinflammatory T cell after autoimmune induction^[73-75]. But unlike T_{reg} cells, there is no "master regulator" being identified in B_{reg} cells, which complicated the process of B_{reg} cell classification. So far, there are several B cell subsets have been identified as B_{reg} cells in mice. They are CD5⁺CD1d^{hi} B (B10) cells and Tim1⁺ B cells^[76-78]. In human, there is CD19⁺CD24^{hi}CD38⁺CD1d^{hi} B cells and CD19⁺CD24^{hi}CD27⁺ B cells^[79,80]. B_{reg} cells control inflammation by suppressing IL-12 secretion from DCs, thus inhibiting Th1 and Th17 differentiation^[81]. Through

the secretion of TGF- β , B_{reg} cells can induce CD4⁺ T cell apoptosis and anergy in CD8⁺ cytotoxic T cells^[82,83]. Recent studies indicated that B_{reg} cells play a role in T_{reg} cell development and function. As B_{reg} cells are one of the major sources of IL-10, which drive Tr1 differentiation, it is not surprising that B_{reg} cells can expand T_{reg} cell population during inflammation. Additionally, when B cell specific IL-10 defective mice (DBA/1IL-10 KO^{-/-} mice) were induced with arthritis, the percentage of Tr1 was significantly decreased, indicating effects of IL-10⁺B_{reg} cells on T_{reg} cell formation^[75]. Besides TGF- β and IL-10, recent studies reported that IL-35 is another pleiotropic cytokine that regulate overwhelming inflammation and autoimmunity^[84,85]. Antigen-driven proliferation assay revealed that IL-35 was able to suppress CD4⁺ T cell proliferation^[86]. Treatment with IL-35 ameliorated disease severity and reduced Th1 and Th17 cells in mice with experimental autoimmune uveoretinitis (EAU)^[85]. More importantly, IL-35 can increase T_{reg} and B_{reg} cell populations. Similar to IL-10, IL-35-induced T_{reg} (iT_{reg}35) cells are Foxp3⁺. However, adoptive transfer of iT_{reg}35 cells to various autoimmune disease animal models has sufficiently alleviated their clinical severity, and the effect was comparable to tT_{reg} cells-treated mice^[35]. On the other hand, when recombinant IL-35 was injected into the EAU mice, the frequency of B220⁺ IL-10⁺B_{reg} cells, IL-35⁺B_{reg} cells and B10 cells were upregulated in the spleen and draining lymph nodes^[85]. Collectively, B_{reg} cells exhibit anti-inflammatory and immunoregulatory effects, at least in part, by secreting multiple anti-inflammatory cytokines (TGF- β , IL-10 and IL-35), promoting differentiation of other regulatory cells, and inhibiting the proliferation and function of effector T cells.

B_{reg} LYMPHOCYTE INDUCTION BY MSCs

Although MSCs do not constitutively express IL-10, and currently there is no evidence to indicate that MSCs produce IL-35, several studies have reported that MSCs induce IL-10⁺B_{reg} cell differentiation in mouse model^[87-89]. Our group studied the effects of human bone marrow-derived MSCs in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, and observed attenuation of clinical severity and neuroinflammation; and excitingly, these were associated with expansion of CD1d^{hi} CD5⁺ B_{reg} cells after MSCs administration^[87]. Subsequently, another study demonstrated intravenous infusion of adipose tissue-derived MSCs to Roquin^{san/san} mice, an animal model of systemic lupus erythematosus (SLE), lead to increased numbers of B10, B10pro and naïve T_{reg} cells^[89]. Moreover, the MSCs-mediated B_{reg} cell induction is not restricted to murine models. Administering MSCs into refractory chronic graft vs host disease (cGvHD) patients have improved patients' overall clinical conditions. Consistent with murine models, MSCs increased the frequency and the function of CD5⁺ IL-10⁺B_{reg} cells by enhancing their proliferation and survival^[88]. Momentarily, we are still not clear about the mechanism regarding to MSCs-

mediated B_{reg} cell induction. It is worthwhile to ask whether the induction is IL-35 or IL-10-dependent since MSCs can induce IL-10 production by T_{reg} cells, DCs, and M2 macrophages, implying the possibility of creating a positive feedback loop for B_{reg} cell generation. Further understanding the mechanisms of how MSCs induce T_{reg} and B_{reg} cells can definitely contribute to the therapeutic development of MSCs and further improve their potential therapeutic efficacy.

THERAPEUTIC POTENTIAL OF GENETIC ENGINEERED MSCs

MSCs contain multiple properties that are suitable for therapeutical use. Wide-spectrum of differentiation capacity made it a perfect candidate for regenerative medicine. MSCs have been used to generate cartilage, bone, liver, intervertebral disc, and cardiac tissue^[90]. Recent reports have suggested using MSCs for neural cell replacement. However, rather than direct neural differentiation, MSCs tend to recruit neural progenitor cells (NPCs) to the injury sites and support NPCs proliferation and differentiation^[91]; Immunomodulatory properties of MSCs are potentially useful for the treatment of autoimmune diseases and GvHD. Transplanted MSCs suppressed the proliferation and activation of T cells and NK cells in type 1 diabetes animal model. Also, the level of IFN- γ and TNF- α were reduced. When MSCs were co-transplanted with pancreatic islets, MSCs protected grafted islets from immunorejection and secreted various trophic factors to promote graft vascular network^[92,93]. Another intriguing advantage of using MSCs to treat immune diseases is that, unlike traditional immunotherapy in which a certain modulator act on a particular pathway, MSCs elicit their suppression on multiple immune cell types *via* various mechanisms. Although the immunosuppressive effects of MSCs appear very promising, further investigations are required to elucidate the underlying mechanisms, so as to prevent complications and maximize the therapeutic efficacy.

One current issue on immunotherapy is that a particular modulator or antibody may be seemingly effective, however, the therapeutic efficacy is limited since such modulator may also compromise certain cells or mediators beneficial to the disease recovery. Rituximab, for example, is a CD20 neutralizing antibody and it is believed to be an effective treatment for B and T-cell-mediated diseases, such as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus^[94-96]. Rituximab-induced B-cell depletion depends on the expression of CD20 on the cell surface, but the expression of CD20 gradually disappeared upon plasma cell differentiation^[97,98]. Moreover, B_{reg} cells were also depleted, thus, exacerbates the disease symptoms^[73]. In EAE, B10 cells play an important regulatory role during the initiative phase whereas they are less involved at the late phase of the disease^[99,100]. Therefore, depleting B cells by rituximab at the early phase have a potential risk of worsening the

clinical conditions. As a consequence, it is necessary to develop an alternative strategy.

The immunosuppressive properties of MSCs on different murine autoimmune disease animal models support its potential clinical application. However, the immunomodulatory secretome of MSCs vary and greatly rely on the host inflammatory environment^[21]. To minimize this uncertainty, a novel therapeutic strategy, in which MSCs are genetically engineered with defined immunoregulatory cytokines, has been developed. Transplantation of IL-10-engineered adipose-derived MSCs attenuated EAE by reducing the number of immune cell infiltration to the CNS, decreasing the secretion level of IL-17A, TNF- α and IL-2, and inhibiting antigen-presenting function of DC^[101]. Since the immunosuppressive effect of MSCs is enhanced if they are placed proximal to the inflammatory area, Liao *et al.*^[102] engineered MSCs with CNS homing ligand genes, P-selectin glycoprotein (PSGL-1) and Sialyl-Lewis^x (SLe^x), along with IL-10 to EAE model. Consequently, EAE was attenuated, CNS homing ability was enhanced and their therapeutic efficacy was increased^[102]. Genetic engineering of MSCs has been well studied in regenerative medicine. Different combination of treatments is documented and aims to redirect the MSCs differentiation propensity. Comparatively, genetic modification of MSCs for the treatment of autoimmune diseases is currently under development. Considering that the effect of MSCs may vary between patients with different severity of neuroinflammation, information on the clinical condition and pathology of the individual patient will probably help to predict treatment efficacy. Moreover, questions like in what phase of a particular disease introducing MSCs can improve the clinical outcome, or to what extent MSCs can elicit their suppressive effect and meanwhile, does not compromise the immunity in response to pathogens or infectious agents, are worthwhile to explore in order to safely use in human patients.

SAFETY AND CONCERNS OF MSCs AS CELLULAR THERAPIES IN PATIENTS

To date, there are nearly 500 ongoing MSC-based clinical trials. They aim to investigate the effectiveness of MSCs on treating different diseases, including GvHD, diabetes, cardiovascular diseases, hematological diseases and neurological diseases^[103]. Although most of these clinical trials reported the patients were well tolerated to the MSC infusion and administration, there are some safety concerns requiring caution^[104]. During *in vitro* expansion, MSCs can give rise to replicative senescence, which may affect the activity of surrounding healthy cells and therefore, reduce the clinical efficacy^[105]. Moreover, although MSCs have low immunogenicity due to the reduced expression of co-stimulatory receptors and major histocompatibility complex (MHC) class II antigens, *in vitro* stimulation of pro-inflammatory cytokines on MSCs can upregulate MHC class I and MHC class II expression, compromising the hypo-immunogenicity property of

MSCs.

CONCLUSION

The immunomodulatory properties of MSCs have been massively studied due to its intriguing suppressive effects on various immunological diseases. Broad-range of immune cells can be regulated by MSCs through a series of soluble mediators stimulation, chemokine attraction, and cell-to-cell interaction. MSCs-induced T_{reg} and B_{reg} cells enhance the immunosuppressive capacity and generate a tolerogenic microenvironment against overwhelmed inflammation. This hypothesis supports the observation that infused MSCs can only survive in the recipient for a short period of time, however, the regulatory effects of MSCs are long lasting, suggesting MSCs may act as an activator or a switcher that initiate certain cells, possibly T_{reg} and B_{reg} cells, to react to the inflammation and at the same time, alter the microenvironment for those cells to sustain their immunosuppressive effects. Although MSCs appear very promising as treatment in experimental models of autoimmune diseases, there are still many challenges need to overcome before MSCs can be widely use in clinical medicine.

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Racial disparity in colorectal cancer: Gut microbiome and cancer stem cells

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Abstract

Over the past two decades there has been remarkable progress in cancer diagnosis, treatment and screening. The basic mechanisms leading to pathogenesis of various types of cancers are also understood better and some patients, if diagnosed at a particular stage go on to lead a normal pre-diagnosis life. Despite these achievements, racial disparity in some cancers remains a mystery. The higher incidence, aggressiveness and mortality of breast, prostate and colorectal cancers (CRCs) in African-Americans as compared to Caucasian-Americans are now well documented. The polyp-carcinoma sequence in CRC and easy access to colonic epithelia or colonic epithelial cells through colonoscopy/colonic effluent provides the opportunity to study colonic stem cells early in course of natural history of the disease. With the advent of metagenomic sequencing, uncultivable organisms can now be identified in stool and their numbers correlated with the effects on colonic epithelia. It would be expected that these techniques would revolutionize our understanding of the racial disparity in CRC and pave a way for the same in other cancers as well. Unfortunately, this has not happened. Our understanding of the underlying factors responsible in African-Americans for higher incidence and mortality from colorectal carcinoma remains minimal. In this review, we aim to summarize the available data on role of microbiome and cancer stem cells in racial disparity in CRC. This will provide a platform for further research on this topic.

Key words: Colorectal cancer; Cancer stem cells; Racial disparity; Microbiome; MiRNA

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Core tip: The role of microbial dysbiosis and cancer stem cells (CSCs) in colorectal cancer (CRC) has been studied extensively, however, their implication in racial disparity is not well known. A number of recent studies have shown that different dietary patterns affect gut microbiome. Likewise, dietary patterns also affect intracellular regulatory events which may affect the function of CSCs. Our objective is to consolidate the available data, on the role of gut microbiome and CSCs in racial disparity in CRC, explore a link between them and lay a foundation for further advances.

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INTRODUCTION

Colorectal cancer (CRC) remains the second leading cause of cancer related mortality in the United States. However, the incidence and mortality of colon cancer is different among various racial and ethnic groups. African Americans (AAs) share the largest burden of CRC in the United States. Data from Surveillance, Epidemiology and End Results (SEER) revealed that the age-adjusted incidence of CRC in AAs, based on cases diagnosed between 2008 and 2012 was 52.3 per 100000 for men and women combined per year, compared to 41.5 per 100000 for men and women combined per year among Caucasian Americans (CAs). Similarly, the age-adjusted mortality from CRC in AAs, based on cases diagnosed between 2008 and 2012 was 21.4 per 100000 for men and women per year, compared to 15 per 100000 for men and women per year in Whites/CAs^[1]. AAs not only tend to be diagnosed at a younger age but also have a worse prognosis than CAs^[2,3]. Many genetic, epigenetic and environmental factors have been reported that are responsible for this racial disparity.

In recent years, there has been an increased focus on differences between microbiota of colon of healthy individuals and of patients with CRC. A relationship between microbial dysbiosis and CRC is now well established^[4-7].

The concept that pluripotent and self-renewing cancer stem cells (CSCs) have a pivotal role in the development and progression of many malignancies, including CRC is now well accepted. We have reported a higher proportion of CSCs (specifically CD44⁺ CD166⁺ phenotype) in AAs, who also had a significantly higher

number of adenomas, compared to CAs^[8]. However, underlying regulatory mechanisms remain unknown.

Recent studies have shown that host can alter gut microbiota through external (diet, obesity, etc.) and internal factors (microRNAs in intestinal epithelial cells)^[9]. MicroRNAs (miRNAs) have also been reported to regulate CSCs^[10]. Thus it is possible that the gut microbiota and CSCs are not entirely isolated domains and in AAs, the higher frequency of unfavorable mutations, through miRNAs, facilitates pathogenic microbiota over commensal bacteria (Figure 1).

DIETARY REGULATION OF MICROBIOTA AND RACIAL DISPARITY IN CRC

Human gut is a major harbinger of a wide variety of microbial cells containing approximately 10^{14} cells estimating 1000 species. The dominant composition is bacteria with 90% of species belonging to Firmicutes and Bacteroides^[11]. These bacteria are in a symbiotic relationship with the intestine, utilizing undigested nutrients as substrates and in return produce various vitamins, amino acids, transform bile salts and assist in maintenance of intestinal barrier, appropriate immune response against pathogens^[12]. This homeostasis is altered in a state of dysbiosis, which is overgrowth of pathogenic bacteria that are normally inhibited by commensal bacteria.

Numerous studies have been performed to examine whether and to what extent the dietary changes may affect gut microbiota. In general, these studies suggest that changes in diet and their interaction with gut microbiota exert profound effects on intestinal homeostasis through various metabolites^[13]. Emergence of metagenomic sequencing has enabled identification of microorganisms not possible with 16S ribosomal RNA gene (*rRNA*) sequence-based methods and traditional culture methods^[13-15]. Collective genomes of the members of a microbial community are analyzed against widely available microbial databases, thus allowing identifying microbial communities, which are virtually uncultivable. This has led to discovery of hundreds of microbial genus not previously known to exist in the human gut.

Qin *et al*^[11] published a comprehensive catalogue of human gut microbial genes in 2010. Among the various conclusions, one was that *Fusobacterium* genus is not an abundant constituent of the normal gut microbiota. It is a genus of anaerobic gram-negative bacilli and has been known to cause periodontal disease. *Fusobacterium* species esp. *F. nucleatum* has been isolated from colon and fecal samples of patients with CRC in multiple studies^[16-18]. Castellarin *et al*^[19] even found a positive association between *Fusobacterium* and lymph node metastases.

Gao *et al*^[4] examined microbiota from cancerous tissues of CRC patients and found a significant abundance of Firmicutes and *Fusobacteria* compared to

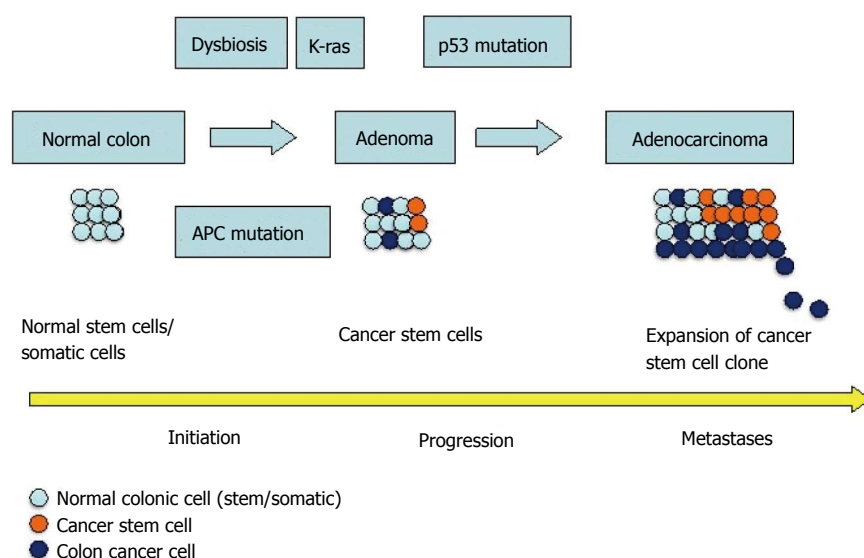


Figure 1 Cancer stem cells during development and progression of colorectal cancer. Schematic representation of the role of dysbiosis caused by microbiome alterations and accumulation of mutations in colonic stem cells leading to development and progression of colorectal cancer. APC: Adenomatous polyposis coli.

healthy individuals. Interestingly they also found that *Proteobacteria* was less abundant in patients with CRC. In the first large series sequencing of stool samples, Sobhani *et al*^[5] reported that *Bacteroides/Prevotella* were markedly increased in patients with CRC.

Dietary components like vegetables, fiber, vitamin D are shown to be associated with a lower risk of colon cancer whereas red meat and a diet rich in saturated animal fat has been shown to be responsible for an increased risk of colon cancer^[20,21]. Two major biotransformation pathways for dietary components mediated by microbiome have been reported.

A diet rich in fiber stimulates saccharolytic fermentation and production of short chain fatty acids (SCFAs) namely butyrate, acetate and propionate. These metabolites, particularly butyrate have anti-inflammatory, anti-proliferative and antineoplastic properties, while a fat rich diet stimulates the synthesis and release of bile acids in the gut^[22,23].

In their study involving four racial groups, Hester *et al*^[24] found that SCFA level was lower in stool from African-Americans than other racial groups. Interestingly, they also found a decreased prevalence of bacteria of *Lachnospiraceae* family in stool from African-American patients. Bacteria of *Lachnospiraceae* family have been previously shown to be associated with butyrate production in colon tissue^[25]. A summary depicting bacteria, whose presence has been shown to have or probably has a positive or negative association with colon cancer in AAs has been shown in Table 1.

It has been widely reported that the higher amount of butyrate is seen in stool of healthy controls than CRC patients^[26]. On the other hand secondary bile acids have been postulated to have a carcinogenic role^[27].

Ou *et al*^[28] examined stool from healthy AAs and from age and sex-matched native Africans and found a higher concentration of fecal secondary bile acid in AAs and a higher concentration of short-chain fatty acids in native Africans. Although the reason(s) for these increases are not known, it is possible that changes in

dietary habits are responsible for these differences.

Majority of the primary bile acids are returned to the liver by the enterohepatic circulation. A fraction of the primary bile acids escapes the enterohepatic circulation and reaches the colon. In the colon, 7-DHC (Dehydrocholesterol), converts primary bile acids into secondary bile acids, like deoxycholic acid and lithocholic acid. There is plenty of evidence to suggest that when exposed to high levels of bile acids, gastrointestinal cells undergo oxidative and nitrosative stress leading to anti-apoptotic and mutagenic properties^[29]. De Boever *et al*^[30] demonstrated the protective effect of *Lactobacillus* against bile salt cytotoxicity. Many studies have found that African-Americans have a lower prevalence of *Lactobacillus*, compared to other racial groups^[31,32].

Moore and Moore^[31] studied the stool microbial composition in populations with higher (CAs, patients with polyp) and lower CRC risk (South African blacks, native Japanese). They found a positive association of *Bacteroides* and *Bifidobacterium*, and a negative association of *Lactobacillus* and *Eubacterium aerofaciens*, with colon cancer risk.

These studies provide ample evidence that a variation in microbial composition between ethnic groups may partly be responsible in colorectal carcinogenesis and that diet plays a role in this microbial diversity.

CSCS AND RACIAL DISPARITY IN CRC

CSCs

According to the stem cell model of carcinogenesis, only some cells in a tissue possess the ability to initiate and sustain tumor growth. These cells, characterized as CSCs have two important properties: Indefinite proliferation and pluripotency (ability to differentiate itself into more than one cell lineage)^[33]. The critical role of CSCs in initiation, development and progression of CRC is now well established^[34]. Mutations in normal stem, progenitor or terminally differentiated cells, are believed to be responsible for origin of CSCs, but the

Table 1 Depicting bacterial genus/families, whose presence has been shown to have or probably has a positive or negative association with colon cancer in African-Americans

Positive association	Negative association
Fusobacterium	Lactobacillus
Firmicutes	Lachnospiracea
Bacteroides	Eubacterium
Bifidobacterium	

processes responsible are not completely known.

Colon stem cells are believed to exist as undifferentiated cells at the base of the crypt of lieberkuhn in the proliferative zone. The undifferentiated cells differentiate in to specialized cells as they move up the crypt-villic axis towards the luminal surface. It is estimated that, in human adults in every square centimeter of colon, there are about 14000 crypts and at a given rate of 5 d for colonic epithelium renewal; over 6×10^{14} colonocytes are produced during the individual lifetime^[35,36]. The lifelong proliferation of the stem cells makes them more prone to accumulation of mutations than other short-lived cells.

Various pathways tightly regulate the processes involved in maintenance of a normal intestinal epithelium. The central among those is the canonical Wnt pathway. Canonical Wnt signals are transduced through an interaction with Frizzled family receptors (Fz) and LRP5/LRP6 (low-density lipid receptor) co-receptor to the β -catenin signaling pathway. In the absence of Wnt signaling, β -catenin becomes a part of a multiprotein degradation complex, containing tumor suppressor gene product adenomatous polyposis coli (APC), scaffold protein Axin and is phosphorylated by casein kinase I α and glycogen synthase kinase 3 β , and then ubiquitinated for subsequent proteasome degradation.

In the presence of Wnt signaling, after signal transduction, Axin is recruited to cell membrane by a Fz-Disheveled (DVL) or LRP5/6 interaction. This leads to degradation of the degradation complex described above and β -catenin buildup in the nucleus. This stable nonphosphorylated β -catenin complexes with several factors and leads to activation of the transcription of several genes like *c-Myc*, *CD44*, *CCND1*, essential for DNA replication, cell cycle control and altered mitosis^[37,38].

Characterization of CSCs: Identification of CSCs is a challenging task given the complexity of the cell surface markers, and their difference between various tissues, apart from the technical issues involved. One of the methods used to identify CSC is by the cell surface markers, also known as epitopes. Colonosphere formation, a functional assay is also used to characterize CSCs.

Colon CSCs have been identified by expression of numerous surface epitopes CD133, CD24, CD44, CD166, EPCAM (epithelial specific antigen/ESA), *etc*^[39]. CD166 expression has been linked with shortened survival^[40]. Similarly, CD44's role in tumor invasiveness

and progression has prompted it to be described as a potential CSC marker for CRC^[41]. It has been shown that CSCs form tumors in SCID mice at much-diluted concentrations, which histologically resemble the primary tumor^[42].

We studied the role of CD44, CD166 and ESA in CRC and reported their expression in premalignant adenomatous polyp^[43] and also showed an age dependent increase in their expression, suggesting their role in tumor development and progression^[43].

We have also recently observed that CD44⁺CD166⁺ cell proportion in the colonic effluent as well as in the colonic mucosal cells is significantly increased in AAs with adenomas than CAs. We also observed that the colonic effluent from high risk AAs (more than 3 adenomas) contained markedly higher proportion of CD44⁺ CD166⁺ cells than low risk AAs (subjects with no adenomas). We were not able to duplicate these results in colonic effluent from white population^[8]. Taken together, the above observations point towards the substantial role of CSCs, not only in higher incidence, but also progression of CRC in AAs.

Racial disparity in miRNAs and signaling pathways regulating CSCs:

According to the well-accepted Fearon and Vogelstein model of CRC progression, development of CRC is an outcome of accumulation of mutations in tumor suppressor genes, oncogenes; and accumulation of changes is more important than the sequence of changes^[44]. This is also the basis of "adenoma-carcinoma model" in which *APC* gene mutation initiates the sporadic CRC, which accounts for 80%-85% CRC, followed by mutations in other genes-notably *K-ras*, deleted in colorectal carcinoma and *p53*^[45]. Mutated *APC*, in association with β -catenin up regulates many oncogenes, notably *CCND1* and *c-myc*^[46,47]. We have recently reported that AAs had higher (48%) number of adenomas, recorded during colonoscopy, than CAs^[8]. These findings confirm the data in separate studies by Corley *et al*^[48] and Lebwohl *et al*^[49]. In line with the Fearon and Vogelstein model, one of the reasons, in AAs, for a higher incidence of CRC could be the higher number of adenomas in them.

We also examined the rates of mutation of APC and β -catenin, in a small cohort of AAs ($n = 10$) and CAs ($n = 10$). The agarose gel electrophoresis of the PCR products of wild type and mutant *APC* gene (hAPC) in colonic biopsies from 5AAs and 6 CAs without adenomas is shown in Figure 2. Out of 10 AAs, 2 showed mutation in *APC* gene, whereas none of the CAs showed mutation in the gene. Similarly, 3 AAs showed mutation in β -catenin, as compared to none of the CAs. This preliminary data clearly supports the role of APC and β -catenin mutations in higher incidence of CRC in AAs.

MicroRNAs (miRNAs) are an expansive class of small non-coding RNAs, 18-23 nucleotides long, and regulate gene expression, either by translational repression or by mRNA degradation through cleavage. MiRNAs are atypically expressed in numerous pathological states,

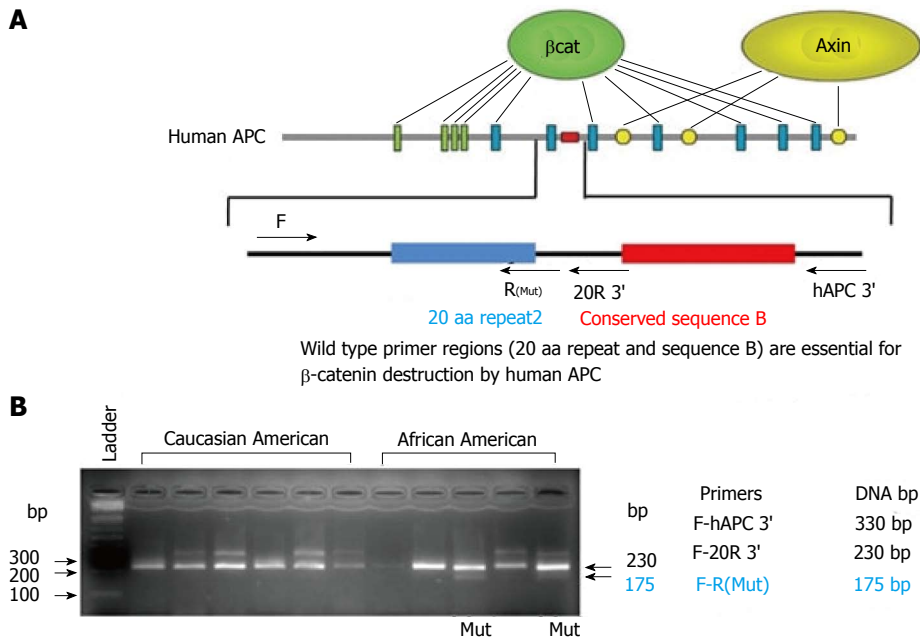


Figure 2 Schematic representation of human APC gene and design of appropriate primers for the wild type and mutant gene. A: Human APC gene with β-catenin (green and blue bars) and Axin (yellow circles) binding sites. Red bar represents conserved sequence of APC gene. Forward (F) and reverse (R) primers were designed to demonstrate mutation in APC gene; B: Agarose gel electrophoresis of PCR products showing higher rate of APC gene mutation (Mut: 175 bp) in the colonic mucosa of AAs without adenomas than their CA counterparts. AAs: African Americans; CA: Caucasian American; APC: Adenomatous polyposis coli.

and depending on the target, may work as oncogenes or tumor suppressors. MiRNAs' role in CRC regulation through CSCs is well researched^[50-52]. We have examined the role of miRNAs 21 and 145 in regulating colon CSCs and reported that the expression of miR-21 is significantly increased and that of miR-145 markedly decreased in chemotherapy-resistant colon cancer cells, highly enriched in CSCs^[53]. In colon cancer cells, forced expression of miR-145, significantly inhibits CSCs and tumor growth, whereas up-regulation of miR-21 augments the same^[8]. We have also shown the role of miR-21 in age related rise of colon cancer. Upregulated miR-145 was associated with reduced levels of CD44, and β-catenin^[53], both of which, we have been shown to be independently associated with racial disparity of CRC.

These observations led us to explore the role of miR-21 in ethnic differences in CRC and/or its precursor, adenoma. Ongoing studies (unpublished data) from our lab revealed that miR-21 levels in normal looking colon mucosa of AAs with adenomas were significantly higher than their CAs counterparts.

Mutation in *K-ras* gene, second most common in CRC progression, is not required for initiation of CRC. Reduced expression of miR-145 has been shown to contribute to CRC development through K-ras expression^[54]. We have reported that K-RAS' lack in chemo-resistant colon cancer cells upregulates miR-145, downregulates miR-21, as well as disrupts the negative cooperation among miR-21 and miR-145^[53]. Epidermal growth factor receptor (EGFR) is another transmembrane protein, whose role is well established in CRC pathogenesis. We have reported that EGFR inhibitor Cetuximab decreases miR-21 expression, suggesting another link between stem cells and definitive

mutations in CRC^[55].

Mutation in *p53* gene has been shown to facilitate not only growth, but also invasiveness in colorectal adenomas. Therefore, *p53* is implicated in the adenoma-carcinoma sequence^[56]. *P53* mutations have also been associated with altered miRNA processing^[57].

We have recently reported a significant increase in miR-1207-5p in colonic mucosal cells cultured in stem cell media (enriched for CSCs) and CD44⁺CD166⁺ cells isolated *via* flow cytometry, from AAs with adenomas. Additionally, colon cancer cell lines HCT-116 and HT-29 showed a significant increase in miR-1207-5p, compared to normal colonic epithelial cells, HCoEpiC and CCD841^[8]. This lays further weight to the role of miRNAs in promoting stem cell-like properties in colon epithelial cells.

A recent whole exome sequencing study on tissues from AAs with CRC identified somatic mutations in APC. This also supports the role of mutations in the key protein in Wnt/β-catenin signaling pathway-APC in pathogenesis of CRC^[58].

Stemness and epithelial to mesenchymal transition: A tremendous problem in management of cancer is cancer recurrence. In spite of modern breakthroughs, in CRC, the degree of recurrence is as high as 40%-60%^[59].

In any cancer, the capacity of few cells to isolate themselves from an initial site and generate a secondary tumor after implantation at a second site, defines the property of recurrence and metastases. A variety of genetic changes take place *via* a process called EMT (epithelial-mesenchymal transition) that equips CSCs to invade other tissues and survive under attachment free

conditions. In addition to mutations in *APC*, *K-RAS*, *p53* described above, activation of signaling pathways like Wnt/ β -catenin, TGF- β , notch, and hedgehog is a very critical step in EMT^[60,61].

The Wnt/ β -catenin signaling described above regulates EMT by downstream controlling of SNAIL, TWIST, SLUG, which in turn control the expression of effectors of EMT like Vimentin, E-cadherin, and N-cadherin^[62-64].

TGF- β signaling is another key pathway regulating EMT progression and is affected by activation of certain transcription factors like TWIST, SNAIL, SLUG and ZEB. In addition to activation of canonical TGF- β signaling, it is also involved in downstream activation of other canonical pathways, including Hedgehog, Notch, and Wnt and for this reason, is considered to be the master switch of the EMT process^[65-67].

Notch signaling is another central mechanism for EMT development. Bao *et al.*^[68] demonstrated that Notch pathway increases ZEB1 expression, which leads to EMT induction by inhibiting miRNA-200. Notch expression has also been correlated with the EMT markers such as, E-cadherin and Vimentin in prostate cancer^[69].

There is ample evidence to suggest that cells that undergo EMT have CSC like properties. After invading the new site, these cells initiate secondary tumor, much like CSCs^[70]. The regulatory role of miRNA-200 in Notch signaling further supports the CSC theory.

Although the specific differential expression of miRNA-200 in AAs and CAs is not yet elucidated, the direct association of notch signaling with miRNA-200 inhibition, opens avenues for further investigation in the area of racial disparity (see miRNA section). We have also shown that the induced overexpression of miR-1207-5p in normal human colonic epithelial cells (HCoEpiC and CCD841) induces stemness, as well as expression of EMT markers TGF- β , CTNNB1, MMP2, Slug, Snail, and Vimentin associated with an elongated cell morphology^[8], indicating its role in regulating stem cell-like properties in colon mucosal cells.

TGF- β stimulation has been shown to cause increased motility in CD133⁺ cells as compared to CD133⁻ cells in non-small-cell lung carcinoma. We have discussed the differential proportion of CD44⁺CD166⁻ cells in the colonic effluent as well as in colonic mucosal cells of AAs and CAs^[8]. CD44 has been shown to be associated with tumor progression and metastases in CRC in various studies^[71].

CONCLUSION

The conventional therapies for colon cancer do not account for CSCs. This has been postulated as one of the reasons for recurrence. It is well known that the recurrence rates are higher in AAs than CAs. In various studies, racial disparity in survival/recurrence is not well explained by differences in socioeconomic conditions, and general patterns of treatment^[72-74].

It is possible that the higher rate of recurrence in AAs is in part due to prevalence of those CSCs with a

less favorable mutation.

The current cytotoxic therapies act by interfering with the cell cycle of rapidly growing cells. This provides selective advantage to the slow replicating stem cells, which in fact may be enriched after chemotherapy. Data from several studies suggest a pivotal role for CSCs in regulating many malignancies, including CRC. Numerous studies have reported that CSCs or CSC like cells are highly enriched in chemotherapy resistant cancer cells. These include glioma, breast cancer, and colon cancer^[75]. Results from our own studies have demonstrated that although the combined therapy of 5-FU and Oxaliplatin inhibited the growth/proliferation of human colon cancer cells (HCT-116 or HT-29), the remaining cells showed enrichment of CSCs^[76].

It is well known that butyrate induces differentiation of colon cancer cells^[77,78]. Forced cell differentiation has not only been shown to deplete CSCs in colon cancer but also to sensitize colon cancer cells to chemotherapy^[79,80]. When these findings are viewed in light of the observations of lower amount of butyrate in stool from AAs with colon cancer than other racial groups (see section on dietary regulation of microbiota and racial disparity), it provides an interesting link between racial disparity, CSCs and CRC.

In order to successfully tackle the disparity and recurrence issues in colon cancer, a better understanding of the biological pathways is needed. Further, the focus needs to be shifted from a uniform treatment approach to a more personalized medicine. An understanding of specific CSC markers responsible for differential initiation, progression and recurrence in AAs, will help develop therapies, which target the same.

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Roles and regulation of bone morphogenetic protein-7 in kidney development and diseases

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Abstract

The gene encoding bone morphogenetic protein-7 (*BMP7*) is expressed in the developing kidney in embryos and also in the mature organ in adults. During kidney development, expression of *BMP7* is essential to determine the final number of nephrons in and proper size of the organ. The secreted BMP7 acts on the nephron progenitor cells to exert its dual functions: To maintain and expand the progenitor population and to provide them with competence to respond to differentiation cues, each relying on distinct signaling pathways. Intriguingly, in the adult organ, BMP7 has been implicated in protection against and regeneration from injury. Exogenous administration of recombinant BMP7 to animal models of kidney diseases has shown promising effects in counteracting inflammation, apoptosis and fibrosis evoked upon injury. Although the expression pattern of *BMP7* has been well described, the mechanisms by which it is regulated have remained elusive and the processes by which the secretion sites of BMP7 impinge upon its functions in kidney development and diseases have not yet been assessed. Understanding the regulatory mechanisms will pave the way towards gaining better insight into the roles of BMP7, and to achieving desired control of the gene expression as a therapeutic strategy for kidney diseases.

Key words: Bone morphogenetic protein-7; Therapeutics; Kidney; Development; Nephron progenitor cells; Disease; Regeneration; Chromatin conformation; Gene expression; Gene regulation

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Core tip: Bone morphogenetic protein-7 (BMP7) plays crucial roles in both the development and regeneration of the kidney. The functions and mechanisms of this protein have been clarified extensively for these processes in the fetus and adult kidney. However, the functional differences of BMP7 secreted from different sites in the kidney remain

undefined. We propose that uncovering the regulatory mechanism underlying *BMP7* expression will help to solve that issue. Moreover, those data should pave the way towards development of a novel therapeutic strategy for kidney diseases *via* hyperactivation of the endogenous action of *BMP7*.

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INTRODUCTION

Bone morphogenetic protein-7 (*BMP7*) belongs to the transforming growth factor- β (TGF β) superfamily. It was first identified and cloned as a human homolog of the bovine osteogenic proteins, and designated as the osteogenic protein-1 (OP-1)^[1,2]. Knockout mouse models of the *BMP7* gene were reported subsequently. Most strikingly, these models exhibited severe retardation of kidney development and died soon after birth due to renal dysplasia. Additionally, these mice exhibited anophthalmia and polydactyly in the hind limbs. Other phenotypic changes were also observed in ribs and craniofacial bones, but the effects were not fully penetrant^[3-5]. Since then, the roles of *BMP7* in the various stages of kidney development have been extensively studied. Interestingly, *BMP7* was found to be expressed not only during embryogenesis but also in the adult organ^[6-8]. Series of studies have now shed light on the protective and regenerative functions of its expression in the mature kidney^[9]. Furthermore, exogenous administration of *BMP7* and its mimetic has been considered as a promising therapeutic strategy for treatment of severe kidney diseases^[10].

Despite the original implication of an osteogenic property for *BMP7*, the *BMP7* knockout mice showed a severe phenotype in the kidney. This finding clearly illustrated that the function of the gene is critically determined by its expression pattern. In support of this, *Bmp4* under the control of the *BMP7* locus rescued the loss of the developmental function of *BMP7* in the kidney in mice^[11]. Thus, uncovering the regulatory mechanism for *BMP7* will be pivotal for gaining a better understanding of its functional roles and to developing therapeutic applications based upon it. In accordance with this perspective, in this review we first summarize the current knowledge regarding the function of *BMP7* in kidney development and diseases, after which we provide an overview of the recent findings in the regulation of its expression, finally discussing the future directions that will most likely advance the knowledge and clinical applications of this field.

BMP7 IN EMBRYONIC KIDNEY DEVELOPMENT

During embryonic development, *BMP7* is expressed in multiple tissues including the kidney, eyes, heart, limbs, forebrain, branchial arches, bones and cartilage^[6,12,13]. In the mouse, expression in the developing kidney appears first in the Wolffian duct, at embryonic day (E)9.5, and persists in the ureteric bud evaginated from the duct^[6]. At E11.5, *BMP7* expression appears in the condensing mesenchyme that is induced by the ureteric bud. Slight expression is found in the uninduced metanephric mesenchyme as well^[12]. At E13.5, the expression area extends to the pretubular aggregates and others derived from the condensed mesenchyme, including the comma-shaped and S-shaped bodies, the distal tubules and the podocytes of the developing glomeruli^[6,12,14], although expression in the comma-shaped and S-shaped bodies and the distal tubules was found by some of the studies to be very weak or absent^[4,14]. By E16.5, when the ureter has developed substantial branching, the expression in the ureteric epithelium becomes weaker in the medullar region, while its expression in the condensed mesenchyme in the nephrogenic zone of the developing kidney remains robust. Podocytes continue to strongly express *BMP7* after their folding in glomeruli^[3,4,12,13,15].

In mice, development of the kidney is severely retarded in the absence of *Bmp7*. In addition to the mutant kidney being smaller in size at birth, the number of nephrons is greatly reduced. These effects are accompanied by abnormal expansion of collecting ducts, which are interspersed by stromal cells and extracellular matrix. Mesenchymal stem cells and glomerulogenesis are also absent in the mutant kidney^[3,4].

These developmental defects appear as early as E12^[4]. As stated, the size of the mutant kidney is smaller than that of the control kidney, with the condensation of the mesenchyme appearing reduced at E12.5, although formation of pretubular aggregates was also observed^[4]. At E14.5, cessation of nephrogenesis becomes apparent with loss of mesenchymal populations in the cortical region^[3,4]. However, the comma-shaped and S-shaped bodies are present at this stage^[3,4] and the ureteric buds are branched^[3]. Moreover, the expression of marker genes, such as *Pax2*, *Pax8* and *Wnt4*, seems more or less normal in the two lineages, as long as the corresponding structures are present^[3,4].

These results suggested that the initial reciprocal inductive interaction between the ureteric epithelium and the metanephric mesenchymal cells takes place in the absence of *BMP7*^[3]. Studies using terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (commonly known as the TUNEL assay) showed massive apoptosis in the uninduced metanephric mesenchymal cells occurring from E13.5 to E14.5, explaining the

loss of the cell population that was observed in the mutant kidneys^[4,12]. Thus, BMP7 is a survival and/or proliferative factor that acts to maintain and expand nephron progenitor cells, the loss of which leads to severe retardation of kidney development^[3,4,12].

The molecular mechanisms that underlie the roles of BMP7 in kidney development have been uncovered by recent studies^[16]. The collective BMPs are known to activate SMAD1, SMAD5 and SMAD8 transcription factors, which are associated with receptors of BMPs and are phosphorylated upon their binding. Following phosphorylation, these SMADs form a complex with SMAD4, which then binds to *cis*-regulatory regions to activate target genes. On the other hand, BMPs can also activate the mitogen-activated protein kinase (MAPK) pathway, mediating their downstream effects^[17].

An *in vitro* study using a primary culture system of nephrogenic progenitor cells revealed that the proliferative role, but not the survival role, of BMP7 in this cell population is dependent on activation of the jun N-terminal kinase (JNK)-MAPK pathway^[18]. Mice with knockout of the *Trps1* gene, which encodes the trichorhino-phalangeal syndrome-1 zinc-finger transcription factor, show reduced epithelialization of mesenchymal progenitor cells^[19]. Interestingly, expression of *Trps1* in the kidney is dependent on BMP7, *via* p38 MAPK activation^[19]. Therefore, a part of the kidney-related phenotype of the *Bmp7*-null mice should be due to the deficiency in *Trps1* activation^[19].

An additional role of BMP7 involving the SMAD pathway was recently reported^[20]. In the nephrogenic zone, the progenitor populations are partitioned into two distinct compartments: One expressing detectable levels of both *CITED1* and *Six2* expression, and the other of *Six2* only. Importantly, the *Six2*-only compartment is responsive to the canonical Wnt9/ β -catenin signaling, showing appropriate differentiation and epithelialization; meanwhile, the *CITED1*⁺/*Six2*⁺ compartment is refractory to it^[20]. It has been shown that BMP7 promotes the transition of nephron progenitor cells from the *CITED1*⁺/*Six2*⁺ compartment to the *Six2*-only compartment *via* the activation of the SMAD pathway. Thus, BMP7 has multiple roles both in the proliferation and differentiation processes of the metanephric mesenchymal progenitor cells, most likely involving distinct signaling pathways.

Use of the two distinct pathways, MAPK and SMAD, in kidney development has also been suggested by the findings from *in vitro* studies. In an *ex vivo* experiment, BMP7 was shown to control branching morphogenesis of the ureteric buds in a dose-dependent manner; specifically, low dosage of BMP7 was shown to induce morphogenesis, while high dosage was shown to exert an inhibitory effect^[21]. A subsequent study showed that while the low-dose BMP7 induced p38 MAPK signaling, the high-dose triggered the SMAD pathway, which in turn caused negative regulation of the MAPK activity^[22]. Such bimodal regulation might also take place in the developmental context. In this sense, it would be intriguing to understand how secretion of BMP7 from

different sites, particularly the ureteric bud or the metanephric mesenchyme, impinges on the differential functional roles in the developmental process. To date, however, this process has remained largely unstudied.

Bmp7 expression continues in the developing kidney, even after the stage when the knockout mice present the severe abnormality. Several studies have aimed to uncover its roles in these later stages. A mouse strain that expresses the Cre recombinase under the promoter of *Nphs2* was used to create a conditional knockout mouse in which *BMP7* has near-complete specific deletion in the podocytes of mature glomeruli^[23]. These mice presented with hypoplastic kidneys, and proximal tubules of markedly reduced size. Concomitantly, phosphorylation of p38 MAPK was significantly reduced in the proximal tubules^[23].

Interesting phenotypes were observed upon deletion of the *BMP7* alleles at E12.5, which was accomplished using a mouse line expressing an inducible Cre^[24]. Deletion after the early stage of nephrogenesis resulted in precocious maturation of glomeruli, as well as increased apoptosis of the progenitor cells. *In vitro* assays further showed that BMP7 inhibits epithelialization of the mesenchymal progenitor cells, which might explain the precocious maturation that was observed^[24]. These findings might appear to be contradictory to the above-mentioned model in which BMP7 is required for shifting the competence of the mesenchymal progenitor cells for the differentiation cue^[20]; however, at an early stage, the metanephric mesenchymal cells do not need the BMP7/SMAD pathway for differentiation^[20]. In fact, the *BMP7* knockout mice can develop nephron structures adequately up to E13.5. Therefore, the reduction of BMP7 at E12.5 might guarantee or even accelerate the early phase of nephron formation. The late stage formation observed in the deletion mice might be attributed to the remaining expression of *BMP7* (around 10% as compared to the controls) after the induction by tamoxifen, particularly as BMP7 exhibits dose-dependency in induction of the downstream cascades^[22]. However, further studies are necessary to clarify this issue.

Overall, BMP7 mainly acts to determine and balance the fates of the mesenchymal progenitor cells, between proliferation and differentiation, to determine the final size of the mature kidney. Distinct pathways are utilized for these different roles. Although the mechanism to switch between the different pathways is largely unknown, dose-dependent regulation might contribute, at least partially. Therefore, regulation of the expression of *BMP7* is expected to play a critical role in the developmental process, and this topic will be discussed later in this review.

BMP7 IN THE ADULT KIDNEY

Kidney-specific expression of *BMP7* persists in the mature organ of the adult^[7]. Its functional significance has been suggested by a series of studies. All the more,

exogenous administration of BMP7 to injured kidneys was also shown to have therapeutic effects, including prevention of fibrosis, inflammation and apoptosis. We first summarize, here, the findings regarding the latter, and then we discuss the endogenous role of the protein at the end of this section.

Systemic administration of recombinant BMP7 to a rat model of ischemia/reperfusion injury was first shown to enhance recovery after acute injury by suppressing inflammatory responses, apoptosis and fibrosis^[25]. In subsequent studies, the administration of BMP7 to a rat model of unilateral ureteral obstruction (UUO) using prevention protocols resulted in blunting of the development of injury^[26,27].

Renal fibrosis is considered as a hallmark of chronic kidney diseases, although functional benefits of fibrosis have been recognized recently^[28]. TGF β 1 is a key mediator of fibrosis in many tissues, including in the kidney in response to injury (reviewed in^[29,30]). Binding of TGF β 1 to its type II receptor, TBR2, triggers the receptor to activate the TGF β receptor type I (TBR1)-kinase, which in turn induces downstream cascades *via* phosphorylation of SMAD2 and SMAD3^[31]. On the other hand, ligand binding to the type I activin-like kinase (Alk) receptors and type II serine/threonine kinase receptors (BMPRII) for BMP7 activates SMAD1/5/8 for SMAD signaling^[31].

Roles of these signaling pathways in renal fibrosis were investigated in an *in vitro* model^[32]. Incubation of mouse distal tubular epithelial cells (NP1) with TGF β 1 led to induction of epithelial-to-mesenchymal transition (EMT) that was associated with nuclear localization of phosphorylated SMAD2/3^[32]. However, addition of BMP7 to this culture system reversed the EMT through phosphorylation and activation of SMAD1, which in turn transcriptionally up-regulated the expression of E-cadherin, an important adhesion molecule in epithelial cells^[32].

Based on these findings, the counteraction of BMP7 against the TGF β 1-induced EMT was further tested *in vivo*. Intraperitoneal administration of BMP7 to a mouse model of progressive chronic kidney injury with nephrotoxic serum nephritis (NTN) led to reversal of the renal pathology and to a decline in the mortality rate^[32]. The same group also showed that the BMP7 treatment could attenuate progression of chronic kidney fibrosis in two genetic mouse models, namely those of Alport's syndrome and lupus nephritis^[33].

Recent studies have revealed involvement of epigenetic regulation in the renoprotective effect of BMP7. *Rasal1*, the gene encoding rasGAP-activating-like protein 1, was shown to be aberrantly hypermethylated in the fibrotic condition that is induced by TGF β 1^[34]. Reversal of fibrosis by the administered BMP7 was also found to be associated with active removal of methylation at *Rasal1* *via* the 10-11 translocation enzyme-3 (Tet3)^[35].

In contrast to the above findings showing the therapeutic effects of BMP7, the function of the endo-

genously expressed molecule in the adult kidney has not been thoroughly assessed to date. This might be partly due to the technical difficulty of eliminating BMP7 specifically in the adult kidney and not in the developing kidney, so as to avoid the developmental arrest that otherwise leads to death. However, several studies have demonstrated the pivotal role of endogenously expressed BMP7 in protection of the kidney from injuries.

Uterine sensitization-associated gene-1 (USAG1) is a BMP antagonist^[36-38], and is abundantly expressed in the adult kidney^[38]. The *Usag1* knockout mice show resistance to apoptosis and fibrosis, and a down-regulation in the expression of inflammatory genes, all of which were reinduced upon administration of neutralizing antibodies against BMP7^[39]. Thus, BMP7 appears to play a renoprotective role endogenously in the kidney, which is negatively regulated by USAG1. As mentioned above, fibrosis and inflammatory responses upon kidney injury seem to have beneficial effects as well for the renal function, serving to sustain the overall structure of the kidney^[28]. In this sense, USAG1 and BMP7 might cooperatively serve to balance the progression of fibrosis, and titration of these two proteins in the progression of kidney diseases might be an exciting approach for therapeutics.

Kielin/chordin-like protein (KCP) is, on the other hand, an enhancer of BMP signaling. Interestingly, the knockout mouse of the encoding gene develops susceptibility to kidney injury, further demonstrating the protective role of BMP7 in the adult kidney^[40].

Activin-like kinase 3 (Alk3) is one of the three type I receptors for BMP7^[31,41]. During the progression of kidney injury, *Alk3* is up-regulated, while the other receptor genes, *Alk2* and *Alk6*, are not. Loss of *Alk3* leads to more severe fibrosis and inflammatory response upon NTN-induced chronic kidney fibrosis, further supporting the theory that BMP7 exerts renoprotective functions through binding to *Alk3* endogenously^[41]. Furthermore, the small peptide agonist THR-123 exhibits therapeutic effects when applied to different models of kidney injuries, through its interaction with *Alk3*^[41].

REGULATION OF *BMP7* EXPRESSION IN THE EMBRYONIC AND ADULT KIDNEY

In the mouse embryo, *BMP7* is expressed not only in the kidney but also in various other tissues. A recent study showed that expression in extra-nephrotic domains is mostly regulated by long-range enhancers that activate gene expression in a tissue-specific manner around the locus^[42]. The previous *in vivo* studies had identified some of the enhancers capable of inducing reporter gene expression in the developing kidney. One such element is located in intron 1 of *Bmp7*, which is strongly conserved in tetrapods^[43]. When heterologously linked to the lacZ reporter under control of a minimal promoter sequence (Hsp68lacZ)^[44], the element induced lacZ expression in the Wolffian

duct, mesonephric tubules, ureteric bud and collecting duct, from E9.5 until E12.5, but not in the metanephric mesenchymal lineage. Of note, this expression could not be recapitulated by the orthologous sequence in *Xenopus tropicalis*. A lacZ reporter construct under control of the endogenous promoter of *BMP7* was also injected into mouse embryos together with the 4-kb upstream and the 3-kb downstream regions of the transcription start site (TSS) at each side, retaining the endogenous context^[43]. This entire construct was able to induce reporter expression in the nephrogenic mesenchymal regions. Interestingly, however, none of the separated individual elements covering the upstream and downstream regions was able to drive the expression when linked to Hsp68lacZ^[43].

Chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) was performed in Six2⁺ nephron progenitor cells^[45]. A region located at 98-kb downstream of the TSS of *BMP7* was found to be co-bound by Six2 and β -catenin. When tested *in vivo*, this element induced gene expression in a compartment of Six2⁺ renal vesicles, a part of the *BMP7* expression domain^[45]. The ChIP-seq also identified a Six2 binding site in intron 1, an evolutionarily conserved region adjacent to the intron 1 enhancer, though a reporter construct including this region did not show enhancer activity in the developing kidney^[43]. These results suggest that *BMP7* expression in the different compartments of the developing kidney, notably the ureteric bud and the metanephric mesenchyme, might be regulated by a distinct set of enhancers that are active in the respective domains, possibly interacting in a cooperative manner with each other. However, to determine whether or not these elements actually contribute to the *BMP7* expression in the kidney, testing by deletion of the respective regions should be performed in future studies.

Bmp7 is highly and specifically expressed in the adult kidney under normal physiologic conditions^[7]. The main expression domains are the ureter, collecting duct, thick ascending limb, distal convoluted tubules and podocytes in the glomerulus^[46,47]. *BMP7* expression in these cell types might also be regulated by *cis*-regulatory enhancers that are embedded around the locus. However, to date, no such enhancer elements have been described for the expression in adult kidney. Acetylation of K27 of histone H3 (H3K27ac) is associated with enhancer activity of the marked regions. We compared released data from the ENCODE project of ChIP-seq for H3K27ac in kidney tissues at different time points, ranging from E14.5 to the adult stage^[48]. In Figure 1, the regions with peaks are more or less common between different stages, but there are some striking differences. Notably, the region of the intron 1 enhancer is highly acetylated at the embryonic stages, but the mark is almost diminished for the adult kidney. These data might suggest that different sets of *cis*-regulatory regions contribute to the expression at different stages.

Once the kidney is damaged, the levels and sites of *BMP7* expression are dynamically altered^[15,47]. The injuries cause dramatic response to the cellular states in the kidney *via* the inflammatory response and other signaling cascades, such as that involving TGF β 1. Such responses are expected to lead to alteration of epigenetic states around the *BMP7* locus; as a result, *BMP7* expression would be dynamically regulated. In the following passages of this review, we review the expression dynamics of *BMP7* in several kidney disease models, as reported in the literature to date, and discuss how they are regulated.

In the kidneys of the ischemia/reperfusion rat model, *BMP7* expression dramatically decreases soon after reperfusion, particularly in the outer medulla and glomeruli^[47,49]. This reduction might be related to the up-regulation of TGF β 1. The immunostain signal of BMP7 increases in proximal tubular epithelial cells, which are devoid of its expression in the normal situation^[50]. Similar up-regulation of *BMP7* in the proximal tubular epithelial cells following ischemia was confirmed in a mouse model^[51]. Proximal tubular epithelial cells from human patients with proteinuric nephropathies also showed up-regulation of *BMP7* as compared to that in healthy controls^[52]. In an experimental model of diabetic nephropathy, *BMP7* expression was decreased^[53], which might be due at least partly to the concomitant increase in *Tgfb1* expression. A tubular injury induced by folic acid resulted in reduced *BMP7* expression at first, but was followed by a gradual recovery of expression in the regenerative phase^[15]. In the cisplatin nephrotoxicity model, however, no or only a subtle increase in *BMP7* expression was scored^[15,54].

It has long been postulated that TGF β 1, which is an inducer of the fibrotic response upon injury, down-regulates *BMP7* expression (as discussed above). On the other hand, MyoR has been implicated in the activation of *Bmp7*^[54,55]. However it has not been assessed adequately to conclude whether these effects are direct or not.

Epigenetic regulation has been studied to understand the direct linkages between various cellular states and *BMP7* expression. In TGF β 1-induced EMT in human renal proximal tubular epithelial cells, *BMP7* is slightly down-regulated^[56]. Treatment with trichostatin A, a histone deacetylase (HDAC) inhibitor, however, led to deposition of acetylated histones around the promoter of *BMP7* and to induction of its expression, thereby counteracting the fibrosis^[56]. Consistently, in an ischemia/reperfusion mouse model, down-regulation of HDAC5 was found to be involved in the activation of *BMP7* during the regenerative phase following injury, probably *via* acetylation of histones^[51].

Another layer of epigenetic regulation might also impinge on the expression of *BMP7* in kidney. The topological conformation of chromatin has recently been recognized as an important determinant of transcriptional regulation of genes. Particularly, a topologically-associating domain (TAD; a compartmentalized block of the genome,

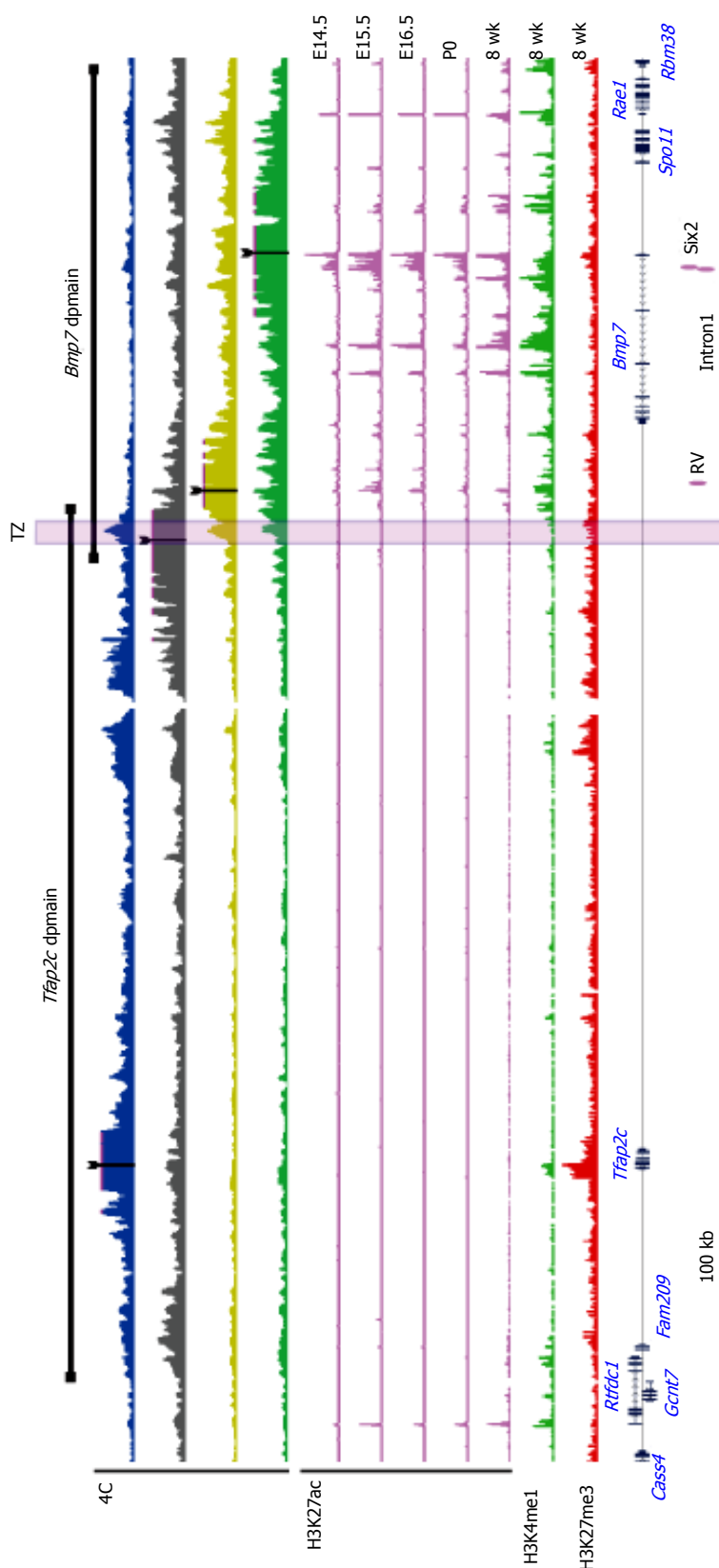


Figure 1 Landscape of the enhancers in the kidney and topological chromatin domains around the *BMP7* locus. Exons and introns of the genes within the locus (chr2: 172,250,000-172,850,000 mm9) are represented by blue boxes and arrowed dashes, respectively. Names of the genes are indicated above or below the respective boxes. The ChIP-seq signals of H3K27ac in the kidney tissue at different stages [E14.5, E15.5, E16.5, postnatal day (P)0 and 8-wk-old; indicated to the right] are shown with pink plots. H3K4me1 and H3K27me3 signals in the adult kidney are shown with green and red plots, respectively. The ChIP-seq data were obtained from the ENCODE project^[48]; the Data Coordination Consortium accession numbers are ENCSR703ZPF, ENCSR000CAF and ENCSR000CFP for H3K27ac, H3K4me1 and H3K27me3, respectively. Enhancer candidates are represented by pink ovals at the bottom: The RV enhancer is bound by Six2 and β -catenin, and induces reporter expression in the Six2-compartment of the renal vesicle^[45]; the intron 1 enhancer drives reporter expression in the developing ureteric buds^[43]; the Six2 binding site next to the intron 1 enhancer was identified by ChIP-seq^[45] but is not sufficient to induce gene expression in the developing kidney^[43]. Note that the H3K27ac mark around the intron 1 enhancer during embryogenesis diminishes in the adult stage. The chromatin domains identified by 4C-seq are shown at the top (indicated by whiskered lines), together with the actual results of the 4C-seq that are shown below^[42]. The viewpoints of the 4C-seq are indicated by arrows on the plots: Tfap2c promoter (blue), transition zone (TZ; gray), next to TZ in the BMP7 domain (yellow), and BMP7 promoter (green). The European Nucleotide Archive accession number of the 4C-seq data is ERP005557^[42]. The TZ between the two domains is indicated by the purple rectangle that spans the entire diagram.

in which the genomic regions preferentially associate with each other) was characterized as a ground-state structure that facilitates and constrains the interaction between enhancers and promoters of genes within it^[57-59].

Bmp7 is flanked by a large intergenic region, at

the other side of which a developmental gene *Tfap2c* is located (Figure 1). A recent study showed that the locus is conformationally partitioned into two adjacent domains, one for *BMP7* and the other for *Tfap2c*, by function of a region at the boundary, termed TZ^[42]. At

this locus, the action of enhancers is limited to genes located within the same domain that they belong to. In Figure 1, the TZ and 4C (circular chromatin conformation capture) plots that describe the physical domain structure were overlaid on the ChIP-seq map. It is apparent that the acetylation only extends within the *BMP7* domain and not to the neighboring one, underlining the importance of the topological structure for the regulation of *BMP7* in the kidney as well (Figure 1).

TADs seem to be more or less stable in different cell types. This might be due to the fact that CTCF, a ubiquitous DNA binding protein, greatly contributes to the formation of the domain structures^[57,60]. However, the topological structure is also a function of other epigenetic modifications, such as transcription of constitutive genes and polycomb group proteins^[57,61]. Indeed, the extent that enhancers can activate genes is sometimes different among different enhancers at the same locus^[42,59]. Therefore, it might be possible that the topological structure is subject to regulation for the dynamic expression of *BMP7* in response to kidney injuries.

CONCLUSION

BMP7 plays an important role in development and diseases of the kidney. In development, *BMP7* is critical both in proliferation and maintenance of the kidney's mesenchymal stem cells and in shifting their competence to respond to differentiation cues. Consequently, *BMP7* is a critical determinant of nephron numbers and the size of the organ. At the adult stage, *BMP7* is implicated in protection and regeneration of the kidney upon injury. Moreover, administration of *BMP7* and its mimetic exerts therapeutic effects in conditions of both acute kidney injuries and chronic kidney diseases.

Precise regulation of the *BMP7* gene is critical to its function in the kidney. At the embryonic stage, the major expression sites are metanephric mesenchyme and ureteric buds, two different lineages that interact with each other. Studies to date have indicated that the active locale of *BMP7* is the mesenchymal cells, rather than the ureteric epithelia, but the functional difference of the expression sites remains elusive. In this sense, it will be insightful to identify *cis*-regulatory elements that induce *BMP7* expression in the different cell populations and to test impacts of mutations in the elements on kidney development. The regulatory mechanisms of the adult kidney also remain elusive. Identification of the enhancers will provide insight into the still opaque role of *BMP7* in the adult kidney.

Understanding the epigenetic mechanism may not only clarify the dynamic regulation of the gene, but also open up a new avenue for therapeutics for kidney diseases through control of the expression of *Bmp7*. Different layers of epigenetic regulation, such as DNA methylation, histone modifications and higher-order chromatin conformation, almost certainly will bear a role in achieving delicate control of the gene's expression. Each of the layers represents a possible

target for therapeutics. Indeed, inhibition of HDAC has already shown a promising effect in augmenting *BMP7* expression in a TGFβ1-induced fibrosis model^[56]. Furthermore, recent advances in genome editing tools, such as the CRISPR/Cas9 system, might allow us to control epigenetic modifications, including higher-order chromatin conformation, in a locus-specific manner to optimize the gene expression^[62]. To this end, it will be beneficial to further deepen our understanding both of the role and regulation of *BMP7* in kidney development and diseases.

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Stem cell-derived exosomes as a therapeutic tool for cardiovascular disease

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Abstract

Mesenchymal stem cells (MSCs) have been used to treat patients suffering from acute myocardial infarction (AMI) and subsequent heart failure. Although it was originally assumed that MSCs differentiated into heart cells such as cardiomyocytes, recent evidence suggests that the differentiation capacity of MSCs is minimal and that injected MSCs restore cardiac function *via* the secretion of paracrine factors. MSCs secrete paracrine factors in not only naked forms but also membrane vesicles including exosomes containing bioactive substances such as proteins, messenger RNAs, and microRNAs. Although the details remain unclear, these bioactive molecules are selectively sorted in exosomes that are then released from donor cells in a regulated manner. Furthermore, exosomes are specifically internalized by recipient cells *via* ligand-receptor interactions. Thus, exosomes are promising natural vehicles that stably and specifically transport bioactive molecules to recipient cells. Indeed, stem cell-derived exosomes have been successfully used to treat cardiovascular disease (CVD), such as AMI, stroke, and pulmonary hypertension, in animal models, and their efficacy has been demonstrated. Therefore, exosome administration may be a promising strategy for the treatment of CVD. Furthermore, modifications of exosomal contents may enhance their therapeutic effects. Future clinical studies are required to confirm the efficacy of exosome treatment for CVD.

Key words: Exosomes; Messenger RNA; Cardiovascular disease; Mesenchymal stem cells; Stem cells; MicroRNA

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Core tip: Exosomes are membrane vesicles that contain and transport specific bioactive molecules, such as proteins, messenger RNAs, and microRNAs, to recipient cells. In this review, we describe the mechanisms of

exosome biogenesis, selective sorting of bioactive molecules into exosomes, and exosome secretion. We also discuss preclinical studies in which stem cell-derived exosomes were successfully used to treat cardiovascular disease (CVD). Finally, we discuss the future possibility of exosome-based clinical treatment of CVD.

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INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide. Owing to recent advances in the treatment of acute myocardial infarction (AMI) using percutaneous coronary intervention or bypass surgery, the survival of patients with AMI has substantially improved. However, many of these survivors develop heart failure (HF) as a result of the death of cardiomyocytes and subsequent tissue remodeling. As the induction of the proliferation and differentiation of the remaining cardiac tissue to regenerate heart structure remains challenging, heart transplantation is still the only treatment option for fatal HF. The development of new therapies for AMI and HF is thus required to improve the outcome in these patients.

Recently, many attempts have been made to improve the outcome of AMI and ischemic HF (IHF) using stem cells in preclinical^[1-4] and clinical^[5-10] studies. Among of the various stem cells, mesenchymal stem cells (MSCs), particularly bone marrow-derived MSCs, have been used to treat patients with AMI and IHF in clinical trials, with their safety and efficacy demonstrated in some studies^[5-10]. The earliest preclinical studies suggested that MSCs have the potential to differentiate into multiple cardiac cell types including cardiomyocytes, vascular endothelial cells, and vascular smooth muscle cells^[1-3]. However, subsequent studies did not demonstrate this remarkable differentiation capacity of MSCs. Rather, it was reported that most intravenously injected cells are trapped in the lung rather than engrafted in the heart^[11,12]. Even when MSCs are administered to the swine heart *via* the coronary artery following AMI induction, only 6% of the injected cells remained in the infarct zones 14 d after AMI induction^[11]. Furthermore, the supernatant of MSC cultures reportedly improves cardiac function^[13-15]. These results suggest that MSCs improve cardiac function *via* the secretion of paracrine factors rather than *via* the direct differentiation of MSCs into cardiac cell types. Furthermore, MSC transplantation has several problems such as low survival rate and stem cell tumorigenesis^[16]. However, if MSC-secreted paracrine factors can efficiently repair and regenerate cardiac tissues, cell-free therapy is possibly a safer alternative in

the future.

Recently, a variety of cell types, including stem cells, have been shown to secrete paracrine factors in not only naked forms but also membrane vesicles, such as exosomes, microvesicles, ectosomes, membrane particles, exosome-like vesicles, and apoptotic bodies^[17]. Exosomes are one of the secreted vesicles (also referred to as extracellular vesicles or EVs) that are 30-100 nm in diameter and contain a variety of biologically active molecules, such as proteins, messenger RNAs (mRNAs), and microRNAs (miRs)^[18]. In this manuscript, we review the characteristics of exosomes and their possible applications in CVD treatment.

EXOSOME ISOLATION AND IDENTIFICATION

Several strategies have been used to isolate exosomes from tissues. These strategies utilize ultracentrifugation, size-based purification, precipitation using polymers, and immunoaffinity purification as reviewed in some reports^[19-21]. Ultracentrifugation is the most established method of exosome isolation which employs sequential centrifugation combined with sucrose density gradient ultracentrifugation. Size-based purification includes ultrafiltration and gel filtration methods. Alternatively, polymers such as polyethylene glycol, widely used to precipitate proteins and viruses, can also be used to precipitate exosomes. As exosomes express specific proteins and lipids on their surface, antibodies recognizing these molecules (frequently conjugated with magnetic beads) are also used in their isolation.

Identification of exosomes is usually achieved by evaluating their morphology and size, their motion in a solution, and the specific molecules they express, as previously reviewed^[22,23]. Electron microscopy is commonly employed to measure the size and assess the morphology of exosomes. The number of particles corresponding to exosome size can be counted by nanoparticle tracking analysis. This method utilizes the phenomenon of Brownian motion in a liquid suspension to measure particle size. Because exosomes are derived from endosomes and are finally released from cells as described in the following section, molecules involved in exosome formation, such as tetraspanins (CD81, CD9, and CD63), are expressed in exosomes. These markers can be used to identify exosomes.

EXOSOME BIOGENESIS, SECRETION, AND UPTAKE BY RECIPIENT CELLS

Exosomes are derived from endosomes that are formed by the inward budding of the plasma membrane (Figure 1)^[18]. The subsequent inward budding of the endosomal membrane results in the formation of intraluminal vesicles (ILVs) into which cytoplasmic molecules, such as proteins, mRNAs, and miRs are sorted^[24,25]. These endosomes containing ILVs, or multivesicular bodies

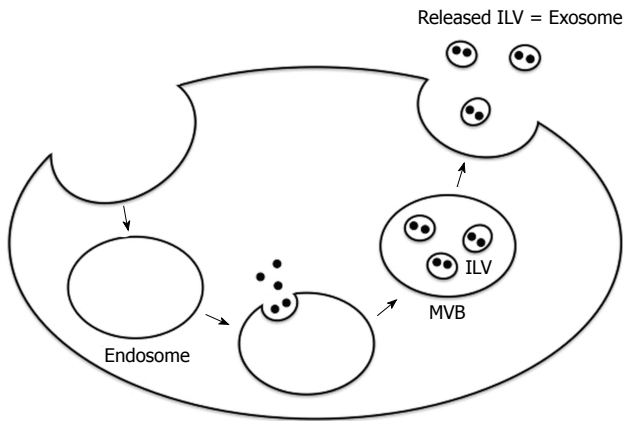


Figure 1 Schematic diagram showing exosome biogenesis and release. ILV: Intraluminal vesicle; MVB: Multivesicular body.

(MVBs)^[18], fuse with the plasma membrane and release ILVs into the extracellular environment by exocytosis. These secreted ILVs containing biologically active molecules are referred to as exosomes.

The mechanisms of exosome formation and processing are just starting to be revealed. The formation of MVBs is reportedly mediated by the endosomal sorting complexes required for transport (ESCRT) system or by systems independent of the ESCRT machinery as summarized in some reviews^[26-28]. The ESCRT machinery comprises four protein complexes, ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, together with accessory proteins. ESCRT-0 recognizes ubiquitinated proteins and is recruited to the endosomal membrane, where it initiates processes leading to the uptake of ubiquitinated proteins into ILVs. ESCRT-0 subsequently recruits ESCRT-I to the endosomal membrane, which in turn recruits ESCRT-II and ESCRT-III. ESCRT-III induces the inward budding of the endosomal membrane and formation of ILVs, while accessory proteins (particularly the vacuole protein sorting gene 4 ATPase or VPS4) are implicated in the dissociation and recycling of the ESCRT machinery. In addition, other molecular pathways mediate ESCRT-independent MVB formation including tetraspanins^[29] such as CD81, CD9, and CD63, and proteolipid proteins such as ceramide^[30].

The docking and fusion of MVBs to the plasma membrane appear to be mediated by soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) proteins such as vesicle-associated membrane protein 7 (VAMP7)^[31]. The release of ILVs (exosomes) from cells following the fusion of MVBs to the plasma membrane is mediated by several mechanisms. The small GTPases of the Rab family (Rab27a/b, Rab11, and Rab35) are the most studied molecules involved in exosome release^[32-34]. Other pathways include WNT5A, glycosphingolipids, flotillins, and stress-induced stimuli such as the increase in intracellular calcium concentration, DNA damage, heat shock, and hypoxia^[35-39]. In addition, an acidic environment has been shown to trigger the secretion of exosomes from cells^[40].

Once released from cells, exosomes bind to target cells *via* ligand-receptor interactions. Molecules, such as integrins, intercellular adhesion molecules, and tetraspanins seem to be implicated in the binding of exosomes to recipient cells^[41-43]. After binding, exosomal contents are reportedly internalized by recipient cells *via* two major mechanisms as summarized in some reviews^[23,44]: (1) exosome fusion with the plasma membrane of recipient cells and direct release of contents into the cytoplasm; or (2) internalization by endocytosis into recipient cells. It has been demonstrated that bioactive molecules in exosomes are not only transferred to recipient cells but also exert functional effects^[45-47].

Although the precise mechanism remains unknown, a specific set of proteins, mRNAs, and miRs are selectively accumulated within exosomes^[48]. It has also been demonstrated that exosomes contain a distinct set of mRNAs compared to the donor cells^[49]. Ubiquitination appears to be required for the uptake of some proteins into exosomes^[50], although ubiquitination-independent accumulation of proteins has also been reported^[51]. The accumulation of miRs into the exosomes of T cells appears to require the recognition of a GGAG sequence located in miRs by the heterogeneous nuclear ribonucleoprotein hnRNP A2B1^[49].

Taken together, accumulating evidence indicates that exosomes are a natural vehicle for the efficient and specific transport of biologically active cargo into recipient cells. These properties may be exploited for the delivery of bioactive molecule such as miRs and chemical compounds such as drugs. For instance, stem cell-derived exosomes may be useful for CVD treatment. We review the potential utility of stem cell-derived exosomes for CVD treatment in the following section.

THERAPEUTIC EFFECTS OF STEM CELL-DERIVED EXOSOMES ON CVD

MSC-derived exosomes

Several preclinical studies have demonstrated the efficacy of MSC-derived exosomes for CVD treatment (Table 1). Lai *et al.*^[52] found that the supernatant of human embryonic stem cell (ESC)-derived MSCs contained small particles (50-100 nm in diameter) corresponding to exosomes. When administered to a mouse model of myocardial ischemia/reperfusion injury, these exosomes remarkably reduced infarct size. The same group also administered exosomes secreted from human ESC-derived MSCs to a mouse model of AMI and demonstrated improved cardiac function^[53]. In addition, they found that the tissue levels of ATP and nicotinamide adenine dinucleotide were significantly increased, while those of reactive oxygen species were significantly decreased after exosome administration. Furthermore, they demonstrated that the phosphorylation of Akt and glycogen synthase kinase 3 (that has anti-apoptotic effects) significantly increased and that of c-jun N-terminal kinase (that has proapoptotic

Table 1 Effects of exosome administration on cardiovascular disease models

Origin of exosomes	Experimental model	Findings	Ref.
Human ESC-derived MSCs	AMI	Reduction in infarct size Recovery of cardiac function Decreased oxidative stress Activation of Akt and GSK3 Inhibition of c-JNK	Lai <i>et al</i> ^[52,53]
Human MSCs	AMI	Reduction in infarct size Recovery of cardiac function Increased angiogenesis	Bian <i>et al</i> ^[54]
Mouse MSCs	AMI	Exosomes were enriched in miR-22 miR22 was implicated in the anti-apoptotic effect of exosomes	Feng <i>et al</i> ^[55]
Rat MSCs overexpressing GATA-4	AMI	Reduction in infarct size Recovery of cardiac function Exosomes were enriched in miR-19a	Yu <i>et al</i> ^[56]
Rat MSCs	Stroke	Recovery of neurological function Stimulation of neurogenesis and angiogenesis	Xin <i>et al</i> ^[57]
Rat MSCs overexpressing miR-133b and those whose expression of miR-133b was knocked down	Stroke	Recovery of neurological function was mediated by miR-133b expressed in exosomes	Xin <i>et al</i> ^[58]
Mouse MSCs	Pulmonary hypertension	Reduction in the progression of pulmonary hypertension and right ventricular hypertrophy	Lee <i>et al</i> ^[59]
Mouse CPCs	AMI	Suppression of apoptosis	Chen <i>et al</i> ^[60]
Human CPCs	AMI	Recovery of cardiac function Suppression of apoptosis Stimulation of angiogenesis	Barile <i>et al</i> ^[61]
Human CPCs	AMI	Recovery of cardiac function Suppression of apoptosis Stimulation of angiogenesis miR-146a was enriched in exosomes and partially mediated their function	Ibrahim <i>et al</i> ^[62]
Mouse ESCs	AMI	Recovery of cardiac function Stimulation of angiogenesis and cardiomyocyte survival Stimulation of the survival and proliferation of CPCs miR-294 was enriched in exosomes and miR-294 promoted the survival and proliferation of CPCs	Khan <i>et al</i> ^[63]
Human CD34+ cells	Matrigel plug assay Corneal angiogenesis assay	Promotion of angiogenesis	Sahoo <i>et al</i> ^[64]
Human CD34+ cells expressing SHH	AMI	Recovery of cardiac function SHH was enriched in exosomes and transferred to recipient cells	Mackie <i>et al</i> ^[66]

ESC: Embryonic stem cell; MSCs: Mesenchymal stem cells; CPCs: Cardiac progenitor cells; SHH: Sonic hedgehog; AMI: Acute myocardial infarction; GSK3: Glycogen synthase kinase 3; c-JNK: c-jun N-terminal kinase.

effects) significantly decreased in cardiac tissue following exosome administration. Bian *et al*^[54] demonstrated the proliferation and migration of human umbilical vein endothelial cells in response to EVs (100 nm in diameter) collected from human MSCs. They also administered MSC-derived EVs to a rat model of AMI and showed that MSC-derived EV administration significantly reduced infarct size, restored cardiac function, and stimulated angiogenesis in the ischemic zone. Feng *et al*^[55] demonstrated that exosomes secreted from mouse MSCs following ischemic preconditioning contained a large amount of miR-22. When administered to mice with AMI, these miR-22-enriched exosomes exerted an anti-apoptotic effect on cardiomyocytes *via* the downregulation of methyl-CpG-binding protein 2. Yu *et al*^[56] used MSCs overexpressing the transcription factor GATA-4 (MSC_GATA-4) and demonstrated that the administration of MSC_GATA-4-derived exosomes restored cardiac function and reduced infarct size in a rat model of AMI. The authors also

showed that MSC_GATA-4-derived exosomes expressed a greater amount of miRs, particularly miR-19a, than control MSCs and that miR-19a appeared to be involved in the cardioprotective effect of MSC_GATA-4-derived exosomes *via* the downregulation of phosphatase and tensin homolog (PTEN) and subsequent activation of anti-apoptotic Akt and extracellular signal-regulated kinase.

Preclinical studies have also reported favorable effects of exosome administration on neurological recovery following stroke induction. Xin *et al*^[57] found that the systemic administration of rat MSC-derived exosomes following the induction of stroke by the ligation of the middle cerebral artery significantly accelerated neurological recovery and stimulated neurogenesis and angiogenesis at the border zone between normal and ischemic tissues. The same group also demonstrated that the administration of MSCs overexpressing miR-133b (MSCs_miR-133b+) enhanced the recovery of neurological function in a rat stroke model whereas MSCs

with miR-133b knockdown (MSCs_miR-133b-) did not^[58]. Furthermore, they showed that the level of miR-133b in exosomes isolated from cerebrospinal fluid was higher in the group that received MSCs_miR-133b+. They also demonstrated that MSC-derived exosomes could be transferred to neighboring cells. Finally, they showed that the expression of connective tissue growth factor (CTGF), a target for miR-133b, was significantly reduced in the ischemic boundary zone following MSCs_miR-133b+ administration, while CTGF expression remained unchanged after MSCs_miR-133b- administration. They concluded that miR-133b derived from exosomes was implicated in MSC-mediated recovery of neurological function in this model.

The beneficial effects of MSC-derived exosome administration have also been reported in a mouse model of hypoxic pulmonary hypertension. Lee *et al.*^[59] demonstrated that the administration of MSC-derived exosomes significantly ameliorated the progression of pulmonary hypertension and right ventricular hypertrophy, possibly *via* the suppression of signal transducer and activator of transcription 3 (STAT3).

Cardiac progenitor cell-derived exosomes

Chen *et al.*^[60] demonstrated that the injection of exosomes isolated from murine cardiac progenitor cells (CPCs) into the murine heart following ischemia/reperfusion injury significantly suppressed apoptosis. Barile *et al.*^[61] demonstrated that the administration of EVs (most of which were exosomes) isolated from human CPCs significantly suppressed apoptosis, stimulated angiogenesis, and improved cardiac function in a rat model of AMI. They also showed that specific miRs, such as miR-210, miR-132, and miR-146a-3p, were enriched in CPC-derived exosomes. Ibrahim *et al.*^[62] reported that the administration of human CPC-derived exosomes in a mouse model of AMI significantly suppressed apoptosis, stimulated angiogenesis, and restored cardiac function. They also demonstrated that miR-146a was enriched in CPC-derived exosomes and that miR-146a administration partially mimicked the beneficial effects of CPC-derived exosomes on cardiac function.

ESC-derived exosomes

Khan *et al.*^[63] reported that ESC-derived exosomes from mouse stimulated neovascularization, enhanced cardiomyocyte survival, and restored cardiac function in a mouse model of AMI. Furthermore, ESC-derived exosomes augmented the survival and proliferation of CPCs. miR-294 was enriched in ESC-derived exosomes and the treatment of CPCs with miR-294 promoted the progression of the cell cycle to the S phase, suggesting that ESC-derived exosomes transferred miRs, such as miR-294, to CPCs, which promoted the proliferation and survival of CPCs.

CD34+ stem cell-derived exosomes

Sahoo *et al.*^[64] isolated exosomes from human CD34+

stem cells (which include endothelial progenitor cells^[65]) and examined their proangiogenic activity. CD34+ stem cell-derived exosomes stimulated tube formation from cultured endothelial cells in Matrigel (*in vitro* assay), and promoted angiogenesis *in vivo*, as assessed by the Matrigel plug assay and the corneal angiogenesis assay. Mackie *et al.*^[66] demonstrated that CD34+ stem cells expressing the pro-angiogenic factor sonic hedgehog (SHH) restored cardiac function in a mouse model of AMI. They also showed that SHH was enriched in exosomes secreted from stem cells and that it was transferred to and expressed functionally in recipient cells, suggesting that exosome-mediated transfer of SHH to recipient cells accounts for the beneficial effects of stem cell administration in this model of AMI.

Collectively, these studies provide compelling evidence that exosomes derived from a variety of stem cells exert beneficial effects on animal models of CVD.

FUTURE DIRECTIONS

Clinical trials

Although clinical trials using exosomes for CVD treatment have not yet started, exosome administration in humans has been tested, particularly for cancer immunotherapy^[67-69]. Phase I and phase II studies have been performed and the safety of the treatment has been confirmed. Future clinical studies will be required to test the safety and efficacy of exosome treatment for CVD.

Modification of exosomes

Given the low toxicity, high stability in the circulation, and high efficiency of transport to donor cells demonstrated by exosomes, several studies have attempted to augment the therapeutic efficacy by modifying exosomal content. For instance, small RNAs such as small interfering RNAs and miRs have been loaded into exosomes during exosome formation using lipofection or following exosome formation using electroporation^[70-74]. These modified exosomes reportedly exerted biological effects in recipient cells^[70-74]. Exosomes have also been used as vehicles to transport exogenous chemical compounds to recipient cells stably and efficiently, because some drugs are condensed in the exosomes of donor cells and transferred to recipient cells. Exosomes enriched in curcumin, an anti-inflammatory agent, or chemotherapeutic agents, such as paclitaxel and doxorubicin, have been used to transport these compounds to recipient cells, with their beneficial biological effects confirmed^[75-78]. Another strategy that has been examined is the modification of exosomal membrane proteins to improve the efficiency of uptake by recipient cells. Alvarez-Erviti *et al.*^[70] prepared dendritic cells that expressed Lamp2b, an exosomal membrane protein, fused to a peptide fragment of neuron-specific rabies viral glycoprotein so that exosomes would be accumulated specifically in the brain. The authors demonstrated that these modified exosomes were specifically taken up by

brain tissues when intravenously administered. Therefore, the modification of exosome structure will enhance the specificity and efficiency of transport and the modification of exosome content (for example, by inclusion of specific miRs) will enhance the therapeutic effect in the future.

Exosome-induced tumorigenesis

It has been reported that MSC-derived exosomes promote tumor growth *in vivo* via the stimulation of vascular endothelial growth factor expression in tumor cells^[79]. In most cases, the stimulation of angiogenesis appears to be favorable for the regeneration of cardiomyocytes after AMI. However, angiogenesis may stimulate tumor growth in other tissues. Therefore, it is desirable to explore a strategy to specifically deliver exosomes to target tissues.

CONCLUSION

Exosomes are one of the secreted vesicles that contain bioactive molecules, such as proteins, mRNAs, and miRs. Exosomes transfer these bioactive molecules to recipient cells, thus exerting biological effects. Preclinical studies have suggested that exosomes can be used for the treatment of CVD such as AMI and stroke. Future clinical studies are warranted to confirm the efficacy of exosome administration for CVD treatment. Furthermore, modifications of exosomal structure and content will enhance the efficacy of exosome administration for such treatments in the future.

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Stem cell therapy for the treatment of Leydig cell dysfunction in primary hypogonadism

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Abstract

The production of testosterone occurs within the Leydig cells of the testes. When production fails at this level from either congenital, acquired, or systemic disorders,

the result is primary hypogonadism. While numerous testosterone formulations have been developed, none are yet fully capable of replicating the physiological patterns of testosterone secretion. Multiple stem cell therapies to restore androgenic function of the testes are under investigation. Leydig cells derived from bone marrow, adipose tissue, umbilical cord, and the testes have shown promise for future therapy for primary hypogonadism. In particular, the discovery and utilization of a group of progenitor stem cells within the testes, known as stem Leydig cells (SLCs), has led not only to a better understanding of testicular development, but of treatment as well. When combining this with an understanding of the mechanisms that lead to Leydig cell dysfunction, researchers and physicians will be able to develop stem cell therapies that target the specific step in the steroidogenic process that is deficient. The current preclinical studies highlight the complex nature of regenerating this steroidogenic process and the problems remain unresolved. In summary, there appears to be two current directions for stem cell therapy in male primary hypogonadism. The first method involves differentiating adult Leydig cells from stem cells of various origins from bone marrow, adipose, or embryonic sources. The second method involves isolating, identifying, and transplanting stem Leydig cells into testicular tissue. Theoretically, *in-vivo* re-activation of SLCs in men with primary hypogonadism due to age would be another alternative method to treat hypogonadism while eliminating the need for transplantation.

Key words: Stem cell therapy; Leydig cells; Primary hypogonadism; Stem Leydig cells; Testosterone; Bone marrow-derived stem cells; Adipose-derived mesenchymal stem cells

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Core tip: Although clinicians are capable of treating primary hypogonadism with exogenous testosterone,

there is no therapy that mimics its physiologic release. Two current directions exist for stem cell therapy in male primary hypogonadism. The first method involves differentiating adult Leydig cells from stem cells of various origins from bone marrow, adipose, or embryonic sources. The second method involves isolating, identifying, and transplanting stem Leydig cells (SLCs) into testicular tissue. Re-activation of SLCs in men with primary hypogonadism due to age would also be an alternative method. As researchers are better able to replicate the differentiation process of androgenic tissue, treatments will hopefully follow.

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INTRODUCTION

Testosterone is an essential hormone that is required for normal male physiologic development. It not only plays a role in the growth of genital organs *in utero*, but also initiates spermatogenesis, as well as the development of secondary sexual characteristics during puberty (Figure 1). Low testosterone, otherwise known as hypogonadism, can result from a primary defect within the testes or secondarily from a disruption in the hypothalamic-pituitary-gonadal (HPG) axis.

Primary hypogonadism can result from a number of disorders, the most common of which is Klinefelter's syndrome, which occurs in one in every 2500 adult males^[1]. Other disorders can be separated into those that are congenital, those that are acquired, and those related to systemic conditions (Figure 2). Congenital disorders, that frequently are associated with hypogonadism, include, among others, myotonic dystrophy, Down syndrome, bilateral cryptorchidism, defects in testosterone biosynthetic enzymes, and luteinizing hormone receptor mutations. Acquired disorders include dysfunction related to aging, trauma, orchitis, and testicular failure secondary to radiation or exposure to chemotherapy. Systemic disorders include chronic liver disease, chronic kidney disease, sickle cell, and vasculitides. Age-related dysfunction, in particular, has become an intensely debated subject as physicians continue to discuss how to properly diagnose and treat hypogonadal men. This debate has led researchers to better understand the role of testosterone in the aging male, and to appreciate just how common this deficiency is in the general population. For example, in the Hypogonadism in Males study, researchers found that when using a testosterone threshold of 300 ng/dL to define hypogonadism, the overall prevalence of androgen deficiency in men over 45 years of age was 38.7%^[2].

The detrimental effects that hypogonadism can have on patients are multiple. Symptomatically, patients can experience fatigue, depressed mood, decreased libido, erectile dysfunction, infertility, alterations in body composition, and decreased cognitive function. Furthermore, there is evidence to suggest that hypogonadal patients are at an increased risk for coronary artery disease, cerebral vascular disease, and metabolic syndrome^[3-5]. Though numerous testosterone formulations have been developed, none are fully capable of replicating the physiological patterns of testosterone secretion from within the testes. With an understanding of Leydig cell development, and an appreciation for the mechanisms that lead to their dysfunction in some of the more commonly encountered etiologies of primary hypogonadism, we can utilize the recent advances in stem cell therapy to provide a long-lasting treatment.

NORMAL LEYDIG CELL DEVELOPMENT

Leydig cells, in the testicle, produce testosterone. They are present in clusters in the interstitium between seminiferous tubules within the testes, totaling 700 million cells. They constitute 2%-4% of testicle volume. The development of Leydig cell function comprises three stages, corresponding to the triphasic development of plasma testosterone levels (Figure 2)^[6,7]. It begins in the sixth to seventh week of gestation, when fetal Leydig cells begin producing testosterone^[8]. This occurs independent of luteinizing hormone (LH), from the anterior pituitary, and human chorionic gonadotropin (hCG), which is secreted from the placenta^[7,9]. After seven weeks, however, hCG and LH are required for Leydig cells to produce enough testosterone for masculinization of the external genitalia^[10]. The proliferation and differentiation of Leydig cells continues until 19 wk, at which time cells undergo regression^[11]. The second stage begins after birth in the neonatal period, at which time a second testosterone surge occurs that is associated with the development of a second wave of Leydig cells that reach a peak at three months of age^[12]. These cells are believed to be a mixture of developing Leydig cells and fetal Leydig cells. Thereafter, regression of fetal Leydig cells occurs, reaching a nadir at one year of age^[13]. In addition, immature Leydig cells remain within the interstitium of the testes until activated during the third stage of development that occurs during puberty^[13,14]. With the onset of puberty, the immature Leydig cells, visualized surrounding the outer peritubular layer of the seminiferous tubules and vasculature of the interstitial tissue, undergo a cytological transformation that allows for a significant production of testosterone in an LH-dependent manner^[15]. This rise in testosterone will then lead to the development of secondary sexual characteristics and sexual reproduction.

STEM LEYDIG CELLS

Despite the well-understood, temporal progression of

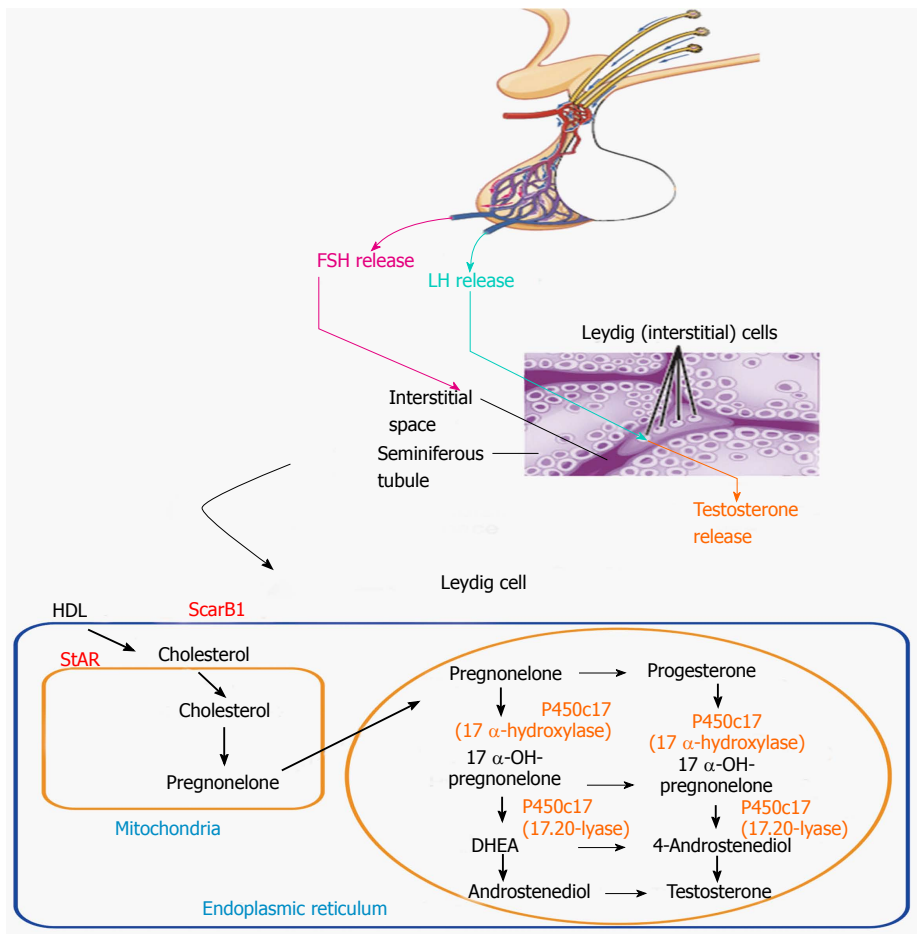


Figure 1 Testosterone biosynthetic pathway. FSH: Follicle stimulating hormone; LH: Luteinizing hormone; HDL: High-density lipoprotein; StAR: Steroidogenic acute regulatory protein.

Causes of primary hypogonadism		
Congenital	Acquire disorders	Systemic disorders
Klinefelter syndrome (XXY)	Bilateral surgical castration or trauma	Chronic liver disease
Myotonic dystrophy	Orchitis	Malignancy (Lymphoma, testicular cancer)
Uncorrected cryptorchidism	Drugs (Spironolactone, ketoconazole, abiraterone, enzalutamide, alcohol, chemotherapy agents)	Chronic kidney disease
Noonan syndrome	Ionizing radiation	Sickle cell disease
Bilateral congenital anorchia		Aging
Polyglandular autoimmune syndrome		Spinal cord injury
Testosterone biosynthetic enzyme defects		Vasculitis, infiltrative disease (Amyloidosis, leukemia)
Congenital adrenal hyperplasia		
Complex genetic syndromes		
Down syndrome		
Luteinizing hormone receptor mutation		

Figure 2 Various possible causes of primary hypogondism.

Leydig cell maturation, the origin of Leydig cells had not been fully elucidated. We now know that stem Leydig cells (SLCs) do exist, and that they are critical to the maturation process. This has been understood through studies that demonstrated the repopulation of adult Leydig cells in rat testes after being depleted by the alkylating agent, ethane dimethanesulfonate

(EDS)^[16-18]. In further support of this theory, it has been shown that the regeneration of cells does not result from quiescent progenitor cells that have already differentiated into Leydig cell lineage, but rather true stem cells that remain undifferentiated and have the ability to proliferate for extended periods of time without expressing Leydig cell markers^[19]. In attempting to

isolate these stem cells in rat models, researchers have not clearly identified the location of these cells. Some studies provide evidence to suggest that they exist within the interstitium as the pericytes and vascular smooth muscle cells along the vessel walls^[20-22]. Others point towards a peritubular location, lying on the surface of the seminiferous tubules^[19,23,24].

To highlight the vascular hypothesis, Davidoff *et al.*^[22] demonstrated that after destroying mature Leydig cells, regeneration was preceded by a proliferation of nestin-expressing vascular smooth muscle cells and pericytes. Expression of nestin, an intermediate filament protein, has not only been observed in stem cells in the nervous system, but in other tissues, including the testes. The proliferating cells in this study were then capable of conversion into steroidogenic Leydig cells. Evidence for the transdifferentiation into Leydig cells was based on the coinciding expression of nestin with steroidogenic genes in newly generated Leydig cells.

In order to elucidate the peritubular hypothesis, it is important to understand that the peritubular compartment contains myofibroblasts, testicular peritubular cells (TPCs), and extracellular matrix^[25]. Specifically, the TPCs contribute to testicular function by secreting paracrine factors and components of the extracellular matrix^[26]. In Stanley *et al.*^[19] researchers isolated cells expressing platelet-derived growth factor receptor- α , but not 3 β -hydroxysteroid dehydrogenase (3 β -HSDneg) from the testes of EDS-treated adult rats. These were later determined to be the SLC. To localize these cells, the seminiferous tubules and interstitium were physically separated and cultured. During culture, the 3 β -HSDneg cells on the tubule surfaces underwent divisions, eventually expressing 3 β -HSD and producing testosterone. Removal of these testosterone-producing cells from the tubule surfaces, followed by further culture of the stripped tubules, resulted in their reappearance. In contrast, the interstitial compartment did not develop 3 β -HSDpos cells or produce testosterone when cultured. The fact that functional Leydig cells are able to differentiate in the absence of interstitium suggests that macrophages and cells associated with blood vessels in the interstitial compartment (vascular smooth muscle cells, pericytes) may not be critical for the development of new Leydig cells, as suggested in some previous studies. These results were further corroborated in a study using human TPCs^[27]. In this study, researchers demonstrated that these cells expressed pluripotency markers, SLC markers, and steroidogenic genes involved in the biosynthesis of sex steroids. Furthermore, these cells were activated to express steroidogenic enzymes that led to the production of pregnenolone and progesterone. Testosterone was not produced, but this may highlight the fact that these progenitor cells have not fully differentiated into a Leydig cell lineage.

It should be noted that the discrepancies in the location of these stem cells might result from differing conditions within the testicular tissue. It may be that SLCs reside in both peritubular and interstitial locales,

as suggested by Chen *et al.*^[28]. For example, Leydig cell regeneration has been shown to occur more rapidly around regressed tubules than near tubules with normal spermatogenesis^[29]. Likewise, in testes with normal spermatogenesis, regeneration appears to occur in proximity to both tubules and the interstitial blood vessels^[30]. A third plausible hypothesis that researchers have put forth is that the adult Leydig cells differentiate not from stem cells, but rather from myoid cells, vascular smooth muscle cells, or pericytes that have transdifferentiated^[28].

LEYDIG CELL DYSFUNCTION

Understanding the mechanisms that cause Leydig cell dysfunction will ultimately lead researchers and physicians to develop therapies that target the specific step or steps in the steroidogenic process that has been damaged, whether it is at the level of the adult Leydig cell, the Leydig stem cell, or beyond. In an attempt to further elucidate these mechanisms, we will cover those disease states that have been comprehensively studied in the lab, and those that may one day be amenable to stem cell therapy. For example, some congenital and systemic disorders leading to hypogonadism often lead to dysfunction at multiple levels in the HPG axis. Our focus will remain specifically at the level of the testis. Likewise, primary hypogonadism due to genetic mutations or enzymatic deficiencies would not be amenable to autologous stem cell transplant because the stem cells themselves would carry the mutation and/or deficiency.

Despite Klinefelter's Syndrome being the most common abnormality of sex chromosomes that invariably leads to testicular failure, researchers have not determined what the mechanisms are that underlie the global degeneration of testicular tissue. In a recent study by D'Aurora *et al.*^[31] researchers conducted testicular gene expression profiling by a whole genome microarray approach using testicular tissue from patients with Klinefelter's Syndrome. They found that the genes responsible for increased apoptotic processes were overexpressed. Furthermore, the data suggested that the dysregulation of genes involved in the inflammation process were responsible for the high degree of fibrosis that is described in the testicular involution process of patients. They also identified the overexpression of genes central to the steroidogenic activity of the Leydig cells. This finding supports the recently demonstrated increase of intratesticular testosterone concentrations in Klinefelter patients in comparison to control patients^[32]. Thus, the low testosterone serum levels commonly seen in these patients could be related to an altered release of the hormone into the bloodstream. Researchers have not yet determined whether the altered release is due to the decreased testicular vasculature commonly seen in these patients, or if it is due to some active transporter that might be involved in testosterone release from Leydig cells^[32]. If it is indeed an issue of

vasculature, then a cell-based therapy that regenerates not only Leydig cells but also the entire testicular micro-environment may be necessary. However, if an active transporter within the Leydig cell is identified then a more cell-specific approach would be feasible.

Interestingly, primary hypogonadism secondary to aging does not result from a loss of Leydig cells. Instead, studies have indicated that it is Leydig cell function that is lost through a process that is independent of LH secretion^[33,34]. Indeed, when LH is administered *in vitro* to Leydig cells from aged rats, testosterone production remains significantly below that of cells from young rats^[35]. Because the steroidogenic process involves a complex interplay of biochemical pathways, researchers have proposed a number of mechanisms responsible for the decreased function^[36].

Critical to function is the interaction between LH, its receptor on the Leydig cell, and the subsequent production of 3',5'-cyclic adenosine monophosphate (cAMP) initiating the steroidogenic process. Researchers have demonstrated a coupling defect of the LH receptor to adenylate cyclase, reducing cAMP production and directly inhibiting testosterone synthesis^[37]. There is also evidence to suggest that increased oxidative stress plays a critical role, not only in the above-mentioned uncoupling defect, but also in cell membrane stability. With increasing age, cells experience increased levels of reactive oxygen species (ROS), due in part to the decreased levels of free radical-scavenging proteins^[38-41]. With increased ROS, lipid peroxidation within the Leydig cell leads to a destruction of membrane stability^[42]. Because steroidogenesis depends on this stability for cholesterol transport, testosterone synthesis is inhibited. Other studies have shown that arachidonic acid positively regulates the effects of LH on steroidogenesis^[43,44]. It, however, can be metabolized by cyclooxygenase 2 (COX2). It has been suggested that with increased levels of COX2 in aged Leydig cells, there is a reduction in arachidonic acid, and thus testosterone^[45]. Further corroborating the oxidative stress hypothesis, researchers have determined that phosphorylation of p38 mitogen-activated protein kinase (MAPK), may serve as the mediating interaction between increased oxidative stress and decreased steroidogenesis^[46]. Relating COX2 inhibition to this theory, it is possible that phosphorylated p38 MAPK increases COX2 synthesis, in turn inhibiting steroidogenic function, although this has not been evaluated in Leydig cells^[28,47].

Hypogonadism is frequently found in men who have undergone chemotherapy. While far less evidence explains how Leydig cells are affected, Al-Bader *et al.*^[48] studied how bleomycin, etoposide, and cisplatin affected the HPG axis in a rat model. They found that chemotherapy induced both Leydig cell hyperplasia and degenerative changes in Leydig cells after exposure. These degenerative changes persisted after 63 d. The question remains as to whether the observed hyperplasia resulted from activated SLCs. Given that the degenerative changes persisted after recovery, this might suggest

that the chemotherapy permanently altered the SLCs. This would stand in contrast to the aging SLCs, which remain quiescent and genomically stable throughout life. Critical to an understanding of these degenerative changes, researchers measured the testicular oxidative stress, which was found to be significantly increased at the end of the chemotherapy, but returned to a normal level after the recovery time. This study went further to evaluate the expression of steroidogenic genes. They found that the two genes critical for completion of the testosterone biosynthesis pathway were downregulated, namely 17 β -hydroxysteroid dehydrogenase and 3 β -hydroxysteroid dehydrogenase, thus explaining the decreased testosterone levels at the end of chemotherapy. Even after the recovery time, the chemotherapy still had inhibitory effects on the transcription of these genes. However, testosterone levels did not show any significant differences with the control group, most likely due to unaffected steroidogenic acute regulatory protein (StAR) expression in the testis, which actually indicated a trend to increase. The StAR protein mediates transmembrane cholesterol transport in mitochondria, an essential rate-limiting step in testosterone synthesis^[49].

Radiation also alters Leydig cell function. Sivakumar *et al.*^[50] evaluated the mechanism behind radiation-induced dysfunction by culturing Leydig cells and exposing them to different doses of fractionated gamma radiation. Researchers found that radiation exposure inhibited Leydig cell steroidogenesis in a dose-dependent manner. They found that at higher doses, radiation exposure impaired Leydig cell steroidogenesis by affecting LH signal transduction at the level of both pre- and post-cAMP generation. Just as in the chemotherapy-treated model, radiation seems to directly alter the steroidogenic pathways of the Leydig cells. However, it has not been determined how radiation affects SLCs. To fully design effective therapies, it will be important to understand the pathologic effects of radiation on SLCs. This will determine the point in the process at which time therapy will intervene.

STEM CELL THERAPY

Multiple stem cell therapies to restore androgenic function of the testes are under investigation (Table 1). Leydig cells derived from bone marrow, adipose tissue, umbilical cord, and the testes have shown promise in future therapy for primary hypogonadism. An initial study by Lue *et al.*^[51] injected unfractionated bone marrow cells into the seminiferous tubules and testicular interstitium of mice. The results demonstrated that the murine bone marrow cells had the potential to differentiate into germ cells, Sertoli, and Leydig cells *in vivo*. However, it was unknown which precursor cell from the bone marrow differentiated into each end testicular cell type. Lo *et al.*^[52] demonstrated that murine testicular stem cells, isolated from the interstitial space of the testis and transplanted into the interstitial

Table 1 Summary of preclinical trials showing successful differentiation of various stem cell lines into steroidogenic Leydig-like cells

Ref.	Stem cells used	Study type	Design	Results
Lo <i>et al</i> ^[52]	Mouse mixed testicular stem cells (SP) containing spermatogonial, leydig cell, and myoid stem cells	<i>In vivo</i>	SCs were injected into the testes of sterile sertoli-cell only transgenic mice and transgenic mice with a targeted deletion of 4-kb pairs of the LH receptor gene	SP cell transplanted mice had increased time-dependent serum testosterone and spermatogenesis compared to non-SP cell transplanted mice
Yazawa <i>et al</i> ^[53]	Rat BM-MSCs	<i>In vivo</i>	BM-MSCs were injected into the testes of 3-wk old Sprague-Dawley rats	BM-MSCs differentiated into steroidogenic cells similar to Leydig cells
	Mouse MSCs		MSCs were transfected with Sf-1 followed by treatment with cAMP and cultured in Iscova's MEM or DMEM with 10% fetal calf serum	Transfected cells differentiated into Leydig cells
Lue <i>et al</i> ^[51]	Unfractionated mouse bone marrow stem cells	<i>In vitro</i>	SCs were injected into the testes of busulfan treated mice and c-kit mutant homozygous mice	SCs differentiated into Leydig, Sertoli, and germ cells after 12 wk. Though germ cells were lacking in c-kit mutant mice
Gondo <i>et al</i> ^[63]	Mouse AMCs	<i>In vitro</i>	AMCs and BMCs were transfected with SF-1 and cultured with Medium A	AMCs were more likely to differentiate into adrenal-type steroidogenic cells with increased production of corticosterone
	Mouse BMCs			BMCs were more likely to differentiate into gonadal-type steroidogenic cells with increased production of testosterone
Yazawa <i>et al</i> ^[54]	Human BM-MSCs	<i>In vitro</i>	BM-MSCs were transfected with LRH-1 followed by treatment with cAMP and cultured in DMEM with 10% fetal calf serum	Transfected cells expressed CYP17 and produced testosterone
Yazawa <i>et al</i> ^[55]	UC-MSCs	<i>In vitro</i>	UC-MSCs were transfected with SF-1 followed by treatment with cAMP and cultured in DMEM/Ham's F-12 supplemented with 0.1% BSA	Transfected cells differentiated into cells with similar characteristics to granulosa-luteal cells
Wei <i>et al</i> ^[62]	Human UC-MSCs Human BM-MSCs	<i>In vitro</i>	UC-MSCs and BM-MSCs were transfected with SF-1 and cultured in the presence of cAMP	Differentiated UC-MSCs had higher expression of steroidogenic mRNAs. They also secreted significantly greater amounts of testosterone and cortisol than BM-MSCs
Yazawa <i>et al</i> ^[64]	Rat BM-MSCs	<i>In vivo</i>	BM-MSCs were transplanted into prepubertal testes	MSCs were able to differentiate into steroidogenic Leydig cells <i>in vivo</i> . SF-1 expression was also detected
Yang <i>et al</i> ^[56]	Rat ADSCs	<i>In vivo</i>	ADSCs were injected into Sprague-dawley rats that had been treated with D-gal (aging model) or saline (control) for 8 wk	ADSCs migrated to damaged areas, reduced the number of apoptotic Leydig cells, and upregulated enzymes to increase testosterone levels in the testis in those treated with D-gal
Yang <i>et al</i> ^[58]	Mouse ESCs	<i>In vivo</i>	ESCs were cultured with cAMP, SF-1, and FSK. These derived Leydig-like cells were then injected into Sprague-dawley rats treated with EDS	FSK enhanced the differentiation of mESCs into Leydig-like cells. Subsequent treatment with these newly differentiated cells led to increased testosterone levels in EDS-treated rats
Hou <i>et al</i> ^[57]	Human BM-MSCs	<i>In vitro</i>	Experimental - BM-MSCs were cultured in conditional medium with different concentrations of HMG/LH Control - BMSCs were cultured in FBS in DMEM medium with normal sodium	Experimental culture medium induced the differentiation of BMSCs into Leydig cells
Zhang <i>et al</i> ^[59]	Rat SLCs	<i>In vitro</i>	SLCs were cultured in a seminiferous tubule model using media containing NGF. The proliferative capacity of SLCs, along with testosterone production, and steroidogenic gene/protein expression was measured	NGF significantly promoted the proliferation of stem Leydig cells and also induced steroidogenic enzyme gene expression and 3 β -HSD protein expression
Odeh <i>et al</i> ^[60]	Rat SLCs	<i>In vitro</i>	SLCs were cultured on the surfaces of seminiferous tubules in a media containing PDGF-AA or PDGF-BB for up to 4 wk. SLC proliferation and differentiation were measured	Both PDGF-AA and PDGF-BB stimulated SLC proliferation during the first week of culture. After this first week, PDGF-AA had a stimulatory effect on SLC differentiation. PDGF-BB began inhibiting differentiation after this first week

Li <i>et al.</i> ^[61]	Rat SLCs	<i>In vitro</i>	SLCs were cultured on the surface of seminiferous tubules to assess the ability of factors from the seminiferous tubules to regulate their proliferation and their subsequent entry into the Leydig cell lineage	SLC proliferation was stimulated by DHH, FGF2, PDGF, and activin. Differentiation was activated by DHH, lithium-induced signaling, and activin, and inhibited by TGF- β , PDGF-BB, and FGF2
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BM-MSCs: Bone marrow-derived mesenchymal stem cells; AMCs: Adipose derived mesenchymal cells; BMCs: Bone marrow cells; UC-MSCs: Umbilical cord mesenchymal stem cells; ADSCs: Adipose-derived mesenchymal stem cells; ESCs: Embryonic stem cells; SLCs: Stem leydig cells; LH: Luteinizing hormone; HMG: Human menopausal gonadotropin; FBS: Fetal bovine serum; PDGF-AA: Platelet-derived growth factor alpha; DHH: Desert hedgehog; FGF: Fibroblast growth factor; LRH-1: Liver receptor homolog-1; SF-1: Steroidogenic factor-1; BSA: Bovine serum albumin; cAMP: Cyclic adenosine monophosphate; EDS: Ethane dimethanesulfonate; NGF: Nerve growth factor.

space of LH receptor knockout mice, yielded a time dependent production of testosterone in a hypogonadal murine model. Yet, these cells were derived from a side population and contained stem cells of multiple lineages including spermatogonial stem cells, SLCs, and possibly myoid stem cells. As in the previous study, it was difficult to determine which cell lineage led to the final end testosterone-secreting cell.

Yazawa *et al.*^[53] injected murine bone marrow-derived mesenchymal cells (BMSCs) into murine testis and demonstrated their differentiation into Leydig cells. They also demonstrated that the same murine BMSCs, when cultured *in vitro* with steroidogenic factor -1 (SF-1) followed by cAMP stimulation, underwent differentiation into Leydig cells. However, when this group cultured human BMSCs with SF-1 followed by cAMP, the cells differentiated into human-derived steroidogenic cells that preferentially produced glucocorticoids, rather than testosterone. Furthermore, when the group injected human BMSCs into murine testis, the cells did not survive long enough for analysis. Researchers hypothesized that the differing steroidogenic products observed in the mouse and human BMSCs were due to heterogeneous populations of stem cells that had different differentiation potentials. Thus, the mouse BMSCs had already committed to the gonadal lineage, whereas the human BMSCs were already committed to the adrenal lineage. This group later demonstrated that human BMSC differentiation into steroidogenic cells was possible with cAMP and liver receptor homolog-1 (LRH-1), rather than cAMP and SF-1, indicating another possible regulator of Leydig stem cell differentiation^[54]. Interestingly, when this group used the method of SF-1 and cAMP on umbilical cord blood-derived MSCs, these steroidogenic cells had similar characteristics of granulosa-luteal cells^[55]. These studies highlighted the fact that stem cells from multiple species have the potential to differentiate into different types of steroidogenic cells.

Yang *et al.*^[56] administered adipose-derived mesenchymal stem cells (ADSCs) into the caudal vein of a D-galactose aging rat model. D-galactose accelerates aging and causes symptoms simulating natural senescence, thus creating an ideal pathophysiological model for evaluating stem cell therapy. This group found that ADSCs migrated to damaged areas of the testes, reduced the number of apoptotic Leydig cells, and

increased serum testosterone. The authors suggested that the ADSCs might prevent ROS production and reduce SLC apoptosis. Supporting this line of reasoning, they found that the increased testicular lipid peroxidation in the aged model was reversed by a subsequent increase in antioxidant enzymes after ADSC therapy. As has been observed in other disease states treated by ADSCs, the mechanism of action is more likely due to a secretion of cytokines and growth factors, with little direct effect on stem cell differentiation. As proof, only a few ADSCs differentiated into new Leydig cells based on labeling and 3 β -HSD expression, while serum testosterone concentrations increased progressively. The immunohistochemical results of the present study suggest that the treatment effect of ADSCs is mediated, at least in part, by a decrease in intracorporal tissue apoptosis and increase in sinusoidal endothelial cells.

Researchers have also been able to manipulate the hormonal milieu to induce the differentiation of human BMSCs into Leydig cells *in vitro*. By using a medium containing human menopausal gonadotropin/luteinizing hormone, hCG, platelet-derived growth factor, and interleukin-1 α , they were able to promote the differentiation of human BMSCs into Leydig cells. However, the cells exhibited senescence and, thus, androgen decline after three weeks of culture. These results highlight the aforementioned problem that Leydig cells are mitotically inactive and that the primary immature Leydig cells lose their desired characteristics during prolonged cultures^[57]. Using murine embryonic stem cells, one study used SF-1, 8-bromoadenosine-3',5'-cyclic monophosphate (8-Br-cAMP), and forskolin to direct differentiation towards Leydig like cells. *In vitro*, these cells produced progesterone and testosterone. When injected into EDS-treated rat testes, these cells improved serum testosterone levels. However, this research group was plagued by a difficulty in obtaining a large enough number Leydig-like cells given that they do not proliferate as readily as the undifferentiated cells^[58]. Despite using different stem cells reservoirs, bone marrow, and embryonic stem cells, neither group was able to produce mitotically active Leydig cells.

And while there have been numerous studies evaluating the use of stem cells from various tissue origins to regenerate mature Leydig cells, few have attempted to reactivate SLCs. However, there are select studies that in exploring the mechanisms that underlie the SLC

maturation process, have found growth factors that lead to reactivation. An initial study explored the role of nerve growth factor (NGF) during SLC differentiation^[59]. They found that in an *in vitro* model, NGF significantly promoted the proliferation of SLCs and also induced steroidogenic enzyme gene expression and 3 β -HSD protein expression. Another group evaluated platelet-derived growth factor alpha (PDGF-AA) and beta (PDGF-BB)^[60]. They found that both ligands stimulated SLC proliferation during the first week of culture. After this first week, PDGF-AA had a stimulatory effect on SLC differentiation. In contrast PDGF-BB, began inhibiting differentiation after this first week. Corroborating some of the results of this study, another group developed an *in vitro* system of cultured seminiferous tubules to assess the ability of factors from the seminiferous tubules to regulate the proliferation and differentiation of SLCs^[61]. SLC proliferation was stimulated by Desert hedgehog (DHH), basic fibroblast growth factor (FGF2), platelet-derived growth factor (PDGF), and activin. Differentiation of the stem cells was activated by DHH, lithium-induced signaling, and activin, and inhibited by TGF- β , PDGF-BB, and FGF2. Building upon these initial studies, it will be necessary to evaluate these growth factors in an *in vivo* animal model.

CONCLUSION

There appears to be two current directions for stem cell therapy in male primary hypogonadism. The first method involves differentiating adult Leydig cells from stem cells of various origins from bone marrow, adipose, or embryonic sources. The second method involves isolating, identifying, and transplanting SLCs into testicular tissue. The first method's shortcomings that should be resolved in future studies include decoding and promoting stem cells to become testosterone-producing steroidogenic cells and improving the mitotic activity of differentiated Leydig cells. One study compared steroidogenic cells from BMSC to those of umbilical cord mesenchymal stem cells and found that umbilical cord mesenchymal stem cells have a greater steroidogenic potential^[62]. However, as previously mentioned, the addition of SF-1 and cAMP *in vitro* to umbilical cord stem cells has been shown to yield cells resembling granulosa-luteal cells, not Leydig-like cells^[55]. Another study demonstrated that the addition of SF-1 and cAMP to ADSCs yielded cells that preferentially produced corticosterone, rather than testosterone^[63]. Undoubtedly much remains unknown about the cellular environment needed to produce specific steroidogenic cell types^[64]. Additionally, this type of therapy may not be durable due to adult Leydig cell senescence and androgen production decline. Younger patients who have undergone premature Leydig cell dysfunction due to chemotherapy and radiation may find long-term success with the transplantation of cells with more regenerative capacity. Alternatively, in the aging population, it might be feasible to differentiate mesenchymal stem cells into

SLCs. If this strategy would address the issue of growth arrest, the use of mesenchymal stem cells may be in the best interest of these patients, whose SLCs are likely damaged. Finally, there are also concerns about the delivery of SF-1, which is currently performed episomally or virally. Efforts are underway to determine a method of gene-free delivery of inducing SF-1 and LRH-1 expression^[64,65].

The second method for stem cell therapy involves the transplantation of SLCs into hypogonadal testicular tissue, with the idea that this therapy's regenerative capacity will be self-fulfilling and could be used for younger patients. However, it is currently troubled by the technique for identification and isolation of SLCs, which is in its infancy^[66]. Additionally, if the transplant were to be autologous in these men, SLCs could be extracted prior to chemotherapy and radiation, as it is likely that these treatments irreversibly damage SLCs. However, another method that has been proposed includes harvesting SLCs in the hypogonadal male, and then amplifying and differentiating these cells into adult Leydig cells *in vitro*, then transplanting autologously into the same man^[66]. Theoretically, the *in vivo* re-activation of SLCs in men with primary hypogonadism due to age would be an alternative method to treat hypogonadism, while eliminating the need for transplantation.

These proposed mechanisms all have the advantage of being subject to physiologic cues, standing in contrast to the current option of a lifetime of exogenously administered testosterone. Current and future research collaborations in the field of male hypogonadism and the regeneration of steroidogenic tissue will influence which modalities will become clinical realities for this patient population.

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Update on acute myeloid leukemia stem cells: New discoveries and therapeutic opportunities

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Abstract

The existence of cancer stem cells has been well

established in acute myeloid leukemia. Initial proof of the existence of leukemia stem cells (LSCs) was accomplished by functional studies in xenograft models making use of the key features shared with normal hematopoietic stem cells (HSCs) such as the capacity of self-renewal and the ability to initiate and sustain growth of progenitors *in vivo*. Significant progress has also been made in identifying the phenotype and signaling pathways specific for LSCs. Therapeutically, a multitude of drugs targeting LSCs are in different phases of preclinical and clinical development. This review focuses on recent discoveries which have advanced our understanding of LSC biology and provided rational targets for development of novel therapeutic agents. One of the major challenges is how to target the self-renewal pathways of LSCs without affecting normal HSCs significantly therefore providing an acceptable therapeutic window. Important issues pertinent to the successful design and conduct of clinical trials evaluating drugs targeting LSCs will be discussed as well.

Key words: Leukemia stem cells; Cancer stem cells; Acute myeloid leukemia; Stem cell niche; Xenotransplantation; Plerixafor; NF- κ B; C-X-C chemokine receptor type 4

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Core tip: Leukemia stem cell (LSC) directed therapy targets: (1) Cell surface markers expressed on LSC: CD33, CD44, CD123, CD47, *etc.*; (2) Crucial pathways for maintenance of their stemness: NF- κ B, PI3K/AKT/mTOR and bcl-2; and (3) Interactions between LSC in the bone marrow niche: LSC mobilization with granulocyte-colony stimulating factor and inhibition of LSC homing to the bone marrow by interrupting the C-X-C chemokine receptor type 4-CXCL12 and VCAM-VLA4 axis.

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INTRODUCTION

Despite extensive research efforts in myeloid malignancies, minimal progress has been made in introducing new effective treatment strategies for acute myeloid leukemia (AML) since the introduction of the anthracycline-cytarabine combination chemotherapy regimens (known as 7 + 3) more than 40 years ago^[1]. Despite achieving complete remission (CR) with intensive induction chemotherapy in about 70% of patients with AML, relapse is frequent and the rate of 5-year disease free survival is only about 30%-40%. It has been long proposed that the high rate of relapse is due to the persistence of a rare subset of malignant cells that are not effectively eliminated by current treatment regimens, the so called leukemia stem cells (LSCs)^[2-4]. LSC were first identified but tumor cells with stem cell-like behavior were later found to be also present in a variety of solid tumors^[5-9]. LSC remain the best studied and characterized cancer stem cell (CSC) due to the easy accessibility of tumor tissue for (*i.e.*, blood and bone marrow) and the availability of a number of cell surface markers that allow their prospective identification and isolation by flow cytometry followed by assays to examine their function both *in vitro* and *in vivo*^[10]. This review will focus on the biology of LSC, the impact they have on current leukemia diagnosis and prognosis and treatment as well as future directions of leukemia therapy based on targeting LSC^[6].

CSC VS CLONAL EVOLUTION THEORY

It is now well understood that not only tumors from different patients but also cells within a single tumor are characterized by heterogeneity in terms of the morphology, cell surface markers, genetic variations and response to therapy^[11]. Why there is significant variation in genetic and epigenetic abnormalities between different cells or locations within a tumor despite the clonal origin of all tumor cells, is a question that has puzzled researchers for decades. There are essentially two different explanations for this fundamental problem of cancer biology: The hierarchy or CSC model vs the stochastic or clonal evolution model^[6]. In the stochastic model, all cells in a tumor have a similar biological function but are heterogeneous (*e.g.*, expression of cell surface markers) because of clonal evolution resulting in small but entirely random/stochastic variations triggered by external and internal factors based on Darwinian principles. Importantly, all cells within the tumor have an equal sensitivity to both

intrinsic (transcription factors and signaling pathways) and extrinsic (host factors, tumor microenvironment and immune response) factors^[10]. In the cancer stem cell (CSC) model, a tumor follows the principles of normal, healthy tissue development with a stem cell at the top of the hierarchy, which gives rise to all other cells in the tumor. In this model only these rare population of CSCs are able to initiate tumor growth: They possess self-renewal capacity and can be isolated from the bulk non-tumorigenic population. Importantly, both models appreciate the existence of a CSC but differ in their assessment what cells within the tumor can be CSCs. In the stochastic model CSCs are created randomly and every cell has the potential to be a CSC, whereas in the CSC model only a subset of cancer cells has the potential to behave like a stem cell^[11].

Whether the stochastic model or the CSC model best reflects tumorigenesis/leukemogenesis, has significant impact on how cancer/leukemia should be treated^[10]. In the stochastic model, the cells within a tumor are relatively homogeneous in terms of genetic makeup and function and therapy can be uniformly directed at the bulk of tumor cells. However, per the CSC model, tumorigenic pathways might operate differently in CSCs compared with the bulk cells and therapy must specifically target the CSCs in order to be truly effective. Most of the current targeted therapies against leukemia and cancer focuses on inhibiting the molecular drivers found in all cancer cells but do not necessarily target CSCs^[11].

BIOLOGY OF LSCS

CSC characteristics

The definition of a LSC is adapted from normal HSC: It is a cell that possesses the capacity to self-renew, proliferates and gives rise to leukemic blasts, which are morphologically homogeneous but biologically heterogeneous^[12]. Apart from self-renewal potential, dormancy/quiescence and a protective stem cell niche are shared characteristics between HSCs and LSCs.

Self-renewal capacity: As the definition of CSCs is a functional definition, CSCs can thus only be defined experimentally by their ability to recapitulate the generation of a continuously growing tumor. Immuno-deficient mice, such as the non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mouse and newer generations of xenograft models, are used to functionally define human hematopoietic stem and progenitor cells as well as LSCs^[13]. Long-term repopulating cells, thought to be LSC are able to be successfully engrafted in these mice over prolonged periods as well as in secondary recipients^[2,14]. Bonnet *et al.*^[15] in the John Dick laboratory isolated subpopulations of cells from primary human AML bone marrow based on their immunophenotype and xenotransplanted them into NOD/SCID mice. It demonstrated that the CD34⁺CD38⁻ expressing sub-population of AML cells

were capable of being serially transplanted in these immunodeficient mice^[15,16]. Reflecting the emphasis on functional assessment, these cells were named as SCID leukemia-initiating cells (SL-IC) and are considered the equivalent of LSC.

Symmetrical vs asymmetrical cell division: Similar to HSCs, LSCs have the ability to undergo symmetrical self-renewing cell division, generating identical daughter stem cells that retain self-renewal capacity (expansion), or an asymmetrical self-renewing cell division, resulting in one stem cell and one more differentiated progenitor cell (maintenance)^[12,17-19]. Normal stem cells are able to switch between symmetrical and asymmetrical division based on the demands of the tissue they are meant to maintain. During early embryogenesis normal stem cells undergo symmetrical cell division in order to expand the total pool of stem cells giving rise to tissues whereas in adult tissues stem cells give rise to mature cells through asymmetrical cell division^[19,20]. There is increasing amount of evidence that in CSCs this delicate balance seems to be disturbed in favor of symmetric cell division^[19,21,22]. For example, CSCs isolated from ERBB2-expressing breast cancer have been demonstrated to prefer symmetric cell division compared to normal breast tissue stem cells^[23]. Furthermore, the adenomatous polyposis coli tumor suppressor gene (*APC*) has been shown to play a major role in regulating asymmetric cell division in *Drosophila* and its mutational loss is suspected to lead to an expansion of CSCs by symmetric cell division^[22,24,25].

Stem cell quiescence and exhaustion: Normal stem cells need to be quiescent to avoid exhaustion of a stem cell pool and to minimize the risk of oncogenic events^[26]. In fact, stem cell exhaustion has been described as one reason for aging and as a consequence of the attempt of the body to prevent the development of cancer^[27]. Aging leads to an accumulation of DNA damage in all cells of the body, including stem cells, which in turn leads to an increased risk of developing cancer. Aging stem cells are affected by sophisticated mechanisms cells have developed to suppress the development of cancer, mainly induction of senescence and apoptosis, which are mediated through telomere shortening and activation of tumor suppressor genes *p16* and *p53*^[28-30]. The diminished ability of aging HSC to reconstitute the hematopoietic system is demonstrated by prolonged myelosuppression after cytotoxic chemotherapy in older patients as well as age of the stem cell donor being significantly associated with overall and disease-free survival after hematopoietic stem cell transplant^[31,32].

However, normal stem cells are also required to continuously replenish the cells that are lost in a tissue. In order to fulfill both purposes-avoid exhaustion as well as maintaining the cellular integrity of a tissue-stem cells undergo asymmetric cell divisions, which give rise to another stem cell as well as a rapidly dividing progenitor cells. These progenitor cells proliferate quickly for a

limited amount of cell divisions and regenerate all cells in a tissue^[33,34].

Similarly, LSCs are quiescent, which explains the difficulties to eradicate LSCs with standard chemotherapies that preferentially target rapid proliferating cells^[35-37].

Key signaling pathways relevant for retaining stemness: Similar signaling pathways involved in the control of self-renewal of HSCs are also key elements maintaining stemness in LSCs (Figure 1). Among many others, these pathways include PI3K/Akt/mTOR^[38], Wnt/ β -catenin^[39,40], Hedgehog^[41,42], NF- κ B^[43,44], Notch^[45] and Bcl-2^[46,47]. Several drugs targeting these pathways are in different stages of preclinical and clinical development (Figure 1).

Stem cell niche: The bone marrow niche is quintessential for normal HSC to maintain their quiescence but at the same time enable HSC to generate cells in the blood stream to meet the organism's needs^[48]. The stem cell niche is formed by a complex network of different cells including vascular endothelial cells, perivascular mesenchymal cells, megakaryocytes, osteoblastic lineage cells, macrophages and nerve cells^[49-53]. Dysregulation of the bone marrow niche plays an important role in preventing the detection of LSC by the immune system and protecting LSC from the effects of chemotherapy^[48,54]. Similar to normal HSCs, LSCs are retained in the marrow niche by interactions between CXCR4, on stem cells, and CXCL12 (SDF-1), on osteoblasts and mesenchymal cells in the bone marrow niche^[55,56]. Chemokine interactions through CXCL12 can lead to up-regulation of vascular cell adhesion molecule-1 (VCAM-1) and very late antigen-4 (VLA-4) expression, which further strengthen LSC retention in the marrow niche^[57,58] (Figure 1). The significance of the interaction between LSCs and the protective bone marrow niche is exemplified by the fact that elevated levels of CXCR4 and VLA-4 have been associated with poor response to chemotherapy and decreased survival^[59-61]. Several therapeutic approaches attempt to break the dormancy of LSCs by induction of stem cell cycling with granulocyte-colony stimulating factor (G-CSF) and inhibition of the CXCR4-SDF-1 axis involved in LSC retention in the protective bone marrow niche^[62,63] (Figure 1).

Identification of LSCs by surface markers

Recent studies have shown that LSCs may reside not only in CD34⁺CD38⁻, but also in CD34⁺CD38⁺ and CD34⁻CD38⁺ compartments demonstrating the lack of a definitive phenotype for LSCs^[64-66]. Several studies have shown that the CD34⁺CD38⁺ fraction has repopulating ability when immunosuppression is applied^[18,67,68]. It was demonstrated that by treating mice with immunosuppressive antibodies, the CD34⁺CD38⁺ fraction of AML samples is able to initiate leukemia in immunodeficient mice^[64]. Furthermore, by transplanting sorted

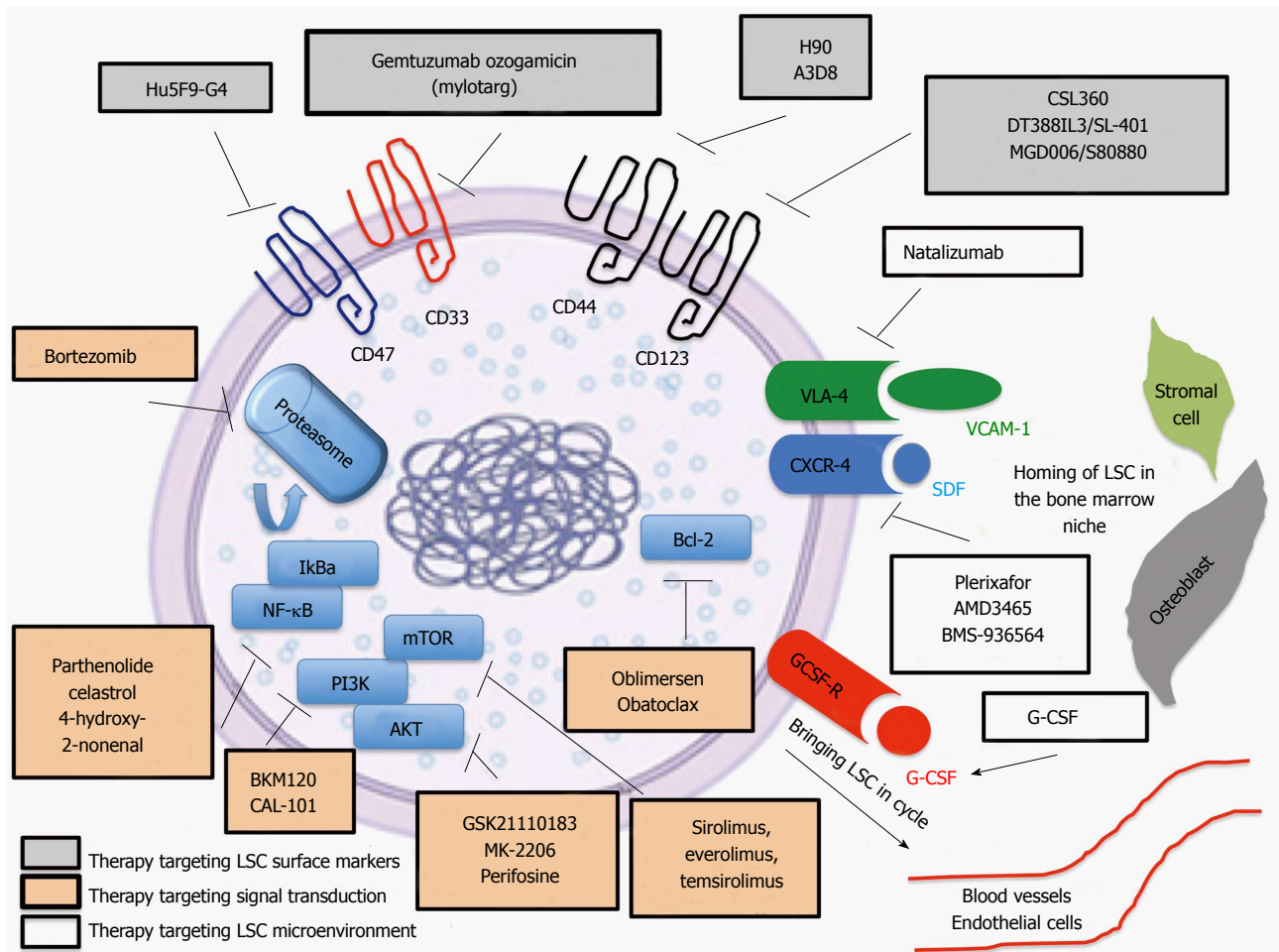


Figure 1 Leukemia stem cells biology and selected therapeutic strategies/agents targeting leukemia stem cell. Leukemia stem cells (LSC) directed therapy targets cell surface markers expressed on LSC (grey boxes), crucial pathways for maintenance of stemness (orange boxes) and interactions between LSC and the bone marrow niche (white boxes). Important LSC surface markers are CD33, CD44, CD123, CD47. Essential pathways are NF- κ B, PI3K/AKT/mTOR and bcl-2. LSC mobilization is accomplished with G-CSF and LSC homing to the bone marrow is regulated by the CXCR4–CXCL12 and VCAM–VLA4 axis. VCAM-1: Vascular cell adhesion protein-1; VLA-4: Very late antigen-4; CXCR4: C-X-C chemokine receptor type 4; SDF: Stromal cell-derived factor 1; PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT: Protein kinase B; mTOR: Mechanistic target of rapamycin; bcl-2: B-cell lymphoma 2; G-CSF: Granulocyte-colony stimulating factor.

fractions of primary *NPM*-mutated AML into immuno-deficient mice, it was shown that approximately one-half of cases had LICs exclusively within the CD34⁺ fraction, whereas the CD34⁺ fraction contained normal multilineage hematopoietic repopulating cells^[66]. Most of the remaining cases had LICs in both CD34⁺ and CD34⁺ fractions and when samples were sorted based on CD34 and CD38 expression, multiple fractions initiated leukemia in primary and secondary recipients (Table 1).

Heterogeneity within the LSC population

Over the last years several groups have found a wide variety of other markers that appear to be expressed higher in LSCs than normal HSCs^[14].

These include CD123, CD96, CLL-1, TIM3, CD33, CD13, CD44, CD47 and others^[69–75] (Table 1). In essence, these studies suggest that leukemogenic activity is not restricted to the CD34⁺CD38⁺ fraction and there is heterogeneity among patients in leukemogenic cell phenotype. Over the last years, there has been significant advancement in the understanding of the

complexity and heterogeneity of human LSC. Several important observations have been made along the way of discovery.

LSC heterogeneity within a patient: First, there is heterogeneity of the stem cell population within the same patient as not all LSC have the same self-renewal capacity^[10,76]. Use of lentiviral gene marking to track the behavior of individual leukemia initiating cells following serial transplantation has revealed heterogeneity in their ability to repopulate secondary and tertiary recipients and this enabled researchers to classify long term (LT-LSC) and short term (ST-LSC) LSCs^[76,77]. LT-LSCs are defined by a long-termed persistence in xenotransplantation models given an extensive self-renewal capacity while ST-LSCs have a reduced self-renewal capacity and only a transient repopulation capability in xenotransplantation models.

LSC heterogeneity based on the specific xeno-transplantation model used: The LSC phenotype

Table 1 Markers of leukemia stem cells

Cell surface markers	Patient samples used	Mouse model used	Ref.
CD34 ⁺ CD38 ⁻ CD34 ⁺ CD38 ⁺	FAB M1, M4, M5 CN-AML, MLL-ENL	NOD/SCID NOD/SCID + IVIG or anti-CD122	[15,16] [18,64,67,68]
CD34 ⁺ CD38 ⁺	AML with NPM1 mutation	NOD/SCID β -2 microglobulin NOD/SCID IL2 receptor $\gamma^{-/-}$ + IVIG	[66]
CD34 ⁺ CD123 ⁺ CD34 ⁺ CD38 ⁺ CD96 ⁺	FAB M1, M2, M4 CK-AML, CBF- MYH11, PML-RARA, AML1-ETO, FAB M4	NOD/SCID Rag2 ^{-/-} IL2RG ^{-/-}	[69] [70]
CD34 ⁺ CLL1 ⁺	AMLs with FLT3- ITD	NOD/SCID	[71]
TIM3 ⁺	FAB M1, M2, M4	NOD/Rag1 ^{-/-} IL2RG ^{-/-}	[72]
CD34 ⁺ CD38 ⁻ CD33 ⁺ CD13 ⁺	CN-AML, CBF-AML, MLL-ENL	NOD/SCID	[73]

FAB: French-American-British classification system; CN: Cytogenetically normal; CK: Cytogenetically complex; MLL-ENL: Mixed-lineage leukemia-eleven nineteen leukemia; NPM1: Nucleophosmin 1; CBF-
MYH11: Core binding factor beta unit-Myosin heavy chain 11; PML-
RARA: Promyelocytic leukemia-retinoic acid receptor alpha; AML1-ETO:
Acute myeloid leukemia 1 protein- eight twenty one; FLT3-ITD: Fms-
like tyrosine kinase 3 Internal tandem duplication; NOD/SCID: Non-
obese diabetic/severe combined immunodeficiency; Rag: Recombination
activating gene; IL2RG: Interleukin 2 receptor subunit gamma.

depends on what mouse model is used for functional assessment of stem cell properties of human AML cells^[14] (Table 1). The bone marrow niche in mice differ from that of humans in terms of architecture, stromal cells, cytokines, growth factors, adhesion molecules and most importantly the immune cell composition, which potentially impairs growth of human HSC or LSC in the mouse bone marrow^[78]. Normal HSCs and LSC are therefore more likely to be detected in more highly immunodeficient mice. As different xenotransplantation mouse models display different levels of immunodeficiency they are associated with different levels of engraftment of normal human HSCs and LSCs^[6,14]. In nude mice T cells are absent whereas in severe combined immunodeficiency mice (SCID mice) both B and T cells are inactivated. NOD/SCID mice, which harbor defects in T, B, and macrophage activity, support higher levels of human engraftment^[14]. NOD/SCID gamma (NSG) mice have almost no murine immune system left as a complete null mutation in the gene encoding the interleukin 2-receptor gamma chain blocks NK cell differentiation^[79]. Similarly, NK cells can be depleted by treating NOD/SCID mice with anti-CD122 antibodies^[80]. In creating a supportive bone marrow niche for engraftment of human AML cells not only a suppression of the hosts immune system is essential but

also a recreation of the cytokine environment supporting stem cell self-renewal and quiescence^[14]. This has led to the development of mice models that express human cytokines like human SCF, GM-SCF, IL3 and TPO^[13,81].

LSC heterogeneity between patients: It has become increasingly evident that the LSC phenotype varies between patients based on the specific subtype of leukemia that they suffer from (Table 1). As mentioned above, the majority of AML cells express CD34, however in AML cells carrying a mutation in NPM1 the CD34⁺ percentage is very low and LSC activity is exclusively restricted to the CD34⁻ population^[66]. Furthermore, specific subtypes of AML (in particular less aggressive subtypes) are significantly more difficult to be engrafted as they may have low progenitor cell frequency or are particularly sensitive to a specific cytokine or cell type missing in the particular xenotransplantation model^[14]. For example, AML samples with a t(8;21) translocation were shown to be difficult to be engrafted and found to be dependent on signaling through the TPO/mpl pathway^[82,83]. Subsequently, human TPO knock-in mice were shown to have improved engraftment for t(8;21) AML samples^[84].

Cell of origin of LSCs

It is important to distinguish the concept of the cell of origin from the CSC^[10]. The CSC has stem cell like properties and is capable of initiating and sustaining tumor growth, whereas the cell of origin refers to the normal cell in which the initial transforming event occurs. Importantly, cancer and LSCs do not have to arise from a normal stem cell, in fact, it is not entirely clear what the cell of origin for most LSCs is^[11,12]. One hypothesis is that LSCs are only able to arise from normal HSCs but not from committed progenitor cells^[10,15]. This theory is supported by the observation that LSCs and HSCs share many characteristics like self-renewal capacity controlled by genes like *Bmi1* and *PTEN* and quiescence^[35,85,86]. On the contrary, transformation might occur in a variety of cell types in the hematopoietic hierarchy, including HSCs and committed progenitors^[10,87]. Experimental evidence in mice shows that LSCs may arise either through neoplastic changes initiated in normal self-renewing HSCs or downstream progenitors cells^[10,11,88]. Some oncogenes including *MOZ-TIF*, *MLL-AF9* and *MLL-ENL* can induce LSCs regardless of what target cell population they are expressed in^[88-90]. Other oncogenes like *BCR-ABL*, *FLT3-ITD*, *Hoxa9* and *Meis1* were found to be oncogenic when expressed in HSCs but not when expressed in progenitor cells^[39,89,91]. However, experimental data in murine studies might be confounded by non-physiologic levels of expression from exogenous promoters, such as transgenes or retroviral vectors^[11]. This was demonstrated by the recent finding that in an MLL-AF9 knock-in model of the same construct shown to initiate disease in both HSCs and progenitor cells by retroviral expression only initiated leukemia from HSCs when expressed from the endogenous MLL

promoter^[92]. *In vivo* clonality studies in humans suggest variations in the cells of origin and it was demonstrated that in patients with t(8;21) AML primitive CD34⁺CD90⁻CD38⁻ HSC like cells from leukemic bone marrow give rise to normally differentiating progenitors, whereas more mature CD34⁺CD90⁻CD38⁺ multi-potent progenitor like cells form exclusively leukemic blast colonies^[93-95]. These observations suggest that the truth about the cell of origin might be reflected by a combination of both theories depicted above: Although the initial genetic mutation might happen in HSCs subsequent events occur in the committed progenitor pool, giving rise to LSCs^[11].

IMPACT OF LSC ON CURRENT TREATMENT AND PROGNOSIS

Impact on prognosis

The LSC burden of AML patient is suggested to be a strong biomarker for clinical outcome in AML^[96-100]. The ability of cells from AML patients to engraft NOD/SCID mice and the LSC frequency (simplistically characterized as CD34⁺CD38⁻ frequency) are associated with worse clinical outcomes^[99-101]. AML patients with greater than 3.5% of CD34⁺CD38⁻ AML cells show a median relapse free survival of 5.6 mo vs 16 mo in those with a lower percentage of CD34⁺CD38⁻ cells^[96]. Furthermore, poor clinical outcome seems to correlate with the degree to which the LSCs matched normal HSC gene expression^[98].

It is noted that it is controversial whether the simplistically phenotypically defined LSC frequency (characterized as CD34⁺CD38⁻) in AML is prognostic and correlates with xenograft potential^[14]. Also, as described above, LSCs can be found outside of the CD34⁺CD38⁻ cell fraction. An improved characterization of subpopulations of LSCs is expected to be associated with improved prediction of prognosis.

Impact on current therapies

It is thought that LSCs have a significant role in the relapse of leukemia as induction chemotherapy targets the bulk of blast cells but not LSC^[102]. Minimal residual disease (MRD) is an important determinant for relapse and poor outcomes in AML and it is likely that the MRD cell population contains LSCs^[103-105]. Thus, in order to improve outcomes in AML, MRD needs to be reduced to prevent disease relapse. LSCs seem to be only minimally affected by traditional chemotherapy^[35,106]. Several reasons for chemotherapy resistance have been proposed, which are related to the key features of LSCs discussed above. LSCs are quiescent in the G0 phase of the cell cycle but chemotherapy is only effective in killing rapidly cycling cells^[36,37]. LSCs are supported by a stem cell niche in the bone marrow protecting them from the effect of classical chemotherapy^[65]. Furthermore, LSCs express high levels of ATP transporters, which are involved in extrusion of chemotherapeutic drugs from

LSCs^[107-109].

To improve survival in AML, traditional chemotherapy targeting the blast population needs to be combined with therapy specifically targeting LSCs to maintain prolonged remission.

FUTURE DIRECTIONS FOR THERAPY

Despite the recent increased interest in LSCs, experimental studies have not been translated into improved survival outcomes for cancer patients. However, several new agents targeting LSC specific surface molecules and pathways as well as the LSC microenvironment remains under different stages of preclinical and clinical development (Table 2 and Figure 1). To rationally design clinical trials testing drugs for efficacy against LSCs, it is important to appreciate the fundamental differences between drug design targeting blast cells and LSCs^[102]. Principles and challenges faced by targeting LSCs will be discussed first followed by an overview of various new therapeutic options targeting LSCs.

General principles and challenges faced by targeting LSCs

Limiting side effects: As LSCs and HSCs have many similar properties (see above), therapeutic approaches targeting LSCs also have the potential of causing severe side effects by eliminating healthy HSCs. To develop novel therapies with limited side effects, unique properties of LSCs have to be identified^[102,110]. While expression of several surface markers is similar between normal HSCs and LSCs (CD34, CD38, CD71 and HLA-DR), other surface antigens are only displayed on LSCs (CD33, CD90, CD117 and CD123)^[110]. Apart from a similar immunophenotype, HSCs and LSCs share many pathways important for maintaining features of "stemness" like quiescence and self-renewal capacity^[111]. Pathways, which are up-regulated in LSCs compared to normal HSCs, are the ideal target for therapeutic approaches directed towards LSCs. For example, the active form of NF- κ B and bcl-2, which are associated with anti-apoptotic activity in cancer cells, are overexpressed in LSCs compared to normal HSC and drugs targeting both NF- κ B and bcl-2 are in clinical development^[36,46,112].

Using biomarkers for LSC eradication: To assess the efficacy of investigational therapies targeting LSCs, precise diagnostic methods are needed to assess the quantity of LSCs present in leukemia patients. Unfortunately, current characterization of LSC phenotype is not precise enough to permit real-time tracking of LSCs *in vivo*^[113]. As discussed above, current strategies for purification do not yield functionally homogeneous population: The frequency of LSCs within the CD34⁺CD38⁻ fraction in AML ranges from 1 in 10⁴ to 1 in 5 × 10⁶ cells and several other populations contain LSCs as well^[15]. Functional assessment of LSC frequency with xenotransplantation models offers a

Table 2 Emerging therapy targeting leukemia stem cells

Drug	Mechanism	Selected clinical trials	Phase	Ref.
Therapy targeting cell surface markers				
GO	Anti-CD33 monoclonal antibody conjugated with calicheamicin, a potent antitumor anthracycline antibiotic	NCT00882102 NCT01869803 NCT00968071 NCT01409161 NCT00766116 NCT02724163 NCT00658814 NCT02473146 NCT00895934 NCT00006265 NCT00860639 NCT00927498 NCT00085709 NCT00195000 NCT00893399 NCT00017589	Phase I-III	[124,126,130,132,133]
Hu5F9-G4	Anti-CD47 monoclonal antibody	NCT02678338	Phase I	[74,141]
CSL360	Anti-CD123 monoclonal antibody	NCT00401739	Phase I	[69,134]
DT388IL3/SL-401	Anti-CD123 recombinant immunotoxin created by the fusion of diphtheria toxin with a ligand targeting the IL-3 receptor	NCT02113982 NCT00397579	Phase I-II	[69,134,136]
MGD006/S80880	Anti-CD3 and CD123 DART	NCT02152956	Phase I	[137]
H90	Anti-CD44 monoclonal antibody	N/A	N/A	[75,139]
A3D8	anti-CD44 monoclonal antibody	N/A	N/A	[139]
Therapy targeting LSC-specific molecular pathways				
Bortezomib	Proteasome inhibitor inhibits the degradation of the IκBα creating an anti-NF-κB effect	NCT00789256 NCT00382954 NCT01127009 NCT00666588 NCT00703300 NCT01534260 NCT00383474	Phase I-III	[36,143-147,175-177]
Parthenolide	Inhibitor of NF-κB	N/A	N/A	[149]
Celastrol	Inhibitor of Hsp90 and by extension NF-κB	N/A	N/A	[150]
4-hydroxy-2-nonenal	Product of lipid peroxidation, inhibiting the proteasome and NF-κB function	N/A	N/A	[151,152]
BKM120	PI3K inhibitors	NCT01396499	Phase I-II	[38,153,154]
CAL-101		NCT01833169 NCT00710528		

GSK21110183	AKT inhibitors	NCT00881946	Phase I-II	[38,155-157]
MK-2206		NCT01253447		
Perifosine		NCT01231919 NCT00301938		
Sirolimus, everolimus, temsirolimus	mTOR inhibitors	NCT01184898 NCT01611116 NCT01074086 NCT01074086 NCT01154439 NCT00775593 NCT02583893 NCT01869114 NCT01822015	Phase I-II	[38,158]
Oblimersen (Genasense, G3139)	bcl-2 antisense oligodeoxy-nucleotide	NCT00085124 NCT00039117 NCT00017589	Phase I-III	[46,159,160]
Obatoclax	Small molecule bcl-2 inhibitor	NCT00438178	Phase I-II	[161-163]
Mesylate (GX15-070MS)		NCT00684918 NCT00684918		
Therapy targeting the LSC microenvironment				
G-CSF	Mobilization of LSC from the protective bone marrow niche -> increased susceptibility to traditional chemotherapy	NCT00820976 NCT00602225 NCT00199147 NCT01723657 NCT01101880 NCT00943943 NCT00906945	Phase I-III	[165-168]
Plerixafor (AMD3100)	CXCR4 inhibitor Decreased homing to the bone marrow	NCT00943943 NCT00906945 NCT01236144 NCT00512252 NCT01319864 NCT01352650 NCT02416908	Phase I-II	[61,135,178]
AMD3465	CXCR4 inhibitor Decreased homing to the bone marrow	N/A	N/A	[61,135,169,179]
BMS-936564	Anti-CXCR4 antibody Decreased homing to the bone marrow	NCT01120457	Phase I	[172]
Natalizumab	Anti-VLA4 antibody Decreased homing to the bone marrow	N/A	N/A	[174]

GO: Gemtuzumab ozogamicin; DART: Dual-affinity retargeting molecule; N/A: Not available; LSC: Leukemia stem cell; IκBα: Inhibitor of kappa B alpha; CXCR4: C-X-C chemokine receptor type 4; mTOR: Mechanistic target of rapamycin; G-CSF: Granulocyte-colony stimulating factor.

more robust method to evaluate eradication of LSCs but might not be feasible in large clinical trials^[102]. Similarly, methods for detecting MRD might guide decisions by detecting patients who do require additional therapy to prevent relapse. However, detecting MRD does not distinguish persistent LSCs, which may cause relapse, from residual blasts and normal HSCs that do not have tumor-initiating activity. Distinguishing residual LSCs from residual blasts might be accomplished by gene expression analysis showing reactivation of self-renewal

genes in LSCs but not in blast cells^[88,114]. In preclinical development, the recently published Connectivity Map could be investigated for agents that attenuate a stem cell gene signature or induce a differentiated state^[115,116].

Timing of LSC targeted therapy: Therapy targeting LSCs is effective in eradicating a small amount of leukemia initiating cells but not the bulk of blasts cells in the blood and bone marrow^[102]. By combining drugs eradicating LSCs with standard chemotherapy targeting the bulk of the disease, both the aggressive proliferating process as well as the root of the leukemia can be targeted^[117]. An example serves the successful combination of the anti-CD33 immunoconjugate antibody gemtuzumab ozogamicin (GO) with standard chemotherapy^[118]. This is associated with challenges in a meaningful design of clinical trials in terms of the correct timing of these therapies. LSC targeting therapy can either be given after reduction of the bulk population with standard chemotherapy as remission therapy or concomitant with chemotherapy as an induction regimen^[102]. Upfront combination would allow assessing for additive and/or synergistic properties between drugs and would allow targeting of LSCs early on in the disease process, which might improve outcomes^[102]. On the other hand, LSC targeted therapy might be particular valuable as post-consolidation therapy as no current post-consolidation intervention has led to improved OS for patients with AML^[102,119,120]. LSC targeting therapies have the potential to fill the gap as they eradicate the cells responsible for relapses of AML.

Assessing clinical endpoints: Classical response criteria like CR and hematologic improvement might not be the best parameters to assess the efficacy of therapeutic approaches targeting LSCs as these drugs do not eradicate the bulk of blast cells but rather eliminate the rare population of LSCs^[102]. Progression-free survival (PFS), event free survival and overall survival (OS) may be a more relevant endpoint for assessing the effectiveness of LSC elimination than tumor response as they better account for whether the root of the leukemia has been eliminated^[113]. Importantly, while LSC frequency was found to be prognostic for survival, response rates did not correlate with LSC burden^[96]. Subsequently, drugs targeting LSCs may show little activity if tested in traditional phase I/II trials as a proper assessment of endpoints relevant for LSCs, like PFS and OS, is generally only feasible in a phase III trial with a larger numbers of patients and long-term follow-up^[113,121].

One example for the importance of assessing relevant endpoints for LSC targeting therapy, is inconsistency of clinical trials evaluating the efficacy of GO^[102]. Single agent studies of GO showed overall response rates only approaching 30% at best and GO was voluntarily withdrawn from the United States market in 2010 after a study showed no improvements in outcomes when used in combination therapy as well

as increased fatal toxicity^[122-124]. In contrast, other large clinical trials showed improvement in outcomes more relevant for therapies targeting LSC- event-free survival, disease-free survival and OS- despite no differences in disease response rates^[125-128].

Targeting LSC surface molecules

Anti-CD33 antibodies: CD33 is found on LSCs although it is not a consistent feature of all LSCs studied^[73,118,129]. As discussed above, there have been conflicting reports surrounding the efficacy and safety of GO and currently GO is not available on the market in the United States or Europe^[130]. Apart from the different endpoints studied, there are additional explanations for the discrepancies observed: First, the dose of daunorubicin as the combination partner of GO did vary between trials, although it is known that treatment with daunorubicin-based schedules of 90 mg/m² for 3 d is more effective than similar schedules with daunorubicin at 45 mg/m²^[131]. In the SWOG trial, which questioned the efficacy of GO, single bolus combined with daunorubicin at 45 mg/m² was studied against a control group with daunorubicin at 60 mg/m²^[124]. However, the best effect of GO was seen when higher dose of GO (3 d at 3 mg/m² for 2 cycles) was added to a daunorubicin regimen of 60 mg/m² in both comparator groups^[126]. Furthermore, GO seems to be quite active in acute promyelocytic leukemia (APL) as APL cells express high levels of CD33^[132,133]. These results have prompted calls to reconsider the approval status of GO^[130].

Anti-IL-3 receptor (CD123) antibodies: The interleukin-3 receptor alpha chain (IL-3R α or CD123) is strongly expressed in CD34⁺/CD38⁻ LSCs and can be targeted with monoclonal antibodies^[69,134]. The blockage of CD123 has pleiotropic anti-leukemic effects including inhibition of LSC homing to the bone marrow, activation of innate immunity and inhibition of intracellular signaling events^[135]. Several different agents targeting CD123 are currently evaluated in clinical trials: CD123 targeting antibodies can either be naked antibodies or be conjugated to toxins (e.g., diphtheria toxin) or chemotherapeutic agents (chemo-immune conjugates) or be the backbone of a bi-specific T cell engager (BITE, e.g., CD3-CD123)^[134,136,137] (Table 2).

Anti-CD44 antibodies: CD44 regulates interaction between LSCs and the bone marrow niche by controlling cell-cell adhesion and cell-matrix interaction through binding to hyaluronic acid, osteopontin, collagens and others^[138].

Inhibition of CD44 with monoclonal antibodies was shown to reduce the numbers of LSCs in NOD/SCID mice and to increase the survival of the primary recipient mice as well prevent engraftment into the secondary recipient mice^[75,139] (Table 2).

Anti-CD47 antibodies: CD47 is overexpressed on LSCs and high expression of CD47 is associated with

worse outcomes^[74]. By interaction with the extracellular region of signal-regulatory protein alpha (SIRP α) on phagocytic cells, LSCs deliver a “do not eat me” message to these phagocytic cells^[140]. Antibodies blocking the interaction between CD47 and SIRP α promote LSC phagocytosis and are in development (Table 2)^[74,141].

Targeting LSC-specific molecular pathways

NF- κ B signaling pathway: Bortezomib is able to suppress the NF- κ B signaling pathway by inhibiting the destruction of I κ B, a cellular inhibitory protein of NF κ B, by the ubiquitin-proteasome pathway^[142]. Several clinical trials are examining the efficacy of Bortezomib targeting AML LSCs (Table 2): Two clinical trials combining Bortezomib with Cytarabine and Anthracyclines resulted in CR rates of 61% and 65%^[143,144], whereas other trials that co-administrated Bortezomib with other drugs did not show encouraging CR rates^[145-147]. Several other inhibitors of NF- κ B signaling are in different phases of development (Table 2)^[148-152].

PI3K/AKT/mTOR pathway: The PI3K/AKT/mTOR pathway is of utmost importance in regulating cellular growth, survival, and metabolism and is frequently dysregulated in cancers and AML^[38]. A multitude of PI3K inhibitors^[153,154], AKT inhibitors^[155-157] and mTOR inhibitors^[158] is currently investigated for their efficacy targeting LSCs in clinical trials (Table 2).

Bcl-2 pathway: LSCs, similar to other tumor cells, are able to avoid apoptosis due to overexpression of bcl-2^[46]. Currently, bcl-2 inhibition is investigated in clinical trials in form of the bcl-2 antisense oligodeoxynucleotide oblimersen^[159,160] and the small molecule inhibitor of bcl-2 obatoclax^[161-163] (Table 2).

Targeting the LSC microenvironment

Approaches targeting the interactions of LSCs with the bone marrow niche focus on breaking the dormancy of LSCs in the bone marrow in order to make them sensitive to traditional chemotherapy^[62,164].

LSC mobilization: LSC mobilization from the marrow niche can be achieved by nonspecific stimulators like G-CSF, Interferon- α and Arsenic trioxide^[62]. Using the NOD/SCID/IL2rgamma (null) mouse model, Saito *et al.*^[165] showed that quiescent human AML LSCs, at first resistant to cytarabine, start proliferating and become susceptible to cytarabine once exposed to G-CSF. Combining chemotherapy with G-CSF leads to significantly increased survival of secondary recipients after transplantation of leukemia cells compared with chemotherapy alone. Furthermore, they showed that treatment with G-CSF before cytarabine did not increase apoptosis of normal HSCs making this approach a particular attractive option for targeting LSCs but at the same time avoiding side effects from depletion of HSCs. The data from clinic trials using G-CSF priming

in combination with chemotherapy are conflicting. Löwenberg *et al.*^[166] randomized 640 newly diagnosed AML patients to receive cytarabine plus idarubicin with G-CSF (321 patients) or without G-CSF (319 patients) for the first cycle of induction of chemotherapy. Patients in CR after induction chemotherapy plus G-CSF had a higher rate of disease-free survival than patients who did not receive G-CSF (42% vs 33% at four years, $P = 0.02$), owing to a reduced probability of relapse (relative risk, 0.77; $P = 0.04$). Other studies did not show a benefit of adding G-CSF to traditional chemotherapy regimens^[167,168]. These different responses to G-CSF might be explained by differences in the group of patients included in these trials^[63]. In the trial by Löwenberg *et al.*^[166] patients with standard-risk AML benefited from G-CSF therapy whereas G-CSF did not improve the outcome in the subgroup with an unfavorable prognosis. In the trials without improvement with G-CSF, patients had a more unfavorable prognosis based on age, cytogenetic abnormalities or response to previous treatment. Several clinical trials are ongoing to investigate the efficacy of G-CSF in combination of chemotherapy in different risk groups of AML (Table 2).

Inhibition of homing: LSC dormancy can be targeted by specifically interrupting the CXCR4-CXCL12 and VCAM-VLA4 axis as well as inhibiting CD44 and CD123 on LSCs to prevent homing of LSCs to the bone marrow.

CXCR4-CXCL12 axis: SDF-1 was shown to promote survival of AML cells, whereas addition of neutralizing CXCR4 antibodies, SDF-1 antibodies, or AMD3100 significantly decreased their survival^[169]. Furthermore, pretreatment of primary human AML cells with neutralizing CXCR4 antibodies blocked their homing into the BM and spleen of transplanted NOD/SCID/B2m^{null} mice^[169]. Additionally, CXCR4 inhibition with AMD3465 was shown to increase the sensitivity of FLT3-mutated leukemic cells to the apoptogenic effects of the FLT3 inhibitor sorafenib^[170]. Recently a phase 1/2 study examined the efficacy of the CXCR4 inhibitor plerixafor in combination with mitoxantrone, etoposide, and cytarabine in 52 patients with relapsed or refractory AML^[171]. Overall CR was found to be 46% and correlative studies demonstrated a 2-fold mobilization in leukemic blasts into the peripheral circulation without evidence of symptomatic hyperleukocytosis or delayed count recovery. BMS-936564, a fully human IgG4 monoclonal antibody against CXCR4, exhibits antitumor activity in cytarabine-resistant mouse xenograft models of AML and is currently tested in a phase I clinic trial (Table 2)^[172].

VCAM-VLA4 axis: Integrin alpha4beta1 (VLA4) mediates adhesion of LSCs to stromal cells and extracellular matrix in the marrow niche and can be blocked by the monoclonal antibody Natalizumab^[59,69,173]. AML cells were shown to de-adhere from a layer of

immobilized human VCAM1 expressing human stromal cells when exposed to Natalizumab and NSG mice transplanted with human AML cells survived significantly longer when they received intraperitoneal Natalizumab injections^[174].

CONCLUSION

AML remains one of the most difficult malignancies to treat. Despite significant advancements in the understanding of disease biology, this has not been translated yet into new treatment modalities improving outcomes. The relapse of AML is frequent and is responsible for the inability to cure AML. LSCs are understood to be the root of relapse and their presence has been found to be prognostic for the disease course. Unfortunately, LSCs are not easy to target as they are quiescent, able to self-renew and well protected by a supportive bone marrow niche. Furthermore, their inconsistent phenotype and similarity to normal HSCs hamper specific drug development. Nevertheless, a multitude of potential targets have been identified and are currently tested in different phases of clinical and preclinical development. Successful eradication of LSCs will require combination of different strategies including targeting LSC specific surface molecules and pathways as well as interactions of LSCs with the microenvironment. Furthermore, clinical trials have to be designed in a way that they are able to detect a specific effect of LSCs, which is easy to miss in a traditional trial design. Overall, targeting LSCs has the promise to not only effectively reduce disease burden but to eradicate the root of leukemia itself.

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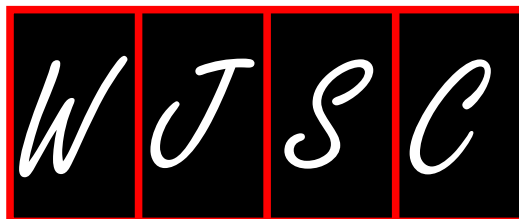
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Stem/progenitor cells and obstructive sleep apnea syndrome - new insights for clinical applications

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Abstract

Obstructive sleep apnea syndrome (OSAS) is a widespread disorder, characterized by recurrent upper airway obstruction during sleep, mostly as a result of complete or partial pharyngeal obstruction. Due to the occurrence of frequent and regular hypoxic events, patients with OSAS are at increased risk of cardiovascular disease, stroke, metabolic disorders, occupational errors, motor vehicle accidents and even death. Thus, OSAS has severe consequences and represents a significant economic burden. However, some of the consequences, as well as their costs can be reduced with appropriate detection and treatment. In this context, the recent advances that were made in stem cell biology knowledge and stem cell - based technologies hold a great promise for various medical conditions, including respiratory diseases. However, the investigation of the role of stem cells in OSAS is still recent and rather limited, requiring further studies, both in animal models and humans. The goal of this review is to summarize the current state of knowledge regarding both lung resident as well as circulating stem/progenitor cells and discuss existing controversies in the field in order to identify future research directions for clinical applications in OSAS. Also, the paper highlights the requisite for inter-institutional, multi-disciplinary research collaborations in order to achieve breakthrough results in the field.

Key words: Obstructive sleep apnea syndrome; Continuous positive airway pressure therapy; Lung resident stem/progenitor cells; Circulating stem/progenitor cells; Lung homeostasis

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Core tip: Obstructive sleep apnea syndrome (OSAS) is a widespread disorder characterized by recurrent upper airway obstruction during sleep, resulting in severe consequences such as increased risk of cardiovascular disease, stroke, metabolic disorders, occupational errors,

motor vehicle accidents and even death. However, the consequences and their costs can be reduced with appropriate detection and treatment. The goal of this review is to summarize the current state of knowledge regarding both lung resident as well as circulating stem/progenitor cells and to discuss existing controversies in the field in order to identify future research directions for clinical applications in OSAS.

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INTRODUCTION

Obstructive sleep apnea syndrome (OSAS) is a prevalent condition with serious undesirable consequences and significant economic burden^[1-4]. It is characterized by recurrent upper airway obstruction during sleep, due mostly to complete or partial pharyngeal obstruction. As a result, sleep fragmentation and repetitive hypoxemia occur, leading to excessive daytime sleepiness, activation of sympathetic nervous system, endothelial dysfunction and hemodynamic changes. Consequently, patients with OSAS are at increased risk of cardiovascular disease, stroke, metabolic disorders, occupational errors, motor vehicle accidents and even death. Health and public consequences, as well as their costs can be reduced with appropriate identification and treatment^[2,5].

The current treatment includes the use of continuous positive airway pressure (CPAP) or oral devices which must be worn at night to help normal breathing. However, the use of such devices raises the problem of patients' adherence to therapy.

Recent development of scientific methods and understandings of stem cell biology have led to an explosion of interest in stem-cell research; consequently, stem cell - based technologies and therapies soon became one of the most rapidly expanding areas, holding a great promise for various medical conditions.

The goal of this review is to summarize the current state of knowledge regarding both lung resident as well as circulating stem/progenitor cells and discuss existing controversies in the field in order to identify future research directions for clinical applications in OSAS.

LUNG RESIDENT STEM/PROGENITOR CELLS

Several varieties of human lung resident stem/progenitor cells have been isolated and identified both *in vivo* and *in vitro*. Broadly, they comprise three major cell types, which are summarized in Table 1: Epithelial

Table 1 Human lung resident stem/progenitor cells

Cell type	Cell markers
Tracheal basal epithelial cells	NGFR ⁺ /ITGA6 ⁺
Type II alveolar cells	HTII-280 ⁺
Airway epithelial cells	CD151 ⁺ /TF ⁺
Airway epithelial cells	SP/CD45 ⁺
Lung epithelial cells	c-kit ⁺ (CD117)
Lung epithelial cells	Ecad/Lgr6 ⁺
L-MSCs	CD73 ⁺ /CD90 ⁺ /CD105 ⁺

L-MSC: Lung mesenchymal stem cells.

stem/progenitor cells, endothelial progenitor cells (L-EPCs) and mesenchymal stem cells (L-MSCs).

Epithelial stem/progenitor cells

Certain cells, which were formerly considered to be differentiated airway or alveolar epithelial cells, have been proved to be able to proliferate and differentiate into other lung epithelial cell types under specific conditions, which suggested that they could be adult lung resident stem/progenitor cells. However, characterization and classification of such cells into a hierarchy could be quite challenging since the terms "stem" and "progenitor" are often used interchangeably^[6,7]. Moreover, data describing putative populations of human adult resident epithelial stem/progenitor cells are limited compared with the large body of evidence in animal models. Several cellular markers have been used alone or in various combinations to identify and isolate stem/progenitor cells in adult human lung.

The first to identify airway epithelial basal cells having stem cell properties in human adult lungs was a group of French scientists in 2007^[8].

By using fluorescence-activated cell sorting, Hajj and collaborators demonstrated that epithelial basal cells, which resided on human adult airway surface and expressed CD151 and tissue factor were able to generate a fully differentiated mucociliary and functional airway epithelium both *in vitro* and *in vivo*, while maintaining their self-renewal potential.

One year later, the existence of a resident side population (SP) cells within the human tracheobronchial epithelium was demonstrated for the first time^[9]. SP cells were identified by verapamil-sensitive efflux of the DNA-binding dye Hoechst 33342. Within SP fraction, CD45⁺ cells represented 0.12% ± 0.01% of the total epithelial cell population in normal airway. Their epithelial phenotype was confirmed by positive immunohistochemical staining for the epithelial markers cytokeratin-5, E-cadherin, tight junction protein ZO-1 and transcription factor Trp-63 (p63) - mainly isoform ΔNp63. In culture, these cells demonstrated sustained colony-forming and clonogenic capacity as well as well-preserved telomere length over successive passages. Moreover, CD45⁺ SP cells were able to generate a multilayered differentiated epithelium in air-liquid interface culture, endorsing their stem cell capacity.

Shortly after, tracheal basal cells have been isolated based on their expression of the markers nerve growth factor receptor (NGFR) and integrin $\alpha 6$ (ITGA6)^[10]. When cultured under appropriate conditions, NGFR⁺/ITGA6⁺ cells gave rise to three-dimensional aggregates (bronchospheres) containing cells positive for transcription factor Trp-63 and cytokeratin 14 (Krt14), luminal cells (Krt8⁺) and also ciliated cells. Hence, human basal cells have been proved to be capable of both self-renewal and generation of differentiated progenies.

In a recent study, Barkauskas *et al.*^[11] isolated human type II alveolar cells (AT2) using fluorescence-activated cell sorting based on a biomarker specific to the apical surface of their membrane (HTII-280). When cocultured with fetal human lung fibroblasts, AT2 cells also formed self-renewing three-dimensional colonies (alveolospheres) composed of a single epithelial layer of HTII-280⁺ cells.

A putative population of c-kit⁺ human lung stem cells nested in niches in the adult distal airways has been identified and characterized as self-renewing, clonogenic, and multipotent both *in vitro* and *in vivo*. When transplanted into damaged mouse lungs, human c-kit⁺ cells not only engrafted, but they were also able to generate human bronchioles, alveoli, and pulmonary vessels structurally and functionally integrated with the host organism^[12]. As appealing as this hypothesis appears - one adult lung cell being capable to give birth to smooth muscle, vasculature, airways and alveoli - it needs further supporting evidence and validation using lineage-tracing during homeostasis and injury^[13].

Shortly after description of c-kit⁺ human lung stem cells, the existence of another population of putative stem cells was reported^[14]. These cells were characterized as positive for E-Cadherin and leucine-rich repeat-containing G-protein-coupled receptor 6 (E-Cad/Lgr6⁺) while being a sub-population of ITGA6⁺ cells. In culture, clonally derived E-Cad/Lgr6⁺ cells formed aggregates capable of *in vitro* indefinite expansion while expressing lung-specific (pulmonary-associated surfactant protein C, Clara cell 10 protein, aquaporin 5), epithelial (E-Cad) and stem cell (Sox9, Lgr5/6, ITGA6) markers. Unlike c-kit⁺ cells, E-Cad/Lgr6⁺ were not able to differentiate into mesenchymal or endothelial cells. E-Cad/Lgr6⁺ single cell transplantations into the kidney capsule generated differentiated bronchioalveolar tissue while retaining the ability to self-renew^[14].

Thus, all these data support the involvement of resident lung stem/progenitor cells in tissue homeostasis, but also in tissue repair after cellular injury.

L-MSCs

Several groups have identified human lung resident cells fulfilling criteria for definition of mesenchymal stem cells^[15-18]. According to Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy criteria, the definition of human MSCs comprises: (1) plastic adherence in standard culture conditions; (2) expression of surface molecules CD73,

CD90 and CD105 in the absence of CD34, CD45, HLA-DR, CD14 or CD11b, CD79a, or CD19 surface molecules as assessed by fluorescence-activated cell sorter analysis and (3) a capacity for differentiation into osteoblasts, adipocytes, and chondroblasts *in vitro*^[19]. Besides these established and generally accepted criteria, another marker which was used to identify human L-MSCs was the ATP binding cassette G transporter^[20].

It is common knowledge that mesenchymal stem cells (MSCs) exhibit high telomerase activity and an extensive secretome that is immunomodulatory, anti-fibrotic, and trophic for endogenous tissue progenitor cells; this robust evidence supports their role as key players in organ homeostasis and repair following injury, and endorse MSCs as ideal candidates for cell-based therapies. L-MSCs demonstrated characteristics similar to other tissue MSCs including paracrine anti-inflammatory properties, suppression of T cell proliferation as well as the ability to differentiate to myofibroblasts^[15,21].

Studies conducted so far - both in animal and human lung tissue - identified MSCs niches colocalized with the alveolar capillary network in the distal lung, suggesting that L-MSCs are anatomically similar to adult angioblasts, pericytes and endothelial precursors^[22,23]. The data regarding the perivascular location of L-MSCs are in agreement with previously reported information which indicates that the distribution of MSCs throughout the post-natal organism is related to their existence in a perivascular niche^[24-26].

Postnatal endothelial progenitor cells

It represent a heterogeneous group of precursor cells, which have been isolated from bone marrow, peripheral and umbilical cord blood, as well as vascular wall, participating in both new blood vessel formation and vascular homeostasis^[27-31].

Unfortunately, no specific marker for endothelial progenitor cells (EPCs) has been identified yet. Cells considered being EPCs share distinguishing features as high capacity for self-renewal and regeneration, fast proliferating endothelial colony-forming units and angiogenic properties. The quest for identifying tissue resident EPCs has not been an easy one since the difficulty to discriminate circulating EPCs from bone marrow and tissue resident EPCs.

Currently, the existence of similar cells in the lungs - resident lung EPCs (L-EPCs) - has been highlighted only in animal models. L-EPCs express classical endothelial cell markers (CD31) and display a microvascular phenotype. Furthermore, similarly to circulating and vessel wall-derived EPCs, mouse L-EPCs express CD34, CD133 and VEGFR-2, while rat L-EPCs are negative for CD133. These cells proved to be highly proliferative and capable of renewing the entire hierarchy of endothelial cell growth potentials. Also, L-EPCs are vasculogenic in Matrigel assays *in vitro* and *in vivo*^[32-34]. These data support the premise that the lung microvasculature is a rich endothelial progenitor niche, with essential role in maintaining vascular homeostasis.

CIRCULATING STEM/PROGENITOR CELLS

To this point, mainly two circulating stem/progenitor cell types are envisaged as having a potential for immediate clinical application in relation to OSAS: EPCs and MSCs^[35].

Circulating EPCs

As previously mentioned, circulating EPCs are bone marrow derived cells that can be found in peripheral and umbilical cord blood.

The phenotype of an accurate EPC, the reliable methods to assess EPCs' quantity and quality as well as their functional status are still under debate. In a review published in 2005, Khakoo *et al.*^[31] described the EPCs having the following characteristics: (1) they are circulating, bone marrow-derived cells that are functionally and phenotypically distinct from mature endothelial cells; (2) they can differentiate into endothelial cells *in vitro*, as assessed by expression profiles and functional characteristics and (3) they can contribute to *in vivo* vasculogenesis and/or vascular homeostasis.

However, since their first mention^[27], the definition of EPCs has come under serious dispute, taking into consideration that further studies have shown that the term "EPC" do not define a single cell type, but rather describe various cell types able to differentiate into the endothelial lineage^[36-40].

According to the timing of their growth in culture, there are at least two morphologically and functionally different endothelial cell populations that originate from circulating mononuclear cells: The so-called "early" and "late" EPCs. The early EPCs are derived from the monocytes and express hematopoietic markers such as CD45, CD14, CD11b and CD11c, while the late EPCs, which are believed to be a subset of CD14⁺ CD34⁺ KDR⁺ cells do not express CD45 or CD14. Although these two types of cells are different-originated with distinct function *in vitro*, both of them contribute to *in vivo* neovascularization in animal models of ischemia^[41-43].

The existence of two different EPCs populations in human peripheral blood, one with high proliferative capacity and the other with lower proliferative capacity, both with comparable efficacy in neovascularization in an ischemic limb model was demonstrated also by the work of Hur *et al.*^[44]. Early EPCs had spindle shape, their growth in culture peaked at 2 to 3 wk and died at 4 wk, whereas late EPCs with cobblestone shape appeared after 2 to 3 wk in culture, showed exponential growth at 4 to 8 wk, and lived up to 12 wk. Late EPCs was different from early EPCs, having strong expression of VE-cadherin, Flt-1, KDR, and vWF. Late EPCs produced more nitric oxide, incorporated more readily into human umbilical vein endothelial cells monolayer, and formed capillary tubes better than early EPCs. However, early EPCs had a more pronounced *in vitro* capacity to secrete

angiogenic cytokines [such as and vascular endothelial growth factor (VEGF), IL8] in comparison to late EPCs.

The final touch in the field (till this moment) was added by Sieveking and collaborators, who emphasized the "strikingly different angiogenic properties of different EPCs: Late-outgrowth endothelial cells directly participate in tubulogenesis, whereas early EPCs augment angiogenesis in a paracrine fashion, with implications for optimizing cell therapies for neovascularisation"^[45]. As for surface markers, their results are consistent with the previous studies, endothelial antigens (*e.g.*, CD31, CD146, VEGFR-2) being expressed by both early and late EPCs. These two populations could be discriminated by CD14 and CD45 expression, with early EPCs showing high expression of these markers (over 95%) whereas late EPCs did not express either marker.

EPCs are able to migrate to the site of injury and participate directly and indirectly to the development of new blood vessels, therefore having a key role in the maintenance of endothelium integrity and function^[46]. Moreover, these cells play an essential role not only in physiological neovascularization, but also in pathological conditions (wound healing, tissue regeneration in ischemia, tissue remodeling in diabetes mellitus and heart failure, growth of tumors)^[47-50]. Studies conducted so far have shown that EPCs' mobilization from the bone marrow is governed by a multifaceted interaction between cytokines/chemokines, proteinases and cell adhesion molecules^[51-55], many of them having abnormal expression in OSAS^[56-58].

In order to minimize the potential interfering factors, studies on EPCs in OSAS have been conducted on subjects free of any other known cardiovascular risk factors. OSAS patients and healthy controls were well-matched for age, sex and body mass index; moreover, they had similar blood pressure, fasting blood glucose and total cholesterol levels^[59].

Even though, the cumulative results regarding the level of circulating EPCs in OSAS are still under debate, since existing studies have reported heterogeneous data (Table 2).

Five studies out of 11 reported a decrease in EPCs in OSAS patients comparative with healthy controls, either adults or children^[60-64]. In contrast with these findings, data from quite similar studies showed that patients suffering of this medical condition had unchanged^[65-67] or even increased number of circulating EPCs in peripheral blood compared with the control group^[68-70].

It is only natural to ask ourselves why there is so much divergence in apparently similar studies. Some possible reasons concerning this heterogeneity have been identified^[59]: (1) Different studies measured circulating EPCs by means of different methods: Flow cytometry vs endothelial colony forming units assay; (2) Different investigators used different marker combinations for the assessment of EPCs (Table 2); (3) Different participants: Adults vs children or male vs men and women; (4) Small number of subjects enrolled, as

Table 2 Circulating endothelial progenitor cells studies in patients with obstructive sleep apnea syndrome

Ref.	Study design	EPCs phenotype	OSAS effect on EPCs number
de la Peña <i>et al</i> ^[60]	Adults, men, flow cytometry	CD34 ⁺ VEGFR2 ⁺	Reduced number
Jelic <i>et al</i> ^[61]	Adults, both genders, flow cytometry	CD34 ⁺ CD133 ⁺ VEGFR2 ⁺	Reduced number
Jelic <i>et al</i> ^[62]	Adults, both genders, flow cytometry	CD34 ⁺ CD133 ⁺ VEGFR2 ⁺	Reduced number
Murri <i>et al</i> ^[63]	Adults, both genders, flow cytometry	CD45 ⁺ CD34 ⁺ CD133 ⁺ VEGFR2 ⁺	Reduced number
Kheirandish-Gozal <i>et al</i> ^[64]	Children, both genders, flow cytometry	CD34 ⁺ CD133 ⁺ VEGFR2 ⁺	Reduced number
Martin <i>et al</i> ^[65]	Adults, both genders, flow cytometry	CD34 ⁺ CD133 ⁺ CD45 ^{dim}	Unchanged number
Yun <i>et al</i> ^[66]	Adults, both genders, endothelial colony forming units assay	-	Unchanged number
Simpson <i>et al</i> ^[67]	Adults, men, flow cytometry	CD34 ⁺ KDR ⁺ CD45 ⁺ CD34 ⁺ KDR ⁺	Unchanged number
Kizawa <i>et al</i> ^[68]	Adults, men, flow cytometry	CD133 ⁺ CD34 ⁺ CD202b ⁺ CD45 ⁻	Increased number
Lui <i>et al</i> ^[69]	Adults, both genders, flow cytometry	CD34 ⁺	Increased number
Chou <i>et al</i> ^[70]	Adults, both genders, flow cytometry	CD34 ⁺	Increased number

EPC: Endothelial progenitor cell; OSAS: Obstructive sleep apnea syndrome.

well as the small number of EPCs circulating in peripheral blood.

Also, taking into consideration the complexity of pathogenic mechanisms involved, it is very possible that - depending upon disease severity and duration - certain mechanisms to prevail.

Potential molecular mechanisms through which OSAS has effects on EPCs were reviewed in the exhaustive work of Wang *et al*^[59]. Briefly, intermittent hypoxia and sleep fragmentation which are key features of OSAS act as triggers of oxidative stress, systemic inflammation and sympathetic activation.

While most mechanisms lead to decreased EPCs mobilization and increased cell apoptosis, there are others with stimulating effect as regards mobilization through hypoxia inducible factor 1 (HIF-1) regulatory pathway activation and upregulation of proangiogenic factors including vascular endothelial growth factor, stromal-derived factor-1 and erythropoietin.

But what is the effect of treatment on EPCs? The current gold standard treatment for OSAS is CPAP therapy, which has been demonstrated not only to significantly improve sleep quality, reduce the risk of comorbidities and increase patient quality of life, but also to minimize risks of accidents and injuries^[71-74]. By effectively diminishing the intermittent hypoxia episodes, CPAP can prevent the activation of pathogenic mechanism that has been shown to affect EPCs number and function.

As depicted in Table 3, CPAP therapy had opposite consequences on circulating EPCs: Normalization in patients having decreased levels^[61-63] - or lessening in patients with high levels^[68,70]. One study reported unchanged values before and after treatment^[67]. Of course, there is an essential factor to consider when assessing rehabilitation effect: Patients' adherence to prescribed therapy. Adherence to CPAP treatment is still a critical and complex issue, subjected to the influence of a wide array of factors^[75-79]. Poor adherence to CPAP is generally acknowledged as a major limiting factor in treating OSAS, with a negative impact on therapeutic success^[80-82]. Studies conducted in the 1990s or even

recently, revealed that approximately 30%-50% of OSAS patients rejected CPAP immediately, the proportion of noncompliant patients reaching 80% within a year^[83-86].

Circulating MSCs

MSCs are located mainly in the bone marrow, but are also found in various tissues and organs. When stimulated by specific signals, these cells are mobilized from their perivascular niche into peripheral blood and home to the target tissues where they contribute to local tissue regeneration and homeostasis^[87-90].

There is only little evidence regarding the number and function of circulating MSCs in peripheral blood in OSAS; only 3 studies in animal models^[91-93], and a single one in humans, in which circulating MSCs could not be detected, probably because their very low number^[94].

In an acute rat model of recurrent airway obstructions mimicking OSAS, Carreras *et al*^[91] demonstrated early release of MSCs into circulation, higher mobility, increased adhesion to endothelial cells and enhanced endothelial wound repair in rats subjected to recurrent obstructive apneas (15 s apnea/min for 3 h), as compared to the number observed in control animals under normoxia^[93]. In addition, in the group of apneic rats subjected to MSCs intravenous injection, MSCs triggered an early systemic anti-inflammatory response by decreasing levels of interleukin-1 beta (IL-1β)^[92]. This property of MSCs has been confirmed in a chronic murine model of OSAS in which atrial fibrosis has been inhibited by the intravenous administration of MSCs as a result of normalization of IL-1β plasma levels^[95].

Considering all this data, one of the main benefits of MSCs therapy in OSAS patients could be the local and systemic anti-inflammatory effect. Besides this, exposure to hypoxia upregulates microRNA-486 (miR-486) expression in MSCs resulting in increased production of angiogenic factors (hepatocyte growth factor and VEGF), increased proliferation and reduced apoptosis^[96].

One of the challenges for cell therapy is that it requires high numbers and good quality of stem cells.

Table 3 Circulating endothelial progenitor cells studies in patients with obstructive sleep apnea syndrome treated by continuous positive airway pressure

Ref.	Study design	EPCs phenotype	CPAP effect on EPCs number
Jelic <i>et al</i> ^[61]	Adults, both genders, flow cytometry	CD34 ⁺ CD133 ⁺ VEGFR2 ⁺	Normalized after 4 wk of CPAP
Jelic <i>et al</i> ^[62]	Adults, both genders, flow cytometry	CD34 ⁺ CD133 ⁺ VEGFR2 ⁺	Normalized after 4 wk of CPAP
Murri <i>et al</i> ^[63]	Adults, both genders, flow cytometry	CD45 ⁺ CD34 ⁺ CD133 ⁺ VEGFR2 ⁺	Values returned to control values after 1 mo of CPAP
Simpson <i>et al</i> ^[67]	Adults, men, flow cytometry	CD34 ⁺ KDR ⁺ CD34 ⁺ KDR ⁺ CD45 ⁻	Unchanged
Kizawa <i>et al</i> ^[68]	Adults, men, flow cytometry	CD133 ⁺ CD34 ⁺ CD202b ⁺ CD45 ⁻	Values decreased after 12 wk of CPAP treatment
Chou <i>et al</i> ^[70]	Adults, both genders, flow cytometry	CD34 ⁺	Mobilization ratio in patients with OSAS tended to decline

EPC: Endothelial progenitor cell; OSAS: Obstructive sleep apnea syndrome; CPAP: Continuous positive airway pressure.

Among factors impairing the quantity and quality of autologous MSCs is age which is associated with progressive loss of cell proliferation and differentiation potential.

Nevertheless, MSCs cultured under hypoxic condition exhibited enriched self-renewing and proliferation capacity even in aged donors compared to normal condition. It was shown that, at low O₂ concentration (such as 1% O₂) MSCs are resistant to apoptosis and do not lose their beneficial paracrine activity, suggesting that they could be transplanted in hypoxia affected tissues without losing their viability or therapeutic properties^[97]. In a very recent study, different profiles of hypoxia-inducible miRNA signatures between young and aged MSCs have been identified and demonstrated to target transcriptional activity leading to enhanced cell proliferation and migration, but also to decrease in growth arrest and apoptosis through the activation of multiple signaling pathways. According to donor's age and culture conditions a therapeutic potential hierarchy of MSCs was established as follows: Young (hypoxia) > young (normoxia) > old aged (hypoxia) > old aged (normoxia)^[98].

Another particular aspect concerning MSCs therapeutic applications is related to their hypoimmunogenic or "immune privileged" status; this unique feature endorses them as suitable candidates for allogeneic transplant.

Human MSCs display low levels of human leukocyte antigen (HLA) major histocompatibility complex class I, lack major histocompatibility complex class II expression and do not express costimulatory molecules CD40, CD80 and CD86^[99-101]. Furthermore, these cells have been shown to have immunomodulatory effects on both the innate and adaptive immune system, being able to suppress the activity of a variety of immune cells, including natural killer T cells, dendritic cells, neutrophils, monocytes, macrophages, B and T cells^[102-105].

PERSPECTIVES

Research regarding the role of SC in OSAS pathology and their potential use in OSAS treatment is still recent and quite limited, requiring further studies in both animal models and humans. Future directions and recom-

mendations to achieve advanced understanding of mechanisms of lung homeostasis and repair have been proposed during expert meetings^[106-108]. In this regard, it is necessary to identify additional cell surface markers to characterize lung cell populations but also to refine the nomenclature used for resident and circulating lung stem cells. Additional studies are required to identify and characterize resident lung stem/progenitors cells and their niches comparatively between different lung compartments and also regulatory pathways guiding their behavior. Mechanisms of recruitment, mobilization and homing of circulating or transplanted cells to various lung compartments have to be elucidated based on disease-specific models (including large animal models).

Maybe the most important take - home messages are those emphasizing the requisite for inter-institutional, multi-disciplinary research collaborations and consortiums. A successful stem cell research requires state-of-the art infrastructure and vast resources. Connecting with existing networks, nonprofit respiratory disease foundations and industry could accelerate clinical applications. Also, joining other clinical trials in related disciplines (*e.g.*, cardiovascular disease) would provide valuable data for development of stem cell research-derived therapeutics.

Last but not least, obtained information must be largely disseminated through existing core services, facilities and web links.

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Basic Study

Model acupuncture point: Bone marrow-derived stromal stem cells are moved by a weak electromagnetic field

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Abstract

AIM

To show the existence of a structural formative role of magnetic fields (MFs) with respect to biological objects by using our proposed model of an acupoint.

METHODS

We introduced a magnetised 10-100 μ T metal rod (needle) into culture dishes with a negatively charged working surface and observed during 24 h how cells were arranged by MFs and by electrical fields (EFs) when attached. Rat and human bone marrow-derived stromal stem cells (rBMSCs and hBMSCs), human nonadherent mononuclear blood cells, NCTCs and A172 cells, and *Escherichia coli* (*E. coli*) were evaluated. The dish containing BMSCs was defined as the model of an acupoint. rBMSCs proliferative activity affected by the needle was investigated. For investigating electromagnetic field structures, we used the gas discharge visualisation (GDV) method.

RESULTS

During 24 h of incubation in 50-mm culture dishes, BMSCs or the nonadherent cells accumulated into a central heap in each dish. BMSCs formed a torus (central ring) with an inner diameter of approximately

10 mm only upon the introduction of the needle in the centre of the dish. The cells did not show these effects in 35- or 90-mm culture dishes or hydrophobic dishes or rectangular cuvettes. NCTCs and A172 cells showed unstable the effects and only up to two weeks after thawing. Moreover, we observed that the appearance of these effects depended on the season. In winter, BMSCs showed no the effects. GDV experiments revealed that the resonant annular illumination gradually formed from 10 to 18-20 s in polar solutions with and without cell suspension of BMSCs, NCTCs and *E. coli* when using circular 50-mm dishes, stimulation at 115 V and switching of the electrode poles at 1 kHz. All these data demonstrate the resonant nature of the central ring. Significant influence of MFs on the rBMSC proliferation rate was not observed.

CONCLUSION

BMSCs can be moved by MFs when in the presence of a constant EF and MF, when the cells are in the responsive functional state, and when there is a resonant relationship between them.

Key words: Stem cell movement; Magnetic targeting; Acupuncture; Model acupoint; Frizzled-related protein; Biology resonance; Cytoplasm movement; Glycocalyx

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Core tip: On the basis of the literature, we propose the simplest possible model of an acupoint. This model that allowed us to move bone marrow-derived stromal stem cells (BMSCs) using magnetic fields (MFs) without any magnetised nanoparticles. This is a newly identified property of BMSCs, which may be involved in the formation, maintenance and regeneration of tissues and organs. The associated movements of BMSCs may occur *via* acupoints, and the meridian system may thus control the processes of structural regeneration and be the most ancient regulatory system. Not until the cells become MF amplifiers (resonators) can MFs move the cells. That is possible within our acupoint model.

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INTRODUCTION

At present, various types of stem cells (SCs) have been successfully used to rescue acquired or congenital defects in human tissues. Tissue grafts (dermal equivalents, biodegradable polymer-based grafts, etc.) are used for large tissue defects or for correcting the disordered structures of hollow viscera. However,

reproducing the structure of parenchymatous organs (e.g., liver, kidney) has not yet been successful^[1].

It has been suggested that the spatial structure of the body and its organs, in their phylogenesis and ontogenesis, were formed not only under the influence of various internal and external physiological factors but also under the influence of certain non-physiological laws of morphogenesis^[2]. Influencing the shape during organogenesis was shown in heterotopic transplantation experiments when grafting under the kidney capsule of a Millipore membrane filter folded with mouse bone marrow stem cells on its inner surface. Not until the filter was present and was sufficiently folded did bone structures form^[3]. Biological fields are believed to play a significant role in these patterns that influence morphology^[4,5]. The nature of these formative fields has been defined in the modern experimental studies^[6] and theoretical constructs^[7] as an electromagnetic. Influences of electric, magnetic and electromagnetic forces in cells, tissues and the whole organism have been addressed in many papers^[8,9]. In particular, there are a number of studies that investigated the effects of electromagnetic fields in the visible and infrared ranges on various types of SCs^[10]. However, the influence of the physical forces stated above at physiological doses on the morphogenesis of biological tissues has not been shown.

Cells somehow know how to line up in space, e.g., during *in vivo* regeneration of surface defects. Their ability to regenerate the external body shape seems amazing, for example, in coelenterates and some vertebrates. That is possible only if there is an EMF mould within at least a very small distance over the surface of the wound, which would determine the directional synthesis of extracellular matrix by the surface cells and then the directional cell movement upon that matrix. It has to be the same in embryo morphogenesis. To date, no structure responsible for establishment of shape-supporting EMFs has been detected in living organisms. The only system that could be qualified for this role is the acupoint and acupuncture channel system. There are many theories about this system, none of which has been completely proven. Moreover, the system has not been proven to exist in the body^[11]. However, acupoints are known to have certain anatomical and physiological characteristics^[12,13]. In particular, relative to surrounding tissues, acupoints appear to have an elevated electrical potential and a reduced electrical resistance^[14,15]. Areas with reduced impedances and higher electric potentials have also been found in plants^[16].

In this study, we sought to show that existing EM forces in the body not only can influence the intracellular, interstitial and intra-organ physiological processes but also can significantly affect the structures of organs and tissues. We primarily used SCs in our work because the processes of shaping in the body connect with its regeneration system. As the electrical matrix, we used

culture dishes with a negatively charged working surface because acupoints have heightened electrical potentials. As a magnetic component, we used a magnetised metal rod. From our perspective, such a culture dish with cells placed in it can be considered the simplest model of an acupoint. This model represents a cross-section of an acupoint. The negatively charged surface of the culture dish models the interior of the acupoint. The hydrophobic walls of the dishes model the transition from the inner space of the acupoint to the surrounding tissues.

Thus, the aim of this work was to show the possible structural formative role of EMFs with respect to biological objects. The main objective was to show that EM forces, which are probably present in acupoints, can greatly affect the spatial arrangement of cells that are in the scope of action of the forces.

MATERIALS AND METHODS

Isolation and culture of rat bone marrow-derived stromal stem cells

In total, 8 outbred white rats at 2-6 mo of age were used for all of the experiments (2 years, one rat per season). The rats were maintained from birth at 23 °C, 50% humidity, with ad libitum access to food and water, and with artificial light from 8:00 to 16:00 and with natural light provided by large windows. Rat bone marrow-derived stromal stem cells (rBMSCs) were isolated as described by Javazon *et al.*^[17] with some modifications. Briefly, rats were anaesthetised with ethoxyethane and then sacrificed by cervical dislocation in accordance with the guidelines approved by the Institutional Animal Care and Utilisation Committee. BM was collected from the femurs and tibias by inserting a 22-gauge needle into the shaft of the bone and flushing it with phosphate-buffered saline (PBS). Clots of cells were broken by syringing. Next, the cells were loaded onto Histopaque-1077 (Sigma, United States) for density gradient centrifugation (500 × g, 20 min). The cells were collected from the interface, resuspended in PBS and centrifuged at 370 × g for 10 min. After centrifugation, the residual cells were resuspended in Eagle's minimum essential medium, alpha modification (α-MEM, Sigma, United States) containing 2 mmol/L L-glutamine, 100 U/mL antibiotic-antimycotic (PenStrep, GIBCO, Canada) and 100 mL/L foetal bovine serum (FBS, HyClone, United States) and seeded at 5-7 × 10⁶ cells per 50-mm culture dish. The cells were cultured in a humidified atmosphere of 50 mL/L CO₂ at 37 °C. After cell colonies arose, nonadherent cells were discarded, and adherent cells were grown to 90% confluence in fresh medium. The cells were detached by trypsinisation, harvested by centrifugation at 500 × g for 10 min, counted using a counting chamber, seeded at 5-10 × 10³ cell/cm² into flasks or dishes (passage 1) and placed in a humidified atmosphere of 50 mL/L CO₂ at 37 °C for amplification. After cells were grown to 70%-80% confluence, they were reseeded.

Isolation and culture of human BMSCs

The procedures were carried out as described by Wolfe *et al.*^[18] with some modifications. BM aspirate obtained from 2 healthy adult donors with informed consent was used in this research. Briefly, after the approval of the local Ethics Committee, human BMSCs (hBMSCs) were obtained from a patient selected for the study protocol: 6 mL of BM was aspirated from the posterior iliac crest and supplemented with 100 U/mL heparin for transportation. Within 1 h after aspiration, the extracted BM suspended in 30 mL PBS and centrifuged at 200 × g for 5 min. The residual cells were resuspended in 10 mL PBS and loaded onto Histopaque-1077 (Sigma, United States) for density gradient centrifugation (500 × g, 20 min). The subsequent procedures were the same as for rBMSCs. Unlike rBMSCs, the culture medium was changed once every three days, and after the first medium change, the nonadherent mononuclear cells (mostly leukocytes) were not discarded. They were cultivated in the same fresh medium separately from the BMSCs.

Culture of stable cell lines and Escherichia coli

The two stable cell lines used in the experiments were provided by the Russian Cell Culture Collection of Institute of Cytology of the Russian Academy of Sciences (St. Petersburg). One was NCTC clone 929 of the cell line L (NCTCs, murine fibroblast-like cells from subcutaneous connective tissue), and the other was A-172 cells (human fibroblast-like glioblastoma cells). The cells were recovered from frozen vials and resuspended in Dulbecco's modified Eagle's medium (Biolot, Russia) supplemented with 100 mL/L FBS, 2 mmol/L L-glutamine and 100 U/mL antibiotic-antimycotic. The cells were grown in a humidified atmosphere of 50 mL/L CO₂ at 37 °C. After cells were grown to 70%-80 % confluence, they were reseeded.

Escherichia coli (*E. coli*) was cultured at 37 °C in Luria-Bertani culture medium: 10 g/L bactotryptone and 5 g/L yeast extract in 10 g/L aqueous solution of NaCl.

Setting a magnetised metal rod (needle) in the culture dish to produce electromagnetic effects on cells

For the experiments, cells at 70%-80% confluence were detached by trypsinisation (2.5 g/L trypsin, 0.2 g/L EDTA: GIBCO, United States) for 2-5 min at 37 °C, flushed with PBS, harvested by centrifugation at 500 × g for 10 min, resuspended in the appropriate fresh culture medium, and plated at 10 × 10³ cell/cm² in 50-mm culture dishes for the proliferation assay and at 20 × 10³ cell/cm² in culture dishes (diameter, 35 mm, 50 mm, 90 mm) to observe the sites of cell attachment to the bottoms of the culture dishes. The volumes of culture medium per dish were as follows: 2 mL per 35-mm diameter dish, 4 mL per 50-mm dish, 10 mL per 90-mm dish. Those volumes were selected to obtain a 3-mm height of liquid in each type of culture dish. The lids of the culture dishes were replaced with lids with openings for the needles, and magnetised needles were inserted into those openings. The cells were incubated

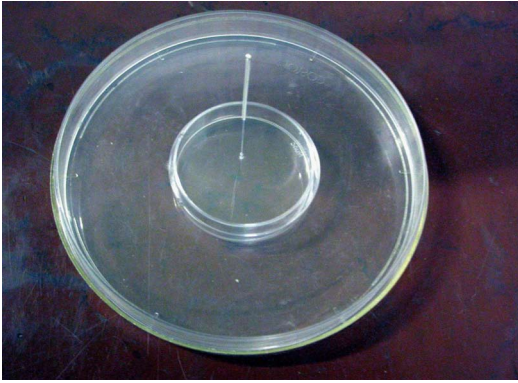


Figure 1 A general view of the culture dish with the needle. The magnetized acupuncture needle is in the center. The needle passes through the cover of the outer dish (the "shirt"), then passes through the cover of the inner dish (the culture dish) and reaches the bottom of the culture dish. The needle is held in an upright position by both the "shirt" cover and the culture dish cover.

in a humidified atmosphere of 50 mL/L CO₂ at 37 °C for 1, 3 or 6 d.

The metal rods were acupuncture needles of surgical steel (Kangnian, China) with a diameter of 0.3 mm and a length of 75 mm (working portion, 35 mm long). Needles of surgical steel are highly resistant to corrosion and do not leave visible traces of metal when introduced into human tissue and left in for at least several days (normal exposure of "buttons" is 3-4 d). Needles were magnetised by a permanent magnet to a residual magnetisation of 10-100 μ T.

We also used Nunclon™ Δ Surface (NUNC, England) culture dishes. These are ordinary dishes used for culturing cells and possess a hydrophilic bottom surface that has negative carboxyl groups (COO⁻) chemically attached to the plastic in a uniform distribution. Meanwhile, the vertical, non-working walls of the dish remain hydrophobic. In addition, 14-cm culture dishes were used as "shirts". Before the experiments, holes the diameter of the needle trough covers of both the culture dishes and the "shirts" were made as follows: In the centre of the "shirt" covers and in the centre or at a certain distance from the centre of the culture dish covers. Before use, the covers and the needles were treated with 700 mL/L ethanol and dried under ultraviolet radiation for at least 10 min. The needle was introduced immediately after cell transfer into the culture dishes. The needles were introduced vertically into the culture dishes, reaching the bottom of the dish. A general view of the culture dish within the "shirt" and with the inserted needle is shown in Figure 1.

Recording the sites of cell attachments to the bottoms of culture dishes

Cells were stained as described previously^[19] with some modifications. Cells were fixed by the addition of 110 g/L glutaraldehyde solution up to a concentration of 10 g/L glutaraldehyde within the culture medium. After gentle shaking for 15 min at room temperature, culture dishes were washed with deionised water and dried.

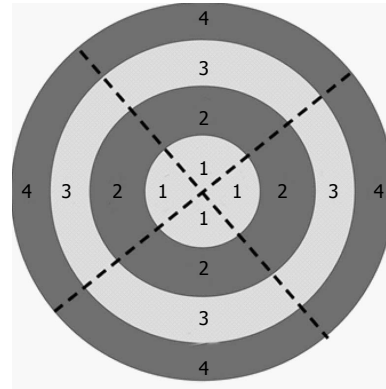


Figure 2 The scheme for photographic fields of the bottom of the culture dish. The numbers mark the concentric regions: The central region (1); the area near the central region (2); the area near the fringe region (3); the fringe region (4). The dotted lines divide the bottom into four sectors. Each sector comprises four photographic fields; thus, overall, there are 16 photographed fields for each culture dish.

Cells were stained by the addition of a 1 g/L solution of crystal violet dissolved in 200 mmol/L MES buffer, pH 6. After shaking for 20 min at room temperature, excess dye was removed by extensive washing with deionised water. The culture dishes with stained cells were dried, and then their vertical walls were removed using special cutting pliers. The bottoms of the dishes were scanned using an HP LaserJet 3392 scanner.

In addition, live cells were filmed within culture dishes. We used lenses of 4 \times and 10 \times magnification and an ocular magnification of 10 \times . For more accurately estimating cell attachments, the culture dish bottoms were divided into four concentric regions of equal width and four equal sectors. Images were collected from all concentric areas of each of the four sectors (a total of 16 fields). The photographed fields are shown in Figure 2.

Gas discharge visualisation Pro Camera assay

The gas discharge visualisation (GDV) method was formulated by KG Korotkov in the book "The foundations of GDV-bioelectrography", which was published in 2001^[20]. The GDV method is based on the visualisation of gas discharge-induced photoelectron emission from the surface of an object placed in a high-tension electric field (Kirlian effect). We used a hardware-software complex called the "GDV Pro Camera" (Biotechprogress, Russia, ktispb.eng). A schematic of this device is shown in Figure 3.

A culture dish with either adherent cells or suspended cells at 10×10^3 - 20×10^3 cell/cm² in 4 mL of culture medium or PBS was placed onto the transparent electrode of the GDV camera. The ground electrode was then placed vertically in the culture dish to the depth of contact with liquid. The voltage mode was fixed at 90, 115 or 125 V. The frequency at which the poles of the electrodes changed was 1 kHz. The exposure time was varied from 0.6 to 32 s. Photography was carried out in the dark. As controls, GDV was performed using culture

Table 1 The examination of magnetized rod action on rat bone marrow-derived stromal stem cells proliferation

Exposure time (d)	The average of three independent replicates of the experiment (the number of cells is $\times 10^3/\text{cm}^2 \pm \text{SE}$)		Experimental t statistic	Standard t statistic (t_{α})	Experimental U statistic	Standard U statistic (U_{α})
	Experiment	Control				
1	10.77 ± 3.31	10.67 ± 1.28	0.06	$t_{\text{st}} = 2.78$ when the number of degrees of freedom $\kappa = 4$ and $P = 0.05$	3	$U_{\text{st}} = 0$ when the number of observations $n_1 = n_2 = 3$, $P = 0.05$
3	20.33 ± 2.36	21.67 ± 0.47	1.78		2	
6	31.77 ± 2.29	30	2.76		3	
from 3 to 6	11.44 ± 3.24	8.33 ± 0.47	1.12			

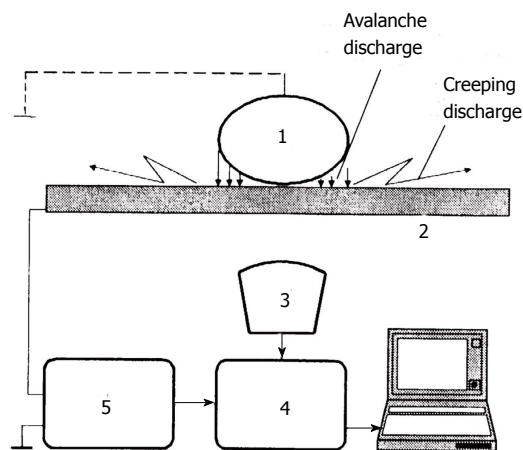


Figure 3 The scheme of a device for studying the characteristics of gas discharge visualisation. An object of a study with an attached grounding electrode (1); a transparent electrode (2); an optical system (3); video converter (4); electronic components (5). The optical system (3) produces photographs of the electron avalanche and the creeping gas discharges through the transparent electrode (2) (cited in Korotkov, 2001).

dishes of the different diameters (35 and 50 mm) or with square cuvettes, with culture medium or PBS without cells, with distilled water, and with suspensions of *E. coli* or nonpolar particles of chalky powder. A general view of the experimental setup is shown in Figure 4. In these GDV experiments, the glow appears to be largely due to photoelectron emission from charged particles within the solutions and to avalanche gas discharges from the bottom of the culture dish.

Statistical analysis

Statistical comparisons of rBMSC proliferation were based on the results of three separate experiments with the same incubation time. Cells were detached and counted using a counting chamber. The result is expressed as the mean value \pm standard error. Statistical significance of differences between the needle-exposed group and the needle-free control group was performed using Student's t test and the Wilcoxon-Mann-Whitney U test, $P = 0.05$.

RESULTS

Proliferation experiments

First, we investigated the effects of the magnetised rod (needle) on the proliferation of rBMSCs. rBMSCs, 10000 cells/cm² (passages 2-3) were placed in culture

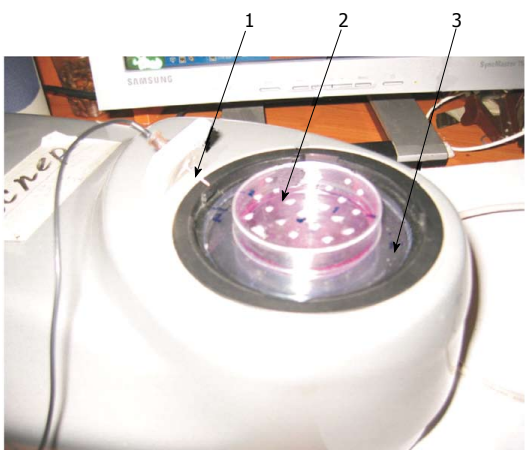


Figure 4 A general view of the experimental setup using the gas discharge visualisation Pro Camera. The grounding electrode (1). A culture dish with culture medium covered with the specially designed lid (2) with openings for the introduction of the grounding electrode (1) at different locations. The dish holder (3). A transparent electrode covers the whole area under the holder (3) and the culture dish (2). To take gas discharge visualisation photos, the grounding electrode (1) is set in one of the lid holes to the depth of contact with liquid. After that, photography was performed in the dark.

dishes of 20 cm² (50 mm diameter). Needles were introduced into the centre of each dish, and the dishes were incubated for 1, 3 or 6 d. Then, the cells were photographed and counted. As a control, rBMSCs were grown without the needles. We did not observe any significant differences between the experimental and control cells (Table 1).

Photographing of living unstained cells

The cells were directly photographed in culture dishes after their incubation. When rBMSCs were cultured for 6 d, they formed a monolayer over which small, rounded cells (approximately 1 μm diameter) were clearly visible. Figure 5A shows that these cells are unevenly arranged on the dish bottom. There are few cells near the needle in region 1. There are more cells in areas 2 and 3, and the number of cells decreases again in region 4, along the outer edge of the dish. After incubating the cells with a needle for 3 d, a similar pattern emerged. However, the small cells were almost entirely eliminated after the third passage. When using hBMSCs, similar results were obtained. On cultivating BMSCs for a day with the needle spread BMSCs were obtained unevenly distributed too. Because the cells do not have time to reproduce any during a day we increased the

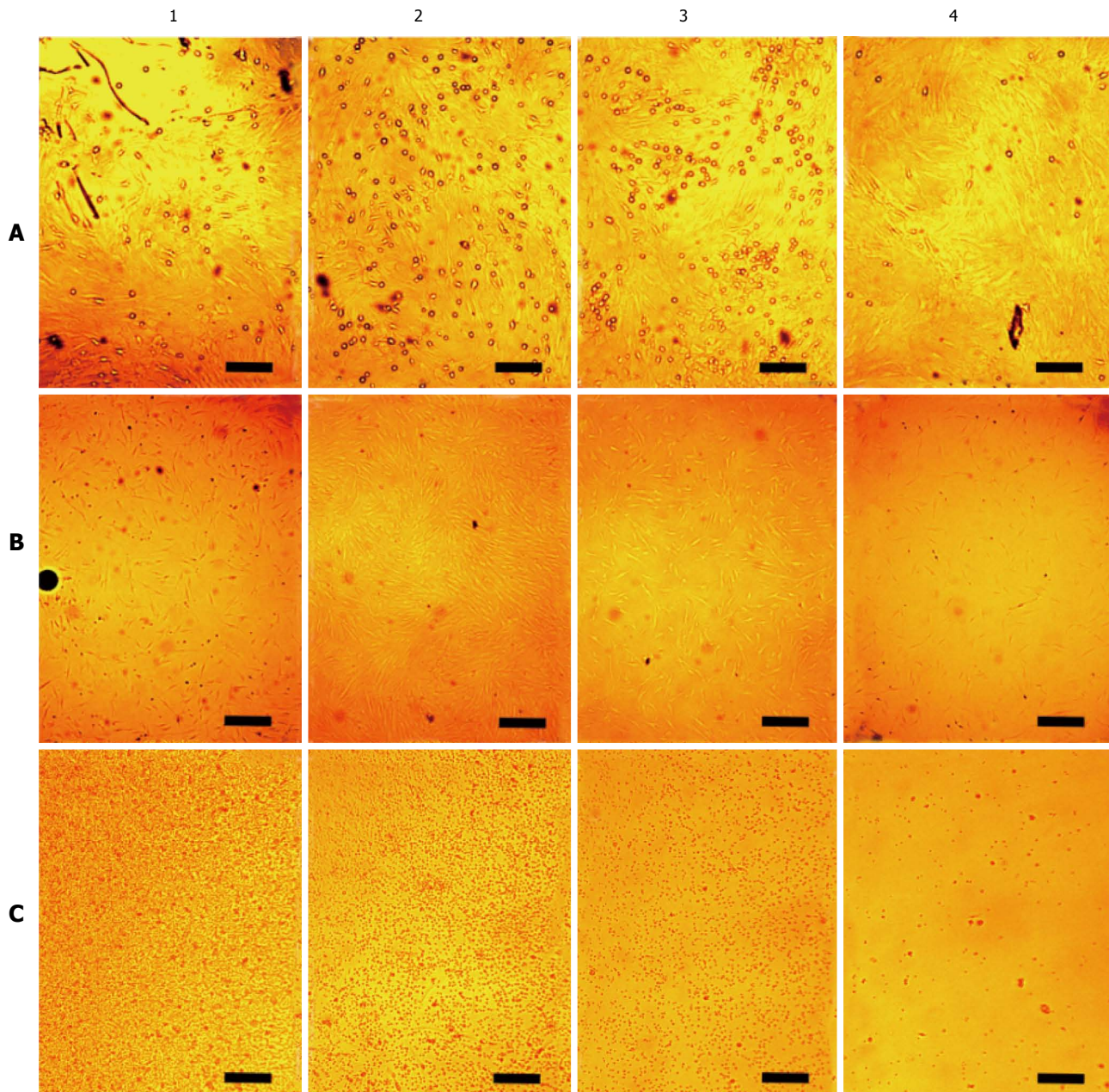


Figure 5 Arrangements of living unstained cells in affecting the magnetized needle. Snapshots were carried out in four perpendicular directions from the center of the cultural dish in four concentric areas fixed width (Figure 2). In columns: The central region (1); the area near the central region (2); the area near the fringe region (3); the fringe region (4). In rows: A: Arrangements at the dish bottom of rBMSCs cultured for 6 d under the influence of the magnetized needle (the needle in the center of the culture dish). The small rounded cells of approximately a micron are seen over the cell monolayer. They are unevenly distributed, depending on their distance from the center of the dish. The bar is 10 μm ; B: Arrangements of hBMSCs cultured for 1 d under the influence of the needle (the needle in the center of the culture dish). The dark round trace of the needle is seen on the left edge of the photo B1. The bar is 25 μm ; C: Migration of human mononuclear leukocytes during a day without exposure to the needle. The bar is 10 μm . rBMSCs: Rat bone marrow-derived stromal stem cells; hBMSCs: Human bone marrow-derived stromal stem cells.

concentration of spread BMSCs up to 20 thousands per square centimetre. Figure 5B shows hBMSCs exposed with the needle during a day. There is most cell density in the region 2.

Figure 5C shows the distribution of suspended human cells without exposure to the magnetised rod. Mononuclear leukocytes made up the majority of the cells, and the cells appear as a heap in the centre of the dish. There are many cells in region 1, fewer in

areas 2 and 3, and practically no cells in region 4. This effect arose during a single day of incubation. We gently redistributed the cells uniformly within the dish. However, the next day, they had again piled up in the centre. We introduced the needle into the cell heap. The next day, the cells had redistributed over the dish bottom more or less uniformly. No heaping was observed for cells cultured in dishes with uncharged, hydrophobic bottoms. Square and rectangular flasks with negatively charged

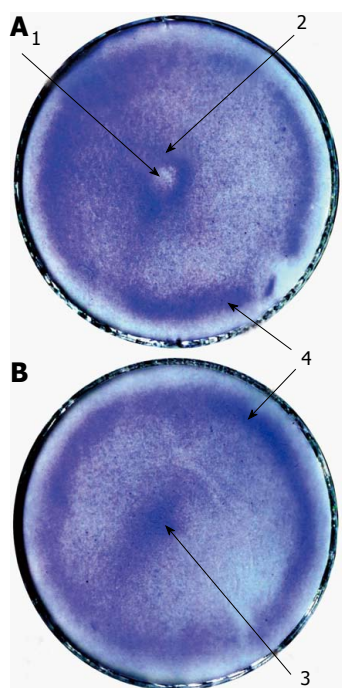


Figure 6 The effect of a metal rod (needle) on the arrangement of rat bone marrow-derived stromal stem cells. rBMSCs in 4 mL of culture medium were plated in 50-mm culture dishes at 20000 cells/cm² and cultured with the needle in the centre of the dishes (A) or without the needle (B) for one day. Cells in the dishes were fixed and stained with crystal violet. The vertical walls of the dishes were removed. The stained bottoms of the dishes were scanned. Arrows mark the central depression (1); the central ring (2); the central heap (3); and the peripheral ring (4). rBMSCs: Rat bone marrow-derived stromal stem cells.

bottoms showed faint clustering of the cells.

Photographing dishes with fixed and stained cells

Photographing living cells allows the cells to be used in experiments again. However, it is obvious that this method is not ideal for establishing exactly how the cells are distributed along the dish bottom. Therefore, to clarify the cell distribution, the cells were fixed in the dishes and then stained, and the entire dish bottoms were scanned. Over the 2 years of experiments, approximately 160 dishes were stained.

In using rBMSCs, positive results were obtained from the first to 11th-12th passages. Figure 6 shows rBMSCs exposed and unexposed (control) to the needle. The needled cells show a rarefaction in the centre of the dish where the end of the needle was (the central depression). Annular crowding of the cells in a 3-5 mm width is observed at approximately 5 mm from the centre of the dish (the central ring). In some dishes, there is another ring-shaped cluster of cells at approximately 5 mm from the edge of the dish (the peripheral ring). The controls may include two clusters of cells: A cell accumulation in the centre of the dish (the central heap) and the peripheral ring. There was only small, inconstant effect involved when using 35- or 90-mm culture dishes. Therefore, only the 50-mm dishes were utilised in the subsequent experiments.

To eliminate the possible influence of metal ions

diffusing from the needle on the formation of the central depression, the effects of the non-magnetised needles and comparable plastic rods on the arrangement of rBMSCs were evaluated. Non-magnetised needles did not cause the formation of the central depression and the central ring, and neither did thin plastic rods. When the non-magnetised needles or plastic rods were inserted away from the centres of the dishes, rarefactions around them remained absent. Further, magnetised needles inserted away from the centres of the dishes also did not form rarefactions (Figure 7B, C and D). However, the central heap seemed to change its shape, becoming irregular and elongated, when magnetised needles were applied in different positions. We next tested the influence of the needles' magnetic polarisation on the arrangement of the rBMSCs, and we did not observe significant differences in these influences. The influence of the north pole seemed to produce a somewhat more vivid picture.

In addition, we observed that the generation of these effects depended on the season. The most pronounced response of BMSCs to the magnetised rods occurred in spring. However, there were always a number of unresponsive cells. In the images, this is reflected by the presence of the background (Figure 6). There were more unresponsive cells in summer and autumn. This was expressed both as a more intense background and as the disappearance of the peripheral ring (Figure 7B, C and D). In winter, from mid-November to mid-January, BMSCs showed no responses to the magnetised needle (Figure 7A). There was always the strongest effect in spring, some effect in summer and autumn, and no effect in winter. Approximately 40 dishes were tested for each season. The 40 dishes for winter (from mid-November to mid-January) showed no effect. The 40 dishes for spring (from March to April) all showed the effect. Consequently, seasonality is present at $P < 0.05$.

In winter, studies were performed to determine the effects on the rBMSCs distribution of both the method of plating the cells and the method of setting the needles. The dishes with just the cells placed in them were moved horizontally on the bench surface back and forth in the 8 cardinal directions, for three times per direction. Thereafter, some of the dishes were moved horizontally in a circle one or three times, creating a torque in the culture medium. Needles were not inserted in these dishes. Notably, these dishes showed no central heaps. In some instances, a formation resembling the peripheral ring appeared. However, in these cases, this "ring" was not uniform and complete, and it was located closer to the edge of the dish than the peripheral ring. To test the method of setting the needles in the culture dishes, needles were introduced perpendicularly into the dishes, heavily or slightly rest against the bottom, and rotated clockwise or anticlockwise. In these cases, we found no central depressions or central rings.

In addition to human and rat BMSCs, in the spring, we used two stable cell lines: Mouse fibroblast NCTC

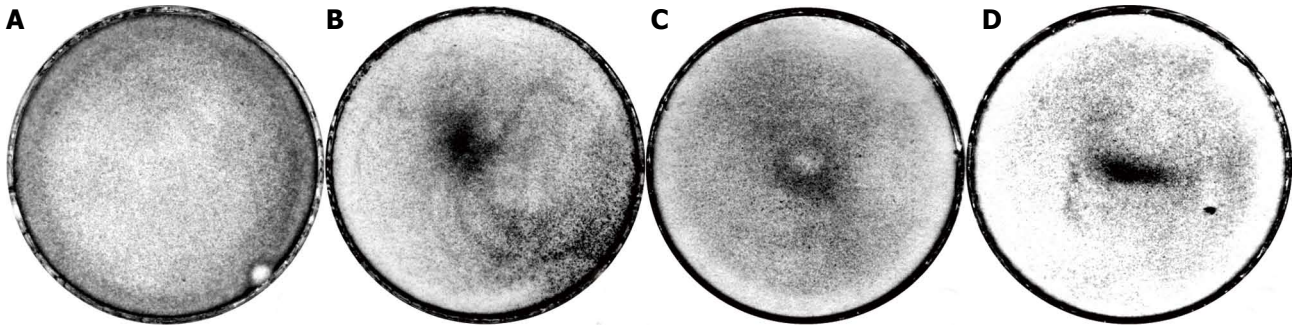


Figure 7 The effect of the needle on the redistribution rat bone marrow-derived stromal stem cells when setting the needle away from the centre of the culture dish. A: The dish in the absence of the effect; B: Control without the needle; C: The needle in the centre; D: The needles away from the centre, with the placement of the needle as indicated by a dot. Explanations are provided in the text.

L929 cells and human glioblastoma A172 fibroblasts. NCTCs, which proliferate rapidly, were used in our experiments beginning at passage 2 (*i.e.*, 4 d after thawing). These cells showed a certain unstable positive result for two weeks after thawing both in the control and in the experiment. However, after that time period, the cells showed no effects of the magnetised needles and distributed uniformly across the dish bottoms both in the control and in the experiment (as in Figure 7A). Similar results were obtained for the A172 cells.

GDV research

We investigated the gas discharge glow (GDG) from circular culture NUNC dishes of diameters of 35 (9.6 cm²) and 50 mm (20 cm²) and from rectangular cuvettes with an area of 20 cm². The emissions at voltages of 90, 115 and 125 V were investigated. The same volumes of water, PBS or culture medium with or without cells were placed in the dishes as in the experiments with the needles. Annular GDG gradually forming from 10 to 18–20 s was observed when using circular 50-mm dishes and a voltage of 115 V (Figure 8C3 and C4). Three peripheral rings were always clearly visible when the location of the ground electrode was in the centre of the dish. The central ring was less pronounced. The average distances between the rings were 5–7 mm (Figure 8B1 and C4). Annular illumination was not observed without the presence of liquid in the dish (Figure 8A1), when the liquid was water (uncharged liquid, Figure 8A2), or from hydrophobic dishes or rectangular cuvettes (Figure 8A3 and A4). The dishes filled with PBS produced annular illuminations more clearly than those filled with culture medium and/or various cells. Three positions of the grounding electrode were evaluated: In the centre, next to the edge and equidistant from the centre and the edge of the dish (Figure 8B1, B2 and B3). Two outer rings were recorded in all cases. The central ring appeared only when the ground electrode was located in the centre of the dish. With a voltage of 90 V, the rings were not formed (Figure 8C2). When using 35-mm dishes, some faint crowded rings around the periphery of the dish were formed at approximately 30 s with a voltage of 125 V (Figure 8D1). The rings were not formed in 50-mm dishes at a voltage of 125 V. Thus,

EM resonances existed in the system.

Patterns regarding the shape and size of the dishes, the location of the grounding electrode and the voltage were detected in both the GDV study of culture medium or PBS and the GDV study of suspended rBMSCs, NCTCs or *E. coli* cells. The rings were the most prominent and stable when using NCTCs. The rings could be obtained by placing these cells in the high-voltage field for 32 s for many consecutive applications. Although the suspension of rBMSCs provided a relatively clear image at approximately 18–20 s of exposure, the glow disappeared within 4–5 s after that and never reappeared. The glow also did not appear in repeated attempts after a while, whether in fresh culture medium or PBS. After a single treatment with the high-voltage field during 32 s, NCTCs and *E. coli* stopped proliferating for several days but then continued to multiply actively. Rat and human BMSCs treated thus ceased proliferating permanently, but when attached to the bottom of the dish, they remained viable for 2–3 wk. A few dishes filled with NCTC suspension after GDV were placed carefully without shaking in an incubator for one day and then fixed and stained. These dishes did not present cell formations similar to the light patterns identified by GDV. When using chalky powder of various concentrations (uncharged suspension), no annular illumination was obtained. In addition, after a day of using a needle to spread the cells into the central depression and ring, we observed GDV. After GDV, the cells were fixed and stained. In these cases, we obtained no annular illumination (Figure 8D2 and D3). In addition, no annular emission was obtained from dishes filled with culture medium and preincubated for a day (Figure 8C1). In most cases, *E. coli* produced GDG that was close to annular (Figure 8D4).

DISCUSSION

Previously, other authors observed that MF increased, decreased or did not affect the proliferation of various types of cells^[21,22]. No clear response patterns of biological systems to the influences of MF were observed. We did not observe significant influence of MFs on the rBMSC proliferation rate. This may indicate that the

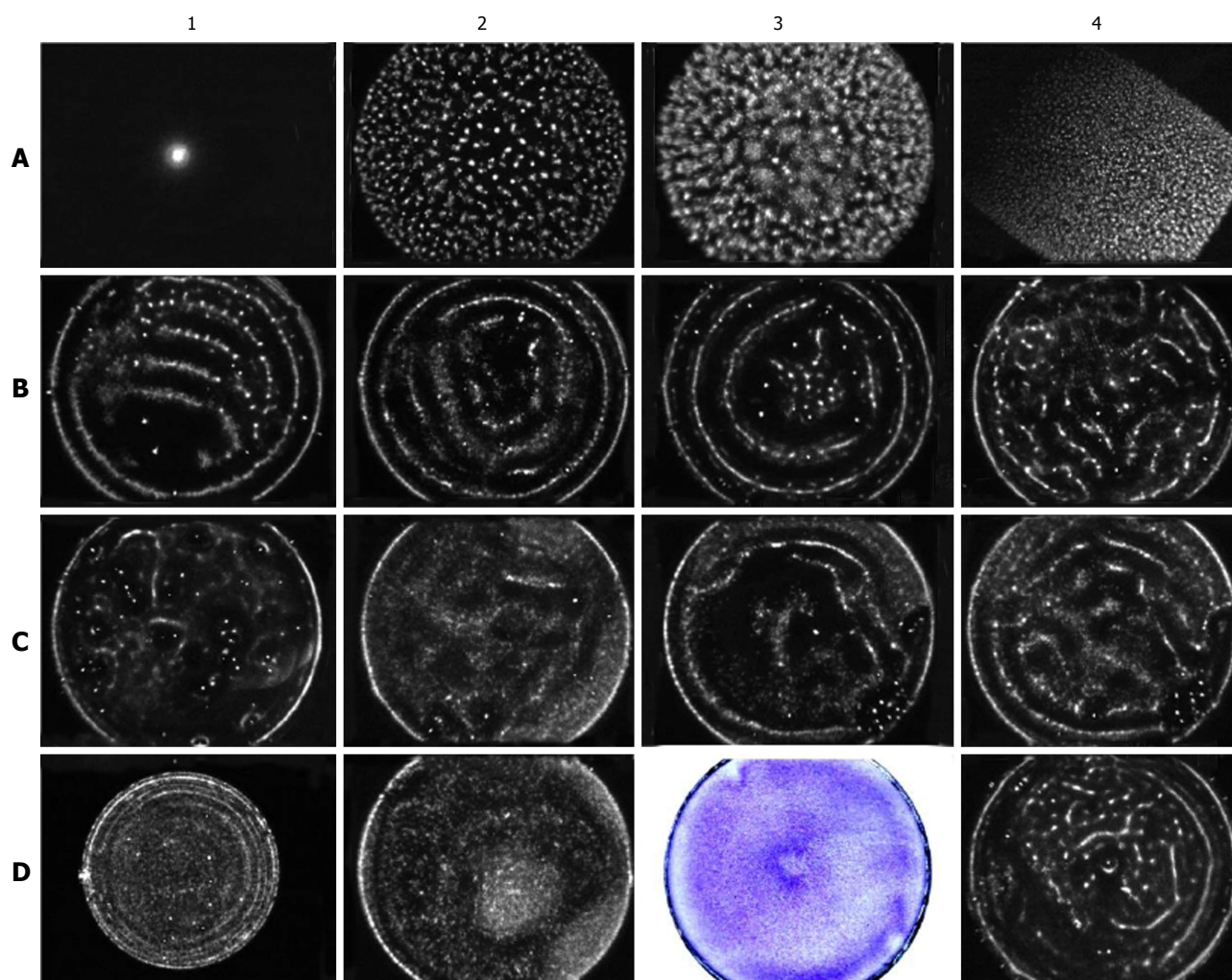


Figure 8 Evaluation of the structure of the electromagnetic field in the dishes using the gas discharge visualisation method. Except where otherwise stated, the experimental conditions are as follows: A 50-mm culture dish with a negatively charged bottom, the grounding electrode in the centre of the dish, a voltage of 115 V, an exposure time of 20 s, and a liquid volume of 3 mL. GDG from an empty dish (A1). Water GDG (A2). GDG from a dish with a hydrophobic, uncharged bottom filled with PBS (A3). GDG from a square culture cuvette filled with PBS (A4). GDG from the dish filled with PBS when the grounding electrode is in the centre (B1, note the gap in the lower left quadrant due to the uneven distribution of the charge on the bottom of the dish), equidistant from the centre and the edge (B2), and at the side (B3) of the dish. GDG from the complete culture medium α -MEM (B4). GDG from α -MEM incubated in the dish at 37 °C for one day (C1). GDG from the dish containing NCTCs at 25×10^3 cells/cm², at a voltage of 90 V (C2) and at a voltage of 115 V, with exposure for 10 (C3) and 20 s (C4). GDG from a 35-mm culture dish with NCTCs at 25×10^3 cells/cm², at a voltage of 125 V (D1). GDG from rBMSCs incubated with the magnetised needle for one day (D2). The same cells after fixation and staining (D3). GDG from *E. coli* at 200×10^3 cells/mL. rBMSCs: Rat bone marrow-derived stromal stem cells; GDG: Gas discharge glow; *E. coli*: *Escherichia coli*.

influence of MFs on cell proliferation is indirect. It may be that the direct effect of MFs is related to morphogenesis. It is logical that structure maintenance of tissues and organs and cell renewal are regulated by different mechanisms.

If fibroblasts are spread on plastic or glass, their attachment occurs only after 30–40 min from placing them into a dish; their spreading continues during the following day^[23]. At 5–10 min after placing cells into a dish, most of the cells have reached the bottom. Upon reaching the bottom of the dish, for approximately half an hour, fibroblasts are in contact with the dish but still not attached to it. At that point, the fibroblast performs a type of “rolling” movement and “releases” and “inhales” its filopodia. Thus, there is a gap between touching and attachment of a fibroblast during which it can be moved by an EF.

We have used culture dishes coated with a uniform negative electric charge on the bottom surface. In accordance with Ostrogradsky’s-Gauss’s divergence theorem, the electric field strength in the plane of the charge, along the dish bottom, decreases from the periphery to the centre of the bottom, where it reaches zero. Thus, the charged particles (cells) situated in the plane of the dish, when of appropriate size and weight, will move to the periphery of the dish (opposite charge, “+”) or to its centre (same charge, “-”). Because culture media are ionic solutions, these electrical forces affect only cells in contact with the bottom of the dish. The forces are absent in the medium column because of an electrical double layer. The participation of the charge of the dish bottom in the cell distribution across the dish has been confirmed by the absence of movement of suspended cells towards the bottom centres when using

dishes with hydrophobic bottoms and by the absence of GDV rings in the dishes with culture medium without cells after a day of incubation (Figure 8C1).

It is widely believed that cells can carry one type of electrical charge or another and can be moved by microelectrophoresis^[24]. In our experiment without the use of the magnetised needles, some BMSCs accumulated in the dish centre (central heap), some accumulated in the dish periphery (peripheral ring), and some did not respond to the EF. Thus, cells within the same population may apparently have either positive or negative charges on their surfaces or may be electrically neutral. This may depend on the availability and structure of the glycocalyx. The glycocalyx, due to its sulfate groups, creates a negative charge on the cell surface, whereas the outer surface of the cell membrane has a positive charge due to ion pumps. The fact that the glycocalyx plays a pivotal role in the cell movement in EFs along the dish bottoms is demonstrated by the accumulation of suspended cells (leukocytes) in the dish centres through one day of incubation. These cells, as they were not subjected to trypsinisation, retained an intact glycocalyx on their surfaces. In addition, these cells cannot crawl from place to place like fibroblasts. BMSCs do not appear to crawl across the dish to produce the effects induced by EFs. Further, one day is insufficient time for BMSCs to crawl the distances involved. That the BMSCs are not crawling is partly illustrated by the positions of their "tails" in all directions after one day of incubation with the needle (Figure 5B). To remove the cells from the substrate, we applied trypsin for 5 min. With this treatment, a large part of the glycocalyx is retained. It has been shown that the glycocalyx is completely removed from the cell surface only after 15 min of incubation in 2.5 g/L trypsin solution^[25]. After trypsinisation during cell isolation from tissue, the complete restoration of the glycocalyx occurred in 7-10 d^[26]. Therefore, by the time the cells were subjected to EMFs (4-7 d after passaging), the cells appeared to have a complete layer of glycocalyx. In spring, rBMSCs trypsinised for 5 or 10 min showed the effect involved. In contrast, rBMSCs during winter and NCTCs and A172 cells at two weeks after thawing showed no such effect. Based on these data, one can conclude that the molecular structure of the glycocalyx is more important than its thickness. In spring, NCTC and A172 cells showed the effect before 2 wk after thawing, but later, they showed no such effect. The behaviour of NCTCs and A172 cells led to the conclusion that the glycocalyx charge depends on protein synthesis. Those proteins are related to some proteins of transformed cells that are not synthesised until the cell's DNA is restored.

Thus, upon reaching the bottom of the dish by gravity, BMSCs begin to move, perhaps by rolling, in response to the EF to the centre or the periphery of the dish, in accordance with the glycocalyx charge, whereby they form the central heap and the peripheral ring.

With the introduction of the magnetised needle, mag-

netic forces are established, and the resultant vector is directed from the centre to the periphery of the dish (note the sparseness around the needle). It is likely that because BMSCs bear negative charges on their surfaces, they move towards the dish centre as long as the magnetic force directed from the centre equals the electric force directed towards the dish centre. It is similar for moving the cells away from the dish centre. Thus, a "donut" (torus) of cells (the central ring) might arise.

Firmly established mechanisms of primary reception of MFs and EMFs in the range of non-thermal effects have not yet been identified. Cytoskeletal rearrangements and intracellular signalling as a result of exposure to EMF on cell surface membrane are assumed to be among the primary magnetoreceptive mechanisms^[8]. However, these mechanisms cannot explain the effects involved here. To date, no one has shown that MFs can move SCs. Currently, to move cells, magnetic nanoparticles are introduced into them^[27]. In our experiments, the cells were moved through MFs over distances of up to 5 mm (the radius of the central ring). As seen from experiments with leukocyte suspensions (Figure 5C), this distance can probably be extended. It is obvious that EFs and MFs affect different cellular mechanisms. However, these mechanisms are somehow associated with each other, given that the cells were not moved in the dishes with the hydrophobic bottoms. Magnetic and electrical mechanisms appear to be nonlinearly related. Therefore, the electrical effects detectable in the controls showed significant variation between experiments. This was manifested in the presence or the absence of the peripheral ring and in the different forms of the central heap. Meanwhile, the magnetic effects, specifically the central depression and the central ring, did not show changeable shapes.

For a MF to move a cell, it must have a directed ion stream. Although there are transmembrane ion currents, these currents are balanced and therefore cannot serve as mechanisms by which MFs can induce cells to move. Protozoan (*e.g.*, the nutrition of *Paramecium caudatum*) and plant cells (*e.g.*, cytoplasmic current in the cambium layer) possess cytoplasmic currents. It is likely that the slow transport in the axons of nerve cells and the circulation of the outer membrane by ruffling are accompanied by directional circular currents of cytoplasm. It is likely that cells can also have circular cytoplasmic currents when detached. If this is actually the case, there is also circular movement of cytoplasm ions, which would produce MFs. Then, the MF of the magnetised needle would affect the cytoplasm, moving it and moving the entire cell together with it. In addition, cells would move in opposite directions in accordance with the direction of their cytoplasmic motion. Our finding that both the N and S needle poles could cause the central depression supports this model. In 1999, Makarevich^[28] used yeast cultures to show that different permanent magnetic poles produced only quantitative differences in cellular responses. We have also shown

that not all cells respond to EM exposure. Thus, there are three types of cells in the same population: Cells with cytoplasm circulating clockwise, cells with cytoplasm circulating anticlockwise, and those with non-circulating cytoplasm. This suggestion accounts for the observation of the same type of central depression in response to either magnetic pole of the needle. The fact that cells of the same population can react differently to the same MF has been shown previously^[29].

The above model cannot explain all of the observed effects. The formation of the peripheral ring remains unclear. Based on the above assumptions, some of the cells should accumulate next to the dish wall and others near the needle. Furthermore, in this model, the phenomena causing the needle MF should be observed when setting the needle outside the centre of the dish. All these reasons led us to explore in more detail the EMFs formed in the culture dishes.

The movement of the cytoplasm create a MF in the range of nT or less. This is likely insufficient to move the cells. Therefore, an amplifier of the MF is needed. In the model system some resonances appear to exist which function as such an amplifier. This assumption is confirmed by findings of the GDV study as follows: The presence of glowing rings; the importance of the shape and size of the dish; the importance of the electric voltage magnitude; and the fundamentally unchanged ring-shaped glow upon setting the ground electrode off to the side from the dish centre (Figure 8). This effect in particular may indicate that it is not necessary to introduce the needle to the acupoint centre only to achieve a therapeutic effect. All of the acupuncture practice has noted this; the exact anatomical centre has not been established for any acupoint. The resonant nature of the field created by the magnetised needle is also demonstrated by the absence of sparseness around the needles set eccentrically and by the poor results when using culture dishes with diameters of 35 and 90 mm. The same findings also demonstrate that there is little or no effect of metal ion diffusion from the needle on the investigated phenomenon. The absence of cell sparseness around the plastic hydrophobic rod demonstrates a very small value of hydrophobic forces produced by the dish wall. Thus, magnetic resonance phenomena are present in the system of the culture dish, the BMSCs, and the needle. There are obvious similarities between the rings obtained by cell staining and observed in GDV (Figure 8C4 and D3). Furthermore, GDV (the same as the cell cultivation in the dishes with hydrophobic bottoms) shows the necessity of a polar liquid or suspension for the effect to exist, with annular GDG being absent from water and from chalky suspensions. We believe that the observed resonance phenomena explain the sparseness around the needle only in the dish centre, the absence of cell accumulation around the needle and forming the peripheral ring. The resonant nature of the meridian system (and, consequently, acupoints) has been proposed previously^[30]. However, experimental

verification of this phenomenon has so far been absent.

In a series of studies, we have shown the fundamental insignificance of the methods of cell application and distribution across the dish bottom and the mode of needle administration for the generation of the central heap, the central depression and the central ring. We have shown that if the cells do not form these structures themselves, we cannot form them by neither some special twisting the needle nor by some rocking or spins the dish as a whole. Clearly, the uniform distribution of cells across the dish bottom may be broken in those cases. Additionally, as shown by GDV, the dish charge sometimes appears to be unevenly distributed. For example, in the bottom left sector, a bit below the centre of Figure 8B1, a gap is clearly visible, distorting the overall picture of the glow. These irregularities in charge also seem to affect the movement of the cells.

In addition, we observed a seasonal pattern in the effects involved. Seasonal phenomena have already been described in the literature in studying whole organisms and their foetuses. Temporary physiological "windows" from minutes and hours to seasons have been reported^[31,32]. Blank *et al.*^[33] showed Na-K-ATPase in various enzyme activity to respond or to non-respond to the same EMF. Laboratories have long empirically known that cell cultures can behave unconventionally during the summer, from mid-July until the end of August, and during winter, from mid-November to mid-January. However, no one has shown the seasonal effect in cell cultures. It is likely that there are transition states from autumn to winter and from winter to spring, but examining those states was beyond the scope of this study.

In conclusion, in this study, we first showed that a magnetic field of 10-100 μ T can move human and rat BMSCs over a distance visible to the naked eye without the introduction of magnetic nanoparticles inside the cells. We have shown that achieving this effect requires a constant EF from one source, a constant MF from another source, and cells in the responsive functional state. We have demonstrated that EM resonances exist in this system. We propose a model to explain the results. We hypothesise that the electrical phenomena depend on the glycocalyx and that the magnetic phenomena may depend on movements of the cytoplasm. Thus, we propose an experimental model of acupoint that may provide the basis for an explanation of EM phenomena in acupoints as well as some features of acupoint treatments (acupoints as a biological MF amplifier). In this study, we also showed that the responses of human and rat BMSCs to EFs and MFs in this system depend on the season. This result is consistent with known features of acupoint function.

From our perspective, aside from the roles of vascular nerve sensors^[13] and connective tissue connectors^[34], acupoints act as MF amplifiers for maintaining the structure of organs and tissues of the body. Based on our present findings, this idea seems less outlandish. Obviously, there may be opportunities for the conditions

in acupoints for MFs structural formative action on cells, tissues, and organs to exist. This possibility needs now to be explored in a whole organism.

Further studies may be advanced on several fronts. If using this model of acupoint one may investigate the influence of various physical and chemical factors such as visible, infrared and ultraviolet light. There is the nature of the resonances observed to be resolved. Solving this problem would allow cells to be moved much further than 5 mm. Verification of the movable cytoplasm hypothesis is also important. If the cytoplasm of the cell genuinely somehow moves directionally, it may be another cellular mechanism for the regulation of cellular functional activity. For example, it has long been known that rearrangement of the actin cytoskeleton first requires its complete disassembly. That cytoskeletal peculiarity may be directly linked to the movement of the cytoplasm. An interesting question is the nature of the glycocalyx charge. The glycocalyx can reach a thickness of 20 nm and thus create tissue electrical potential gradients. Therefore, the model proposed here may prompt various studies in cell biology and physiology.

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COMMENTS

Background

Regeneration occurs *via* two types of processes: (1) forms and structures are reproduced; and (2) the cellular-intercellular mass is reproduced. Stem cells reproduce the cellular mass. It is clear that there must be some mechanism for controlling the reproduction of the form and structure of tissues and organs. This mechanism may be related to the acupuncture meridian system.

Research frontiers

At present, various types of stem cells have been successfully used to treat acquired or congenital defects in human tissues. Tissue grafts are used for correcting large tissue defects or the disordered structure of hollow viscera. However, reproducing the structures of solid organs such as the liver, kidney or heart has not yet been successful. Although the morphological and electrical features of acupoints are known, the nature of the meridian system has yet to be determined.

Innovations and breakthroughs

The authors first showed experimentally that a magnetic field may be involved in the mechanism of structural formative regeneration and the acupuncture meridian system. The authors first showed that when using a magnetic field of 10-100 μ T, human and rat bone marrow derived stromal stem cells can be moved without the introduction of magnetic nanoparticles inside the cells. The authors have shown that achieving this effect requires a constant electrical fields from one source, a constant magnetic fields from another source, and cells in the responsive functional state. The authors have demonstrated that EM resonances

exist in this system.

Applications

To verify the discovery, the authors proposed the acupuncture point as a model. This model allows the investigation of the many cell properties involved in the cell moving by a magnetic field. Of course, it may take considerable effort to develop methods of managing cell movement in the body by means of a magnetic field without magnetic nanoparticles. However, the authors are now confident that these methods exist. Furthermore, the same methods will be able to affect the existing stem cells of a patient to induce the recreation and maintenance of the correct structures of the patient's organs and tissues.

Terminology

BMSCs: Bone marrow derived stromal stem cells. These multipotent cells serve as source for other types of stem cells and regenerative processes. GDV: Gas discharge visualisation is a method based on the visualisation of gas discharge induced photoelectron emission from the surface of an object placed in a high-tension electric field (Kirlian effect). Ultimately, the authors see a glow from the object. This method uses an electromagnetic field of 1 kHz frequency. Thus, the glow is caused by both electrical and magnetic fields.

Peer-review

This is an important research describing about the relationship between BMSCs and electromagnetic field. The manuscript could be of interest in its field due to the novelty of use of acupuncture needles as source of a low magnetic field.

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Basic Study

Characterization and genetic manipulation of primed stem cells into a functional naïve state with *ESRRB*

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Abstract

AIM

To identify differences between primed mouse embryonic stem cells (ESCs) and fully functional naïve ESCs; to manipulate primed cells into a naïve state.

METHODS

We have cultured 3 lines of cells from different mouse strains that have been shown to be naïve or primed as determined by generating germline-transmitting chimeras.

Cells were put through a battery of tests to measure the different features. RNA from cells was analyzed using microarrays, to determine a priority list of the differentially expressed genes. These were later validated by quantificational real-time polymerase chain reaction. Viral cassettes were created to induce expression of differentially expressed genes in the primed cells through lentiviral transduction. Primed reprogrammed cells were subjected to *in-vivo* incorporation studies.

RESULTS

Most results show that both primed and naïve cells have similar features (morphology, proliferation rates, stem cell genes expressed). However, there were some genes that were differentially expressed in the naïve cells relative to the primed cells. Key upregulated genes in naïve cells include *ESRRB*, *ERAS*, *ATRX*, *RNF17*, *KLF-5*, and *MYC*. After over-expressing some of these genes the primed cells were able to incorporate into embryos *in-vivo*, re-acquiring a feature previously absent in these cells.

CONCLUSION

Although there are no notable phenotypic differences, there are key differences in gene expression between these naïve and primed stem cells. These differences can be overcome through overexpression.

Key words: *ESRRB*; *ERAS*; Induced stem cells; Over-expression; C-myc

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Core tip: Derivation and culturing of mouse embryonic stem cells (ESCs) from gene targeting to injection into blastocysts for chimera generation is a lengthy process that is difficult to control. Some stem cells might be in a primed state, having lost some of their characteristics, most importantly their pluripotency. These differences between ESC clones are usually only detected after many months by the failure of chimeric males to transmit the ESC genome through the germline. Here we have determined key expression differences between cells in a primed state and those in a presumed ground state. Detection of these differences will give researchers a powerful tool to quickly distinguish these cells, saving time, money and effort by choosing the best clones to go forward with. Furthermore, we were able to rescue the ground state through overexpression, indicating that the fate of these cells may potentially be controlled.

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INTRODUCTION

Stem cells are in an early undifferentiated state and have the potential to differentiate into a variety of cell types and tissues, both *in-vitro* and *in-vivo*^[1]. There are different types of stem cells. Adult stem cells are multipotent cells that exist within the adult tissue^[2]. Embryonic stem cells (ESCs) have the potential to be differentiated to any cell type (pluripotent), whereas more differentiated stem cells, such as those in the skin, have a more restricted differentiation potential (unipotent)^[3,4]. Induced pluripotent stem cells (iPSCs) can also be differentiated into various cell types^[5-8] but a major advantage of iPSCs is that they can be generated from already terminally differentiated cells, such as skin or fibroblasts, of an individual and do not require isolating cells from embryos^[9]. Findings that the simple over-expression of four transcription factors (Oct4, Sox2, Klf4 and c-myc) was sufficient to induce iPSCs from adult mice^[5] and human^[6] cells made the process of generating stem cells much more tractable in certain species, where it was once difficult to generate stem cells (such as in rats^[10], pigs^[11], and birds^[12,13]). Since then, several strategies have been used to manipulate cells into a pluripotent state^[14,15].

Derivation of mouse ESCs is a lengthy process^[16,17] that often produces cell lines that have all of the features inherent in ESCs, but fail to incorporate into the germline. Similarly, culturing, selection, and expansion of ESC clones for gene targeting experiments results in clones whose potential for germline transmission will only be revealed after months of mouse breeding. This presents a significant limitation, as time invested may not yield the desired results. Identifying the potential of these cells early in the process, in order to make a stop/go decision, could enhance the efficiency in which research is conducted. Furthermore, overcoming identified differences in cells which lost their pluripotency may lead to rescue of valuable cell lines.

Lastly, while the reprogramming of healthy human somatic cells into a stem cell state has been defined^[6,14]; there are still important differences being assessed between pluripotent states in derived ESCs, such as the differences between primed and naïve ground states^[18].

Our work aims to identify differences, molecular and otherwise, between mouse embryonic stem cells which we are defining as naïve (ESCs that result in germline transmitting chimeras, and thus are fully pluripotent) and primed (ESCs that have all of the features of naïve cells, except that they fail to produce germline transmitting chimeras). These included morphological markers, telomerase activity, MTT assays, and microarray analysis, and incorporation into an embryo. Differences in gene expression can be used as a diagnostic tool to determine if the stem cells are in a fully naïve pluripotent state. In addition, we aim to manipulate primed cells, using lentiviral vectors, in order to induce a naïve state. We determined the differential expression patterns in 3 pairs (naïve/primed) of mouse ESC lines derived

Table 1 Naïve and primed mouse embryonic stem cells

Mouse strain of ESC line	Targeted locus	Germline transmission Pluripotent	No germline transmission Not pluripotent
129Sv	POMC	I (QKQR-1E)	II (QKQR-11B)
C57BL/6	IGFR1	III (IGFR1-152)	IV (IGFR1-R13)
C57BL/6	FGF13	V (FGF13-1)	VI (FGF13-15)

ESC: Embryonic stem cell.

from different strains and test the hypothesis that ESC functionality can be restored. Each pair of naïve and primed cell line was generated during a separate gene targeting experiment, each starting from a pluripotent ESC line. Using microarray and bioinformatic analysis, we determined a priority list of differentially expressed genes. The list included genes such as *Esrrb*, *Eras*, *Klf-5*, *c-myc*, *Rnf-17*, *Atrx*, which were significantly downregulated in the primed ESCs; the expression level of these genes was further validated using qRT-PCR. cDNAs for these genes were isolated and used to construct gene cassettes and lentiviral vectors. Primed cells were induced to overexpress some of these genes. Reprogrammed cells were injected into the blastocyst to assess the hypothesis that function, here measured by incorporation into the embryo, could be restored.

MATERIALS AND METHODS

General cell culture

Mouse embryonic fibroblasts were collected at embryonic day 12.5 for 129 Sv and C57BL/6 mice strains (Jackson). Briefly, embryos ($n = 4$) were extracted from the womb, their liver and head were removed, and the remaining contents were minced manually using forceps. The contents were placed in a 15 mL tube and treated with 0.25% trypsin (0.25% Trypsin/EDTA, Gibco; 1-2 mL per embryo) for 30 min at 37 °C, pipetting briefly every 5 min to enhance dissociation. Trypsin was neutralized with complete DMEM media, cells were spun down, counted (hemocytometer), re-suspended in media and plated at a concentration of one embryo per 150 mm dish. When grown to confluent layers, all fibroblasts were passaged in complete media twice before cells were frozen in aliquots. Mouse embryonic stem cells^[16] were cultured using KO-DMEM and standard conditions. Cells from two different genetic backgrounds and from three different gene targeting experiments were paired up after they were revealed as naïve (germline transmitting) or primed (no germline transmission), respectively (Table 1).

RNA extraction

Cells or RNA were spun down and RNA isolated using a standard kit (Promega SV total RNA isolation system, Z3105) as before^[12]. RNA was quantified using a NanoDrop 2000c (Thermo Scientific) and then stored in -80 °C. RNA was used for microarray (methods) and qRT-PCR experiments.

Microarray

Microarray analysis was performed in the Microarray Center (Duke University Center for Genomic and Computational Biology), as per their standard protocols (Affymetrix Exon WT Package). Briefly, total RNA (volume 50 µL) was extracted and submitted to the core for analysis on a Mouse Gene 1.0 ST Array (Affymetrix). Results were analyzed using variance stabilization^[19].

qRT-PCR

Complementary DNA (cDNA) was produced by reverse transcription (RT) in a 20 µL reaction using the supplier's protocol (10 µL of 2 × RT buffer and 1 µL of 20 × Superscript II enzyme; Applied Biosystems). The cDNA was then used as a template to perform PCR gene expression assays in 20 µL reactions containing 1 µL template (approximately 2 µg/µL), 10 µL 2 × Gene Expression Master Mix (BioRad) and forward and reverse TaqMan primer probes (Generated by Applied Biosystems) or in 20 µL reactions containing the same reagents, but in place of TaqMan primers, custom PCR primers and 1 µL SYBR green (BioRad). The reactions were performed in a Cx96 real-time machine (Bio-rad). Cycling conditions were 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. No-template controls were run for each primer set and probe. 18S rRNA endogenous control was run for each sample using TaqMan primers that recognized the RNA in all samples tested (Cat# Eukaryotic 18S RNA HS99999901_S1; Applied Biosystems). The results were normalized to the endogenous 18S expression and to the gene expression level of the control mouse fibroblasts using the 2-DDCT method common for qRT-PCR analyses^[20]. All primers showed efficiency levels above 90%, using the protocol in the MIQE guidelines (minimal information for publication of real-time PCR experiments). For statistical analysis, 2-way ANOVAs were performed on two factors [genes and strain type (C57BL/6 and 129 Sv)] on $n = 3$ independently generated lines (replicates) for each of the groups. Table 2 contains the primer sets utilized in this project.

Viral vector generation

In order to generate vectors, we used the backbone for the STEMCCA Cassette^[20], excising the stem cell genes using restriction enzymes. After evaluating a priority list of differentially expressed genes, we decided to generate cDNAs for two genes, *Eras* (Embryonic Stem Cell Expressed RAS, ENSMUSG00000031160) and Ring Finger Protein 17 (*Rnf-17*, ENSMUSG00000000365) and *Esrrb* (Estrogen Related Receptor Beta, ENSMUSG000000021255) were generated in order to incorporate them into the cassette. RNF17 incorporation was not successful, therefore, only the *ESRRB* and *Eras* genes were used. Cassettes with *c-myc* and *KIF-4* derived from Sommer *et al.*^[20], were also generated. We also generated a cassette with Nanog (NM_028016.1), as a positive control to *ESRRB*.

Table 2 Primers used for quantificational real-time polymerase chain reaction to amplify and quantify expression of differentially expressed genes

Mouse	Gene identification	Fwd primer	Rev primer
Oct-4	NM_013633.2	CCCCATGTCCGCCCGCATACT	AGGCCAGTCCAACTGAGGTC
Sox-2	NM_011443.3	GAAGAACAGCCCGGACCGCGT	ATGAACGGCCGCTTCTCGGT
c-myc	NM_010849.4	ACCCGCTCAACGACAGCAGC	ACTAGGGGCTCAGGGCTGGC
KLF-4	NM_207209.2	TAGTGGCGCCCTACAGCGGT	TCGTGTGTGTGGCCGGTG
KLF-5	NM_009769.4	CACCGGATCTAGACATGCC	ACGTCTGTGGAACAGCAGAG
Pax-6	NM_001244198.1	CACCAGACTCACCTGACACC	TCACTCCGCTGTGACTGTTC
BMP-7	NM_007557.3	CTGAGTAAAGGACAGGGGCG	CTGAGTAAAGGACAGGGGCG
ESRRB	NM_001159500.1	CTACGCCACTCAAGAAGCCA	TGTATGAAGGAGCCGCAACT
Nanog	NM_028016.1	GGCTGCCTCTCTCGCCCTT	GTGCACACAGCTGGGCTGA
ERAS	NM_181548.2	TGCCCTCATCAGACTGCTA	CCAAGCCTCGTACTTTCCT
ATRX	NM_009530.2	CTTGCTTGTTCCTGGCTCT	CTTGTTTCCACTCATGGGCTC
RNF17	XM_006519107.1	CACCTAGTGGAGAGTGACCA	TCTAAATGCCTGTGAGGGGC

Viral generation

Lentiviral vectors were generated in human embryonic kidney 293T cells (Cell Biolabs, Cat # LTV-100), using a third-generation lentiviral system, following a previously described protocol^[12]. Prior to transfection, the cells were plated on 10 cm collagen coated plates at a density that resulted in 60%-70% confluency at the time of transfection. A transfection mix was prepared with either 5, 10 or 15 µg of DNA of the genes generated in vector or control GFP lentiviral vectors (EF1α-GFP; generated in lab), packaging cassette (REV and Gag/Pol, 10 µg) and the VSV-G (5 µg) envelope expression cassette, respectively. The cells were then transduced with the mix, using 40 µL of Lipofectamine (Invitrogen) per plate. Eight hours after the addition of DNA, the transduced cells were washed with PBS and fresh complete media as used for mouse cells. Media with viral particles were collected every 24 h for the next 48 h and stored at 4 °C until complete. Viral particles were separated from cellular debris by centrifugation at 4000 g for 5 min followed by filtration through a 0.45-micron filter. The titer was measured using Quick-Titer (Cell Biolabs Inc, Cat # VPK-112) and promptly stored at -80 °C. If necessary, titer concentrations were increased by ultracentrifugation (SW-29 rotor) at 50000 g for 2 h, followed by re-suspension in PBS (pH = 7.2).

Lentiviral transduction

Transduction was performed in the Comprehensive Cancer Center of Puerto Rico, using the ViraDuctin system, as per supplier's protocol (Cell Biolabs, Cat # LTV-201) in KO medium. Before transduction, cells were thawed and cultured in complete media until 80% confluent. After transduction, cells were grown for 10 d, then passaged (1st passage), and let to grow for approximately 10 d in KO medium. Viral transduction efficiency values were assessed at different vector concentrations in 48 well plates and cell colony-forming units quantified as before (Rosselló *et al.*^[12], 2013).

Proliferation assay

To assess proliferation, we used the MTT [3-(4,5-Dime-

thylthiazolyl)-2,5-diphenyltetrazolium bromide] Quantitative Cell Proliferation Assay (ATCC; Cat# 30-1010K). Briefly, tetrazolium salts are reduced metabolically by the cells, resulting in a colorimetric change. The resulting intracellular purple formazan is solubilized and quantified spectrophotometrically (at 570 nm). Cells were plated at 10000 cells/well (in quintuplets) and incubated for 24 h. Ten microliters of the MTT reaction solution was added to each plate and incubated for 3 h. One hundred microliters of detergent was added to each plate, stored for 2 h in the dark (room temperature), and the absorbance was measured at 570 nm using a Molecular Devices Emax Microplate Reader. ANOVA was performed to test for differences between cells and strain ($n = 5$ lines, per strain). Statistical significance was considered at $P < 0.05$.

Telomerase activity

Telomerase enzymatic activity was determined using the Quantitative Telomerase Detection Kit (BioMax, United States, MT3012), following the manufacturer's protocol. Cell extracts containing proteins and RNA were generated from the ESC, iPSC, and control fibroblast, and then telomerase activity was measured. If telomerase is present, it adds nucleotide repeats to the end of an oligonucleotide substrate of the kit, which is subsequently amplified by real time qPCR. Quantitation was carried out by the PCR software of the BioRad Cx96 system. Positive control (template provided with kit) and negative control (heat inactivated samples) reactions were performed. Cycling conditions for the BioRad Cx96 real-time machine were as follows: 48 °C for 10 min and 95 °C for ten min, followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing/extension). All reactions were performed in quintuplets. Paired *t*-tests were performed to test for differences of telomerase in the induced and control fibroblasts of each cell line. Statistical significance was considered at $P < 0.05$.

Chimera formation

Blastocysts (from strain C57BL/6) were injected with

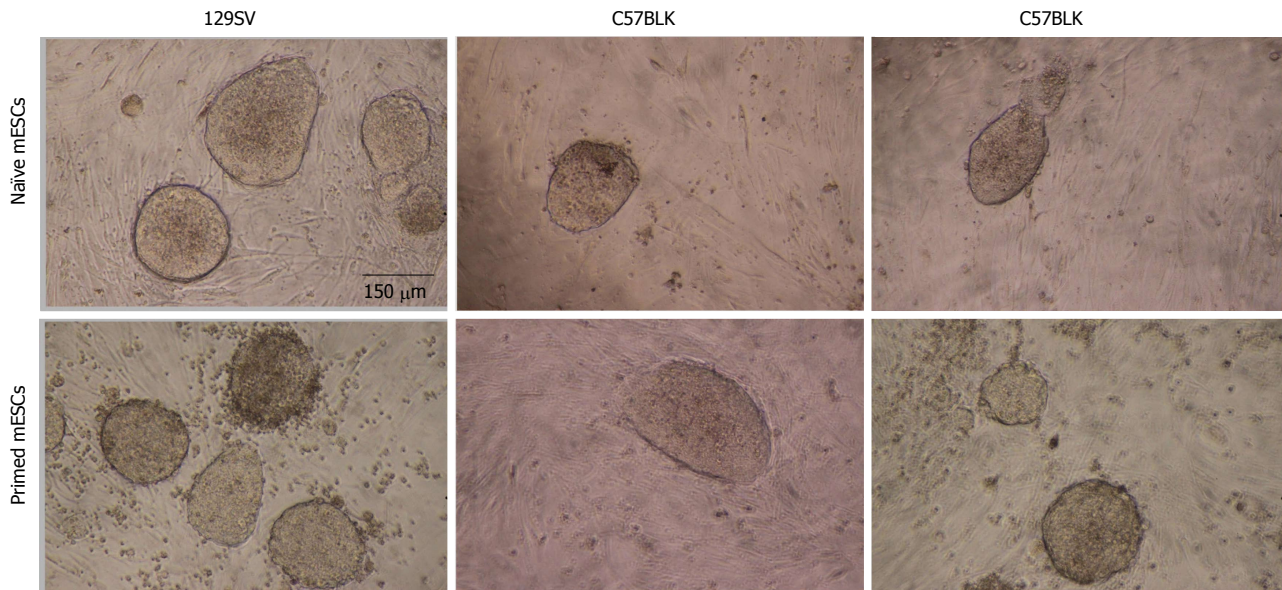


Figure 1 Primed and naive cells from different strains exhibit similar morphology. Cells that turn out to be primed are visually indistinguishable from otherwise fully naive stem cells (scale bar 150 μ m). ESCs: Embryonic stem cells

control fibroblasts, primed ESCs, reprogrammed primed ESCs and positive control naive ESCs and implanted into recipient females of the same strain as has been previously done^[21]. Briefly, we injected blastocysts, isolated from pregnant C57BLK/6 females, with fibroblasts, primed ESCs, reprogrammed primed ESCs, and positive control naive ESCs ($n = 4$). All cells were labeled with GFP through viral transduction. Five days after injection, embryos were extracted and analyzed for incorporation. Embryos were placed in 70% EtOH solution, before being paraffined and sectioned for histological analysis.

Immunohistochemistry

GFP labeling (performed by the Duke University Pathology Lab, as before^[12]) was performed on mouse embryos, or positive control tissue slides (GFP positive), that were cut at 5 μ m on a paraffin block and mounted onto glass slides. These were dried for 40 min at 60 $^{\circ}$ C in an oven. The slides were deparaffinized in 3 changes of xylene (5 min each), 2 changes of 100% EtOH (3 min each), and 2 changes of 95% EtOH (3 min each). Rehydration was performed in dH₂O for 1 min. To block endogenous peroxidase activity, 3% hydrogen peroxide was used for 10 min, followed by a rinse in dH₂O to remove antigens. For the primary antibody [anti-Rabbit GFP Abcam ab290, diluted at 1:100 in PBS (pH = 7.1)], 200 mL of the citrate, pH 6.1, antigen-retrieval buffer from Dako (10 \times concentrate) were used. The buffer was preheated to 80 $^{\circ}$ C in a Black and Decker vegetable steamer for 20 min. The slides were then cooled to room temperature. Slides were thoroughly rinsed in water and placed in TBST. After antigen retrieval, 10% normal rabbit serum was applied to the slides and incubated for 60 min at room temperature. Afterwards, they were washed with PBS and the excess

was drained. After incubation, Vectastain Elite ABC was used, followed by DAB chromagen (Dako), and incubated for 5 min, followed by washing. All slides were counterstained in hematoxylin for 30 s. Slides were rinsed in tap water until clear and coverslipped.

Animal care and use

All appropriate measures were taken to minimize animal discomfort, monitor post operative recovery and establishing humane endpoints per our IACUC protocol A262-12-10.

Statistical analysis

Biostatistics were reviewed by an expert in biomedical statistics, in order to evaluate methods used, as per suggestions. For the gene comparative, the positive log fold change values mean that the gene expression is lower in the pluripotent cells. The same is true for the t value (which the P value is based on that shows the strength of significance). Although P and t values are linked, we use t values to determine differences between populations, in order to measure the size difference relative to the variation.

RESULTS

Morphology, proliferation and telomerase activity

At first glance, all ESC cells exhibit similar morphology. Those that had been determined to be primed, *i.e.*, no germline transmission, showed round, cluster like formation, similar to naive ESCs (Figure 1), as well as alkaline phosphatase activity (not shown). In addition, qRT-PCR was performed on all samples to determine expression of typical stem cell genes (*Oct-4*, *Sox-2*, *Klf-4*, *Nanog*). Here, they exhibited similar profiles (relative to control fibroblasts) (Figure 2). Normalization

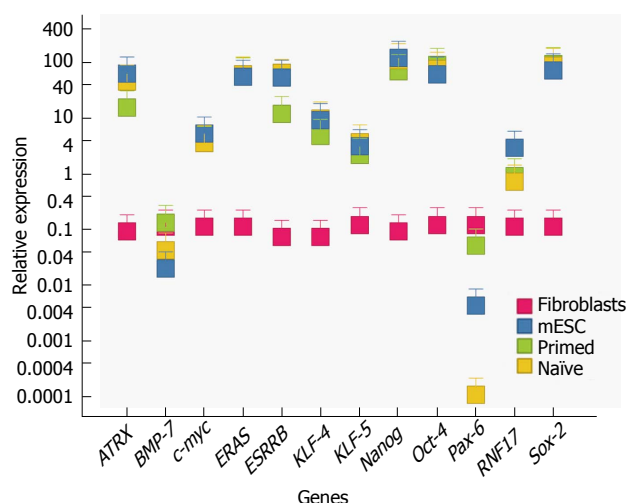


Figure 2 Differentially expressed genes in primed, naive and reprogrammed primed embryonic stem cells. qRT-PCR of known master factor stem cell genes and candidate genes selected from our microarray analysis (Tables 3 and 4, Figure 4). Differences are measured in relative expression levels (to control fibroblasts). Results show that master factor genes such as *Oct-4*, *Sox-2* and *Nanog* are all significantly higher than the control fibroblasts (red) in naive (blue), primed (green) and reprogrammed primed cells (yellow). Primers used are shown in Table 2. *Esrrb*, *Atrx* and *Rnf-17* are all significantly upregulated in naive ESCs and reprogrammed primed ESCs, relative to primed ESCs. *Pax-6* and *BMP-7* are significantly upregulated in primed ESCs. Expression levels were measured in established ESCs and primed ESCs after the 30th passage, in re-programmed primed ESCs two passages after transduction, and in fibroblasts two passages after primary cells were extracted. Error bars indicate SEM within cell populations (Tukey's post hoc, $P < 0.001$; $n = 5$ replicates of independent cell lines). ESCs: Embryonic stem cells.

was performed with 18 s expression levels for each sample. In order to compare the expression levels of the different stem cells relative to fibroblasts, fibroblasts expression values were set at 1 (Figure 2). This normalization allows us to visualize and determine the difference between fibroblasts and the stem cell groups, relative to each other.

Doubling times were observed to be similar in all six cell types (3 naive, 3 primed), but were also put to the quantitative test with an MTT Assay. After 5 passages, there was no significant difference between cells, and they all maintained steady rates (Figure 3A).

Finally, we assessed telomerase activity in all cell types. Telomerase expression is low or absent in most somatic tissues, such as our control fibroblasts, but not in germ cells, stem cells, and tumors. The telomerase binds to a particular repeat sequence TTAGGG present at the ends of chromosomes of most eukaryotic species and extends them during cell replication. While telomerase activity was significantly lower in the control fibroblast cells, there was no significant difference between the naïve and primed ESC groups (Figure 3B).

Differentially expressed genes

The gene array that was utilized (Affymetrix Mouse 1.0 ST Array), evaluated a total of 22690 genes. Our analysis included all of the genes, and a priority list was established for those that were differentially expressed

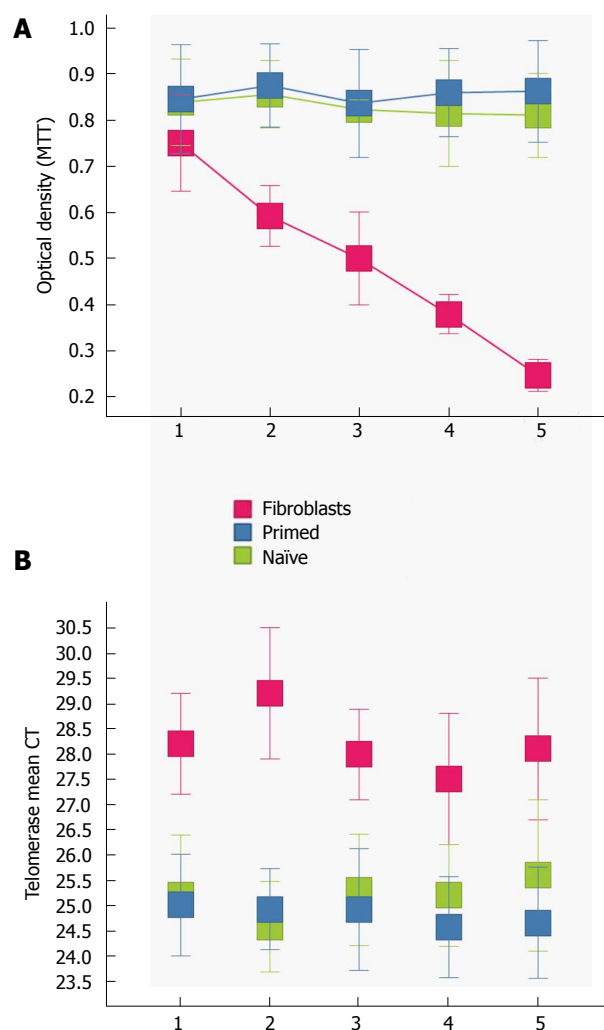


Figure 3 Proliferation and telomerase. Time course of self-renewal and proliferation of stem cells (potential induced pluripotent stem cells-like cells and embryonic stem cells) relative to control fibroblast (red) as measured by the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (read at 570 nm). mESCs (blue) and primed mESCs (green) exhibit similar patterns of proliferation, while fibroblast proliferation diminishes as time passes. Telomerase activity was greatly increased (lower mean CT) in both mESCs and primed mESCs over control fibroblast cells. Error bars, SEM ($n = 5$ independent replicates for both MTT and telomerase data). mESCs: Mouse embryonic stem cells; CT: Cycle threshold.

(Table 3). We used a positive log fold change to evaluate the differences. A positive log fold change indicates that gene expression is lower in the naïve cells. The same is true for the t value (which the P -value is based on that shows the strength of significance).

A gene ontology analysis was performed on the top set of genes using the Ingenuity Pathway Analysis. We have provided a detailed list of all significant gene ontology categories and the genes within (Table 4). Cell proliferation is the most significant gene ontology category. The P -value estimated from the current version of the IPA database (October 2015) is $1.08E-8$. The gene ontology categories we searched are comprised of thousands of complex overlapping hierarchies. Further analysis was performed examining significant sub-categories listed under proliferation. The two sub-

Table 3 Top differentially expressed genes

ID	Gene symbol (HUGO)	t	P value
1420106_at	Siah1a	3.397282751	0.019877842
1451158_at	Trip12	3.397517988	0.019872832
1425223_at	Birc3	3.399188537	0.019837294
1455579_at	Csng	3.407711727	0.019657099
1416670_at	Setdb1	3.40836782	0.019643305
1448406_at	Cri1	3.417799448	0.019446231
1420981_a_at	Lmo4	3.424023676	0.01931741
1417831_at	Smc1l1	3.429111876	0.019212822
1423271_at	Gjb2	3.440985605	0.01897126
1425329_a_at	Dia1	3.444249087	0.018905474
1438223_at	Grid2	3.447575597	0.018838686
1422666_at	Cblc	3.458197089	0.018627226
1434755_at	Coro2b	3.458912253	0.018613086
1422812_at	Cxcr6	3.459767614	0.01859619
1416515_at	Fscn1	3.460527429	0.018581195
1449371_at	Harsl	3.460876887	0.018574304
1415772_at	Ncl	3.461800178	0.018556109
1448389_at	Wdr5	3.473565946	0.018326028
1426389_at	Camk1d	3.474454082	0.018308793
1424840_at	Rbks	3.476474953	0.018269645
1425234_at	1700051I12Rik	3.477915552	0.018241796
1452638_s_at	Dnm1l	3.478750478	0.018225678
1430335_a_at	Pax3	3.480374948	0.018194365
1427854_x_at		3.482574812	0.018152058
1452402_at		3.483987577	0.018124947
1438070_at	Phf3	3.487165588	0.018064131
1454061_at	Thumpd3	3.488257585	0.018043287
1420053_at	Psmbl1	3.488906078	0.018030922
1422546_at	Ilf3	3.508209824	0.017667244
1418909_at	Ermap	3.509477595	0.017643654
1424498_at	5730596K20Rik	3.515771328	0.017527076
1418065_at	Rag2	3.520156261	0.017446374
1425961_at	BC016548	3.520765643	0.017435193
1444953_at	8430423A01Rik	3.524330508	0.017369944
1418227_at	Orc2l	3.526672877	0.017327223
1419179_at	Txn14	3.529589461	0.017274198
1423249_at	Nktr	3.532283103	0.01722539
1427554_at	Hel308	3.53288254	0.01721455
1427643_at	1200009O22Rik	3.543340398	0.01702668
1421869_at	Trim44	3.554867777	0.016822308
1427482_a_at	Car8	3.558911802	0.016751276
1432459_a_at	Rog	3.561412525	0.016707523
1438245_at	Nfib	3.565204959	0.01664142
1422956_at	D1Pas1	3.576076558	0.016453576
1422036_at	Strn	3.57762187	0.016427073
1453683_a_at	1200008O12Rik	3.585757456	0.016288346
1419253_at	Mthfd2	3.586846457	0.016269879
1420974_at	Setdb1	3.587477754	0.016259184
1431686_a_at	Gmfb	3.588593964	0.016240294
1430586_at	2700007P21Rik	3.588969301	0.016233948
1423553_at	Dnajb3	3.599839679	0.016051375
AFFX-18SRNAMur/X00686_5_at		3.612923955	0.015834729
1438748_at	2700078E11Rik	3.613005797	0.015833384
1425605_a_at	Lmbr1	3.614705726	0.015805487
1443589_at	Gpm6b	3.615856465	0.015786634
1425338_at	Plcb4	3.61646714	0.015776639
1428132_at	Cdc42se1	3.625113293	0.015635909
1450541_at	Pvt1	3.628326703	0.015583973
1456565_s_at	Map3k12	3.631282846	0.015536369
1419052_at	Ovol1	3.635287213	0.015472151
1451456_at		3.640820903	0.015383907
1452642_at	Tmem16f	3.646863541	0.015288205
1451021_a_at	Klf5	3.651556285	0.015214353
1421933_at	Cbx5	3.652925906	0.015192876
1419241_a_at	Aire	3.655287704	0.015155921

1456190_a_at	BC031140	3.659632397	0.015088211
1436162_at	C730048C13Rik	3.668343947	0.014953486
1420517_at	2310010I16Rik	3.669025757	0.01494300
1452408_at		3.669339752	0.014938174
1438215_at	Sfrs3	3.674062823	0.014865793
1417757_at	Unc13b	3.696140227	0.014532751
1417270_at	Wdr12	3.706962408	0.014372629
1424942_a_at	Myc	3.714859557	0.014257063
1422135_at	Zfp146	3.722089586	0.014152193
1425270_at	Kif1b	3.723748371	0.014128258
1428045_a_at	Elf2	3.731380905	0.014018724
1427101_at	Metrn	3.749492087	0.013762692
1419940_at	4930488L10Rik	3.761372517	0.01359766
1435626_a_at	Herpud1	3.76528356	0.01354383
1423437_at	Gsta3	3.765317741	0.013543361
1449416_at	Fzd4	3.775661238	0.01340218
1428160_at	Ndufab1	3.778355433	0.013365684
1417029_a_at	Trim2	3.781146538	0.013327996
1455808_at	4922502D21Rik	3.782747632	0.013306432
1430483_a_at	2310042N02Rik	3.791162199	0.013193759
1424083_at	Rod1	3.791959817	0.013183136
1460725_at	Xpa	3.792592186	0.01317472
1452318_a_at	Hspa1b	3.796896622	0.013117602
1421230_a_at	Msi2h	3.812474518	0.012913258
1418349_at	Hbegf	3.817881032	0.012843195
1440192_at	1810054D07Rik	3.854206413	0.012383645
1452243_at	Kcnj14	3.85496781	0.012374217
1451887_at	Lrba	3.857465589	0.012343347
1419900_at	Sin3a	3.88287621	0.012034288
1450193_at	Hcn1	3.891121372	0.011935929
1417905_at	Pr1pf	3.896151709	0.011876375
1434987_at	Aldh2	3.902236841	0.011804791
1449118_at	Dbt	3.905570325	0.011765788
1420982_at	Rnpc2	3.917975152	0.011621945
1424755_at	Hip1	3.936108008	0.011415316
1446914_at	Eif2s2	3.944832819	0.011317406
1455540_at		3.954421033	0.011210923
1456319_at		3.960519659	0.011143796
1438741_at	Rbm13	3.971304363	0.011026221
1449597_at		3.9720719	0.011017908
1416934_at	Mtm1	3.975422707	0.010981701
1426196_at		3.981642563	0.010914857
1427765_a_at	Tcrb-V13	3.988067373	0.010846303
1419277_at	Usp48	3.995642623	0.010766111
1438700_at	Fnbp4	4.007934467	0.010637441
1451739_at	Klf5	4.009661813	0.010619502
1452415_at	Actn1	4.013468651	0.010580091
1421595_at	9630031F12Rik	4.026451959	0.010446942
1423093_at	Incenp	4.056991536	0.010141312
1449729_at		4.059318965	0.010118447
1448371_at	My1pf	4.060423682	0.010107615
1432007_s_at	Ap2a2	4.061118175	0.010100812
1426375_s_at	BC019806	4.061705841	0.01009506
1421267_a_at	Cited2	4.067131851	0.010042127
AFFX-18SRNAMur/X00686_3_at		4.068244684	0.01003131
1424872_at	2310001H12Rik	4.072170362	0.009993261
1448606_at	Edg2	4.076478623	0.009951696
1420930_s_at	Catna1	4.093968451	0.009784998
1438082_at	2310028N02Rik	4.096841999	0.00975792
1418569_at	2410043F08Rik	4.109867254	0.009636263
1417178_at	Semcap2	4.111817999	0.009618195
1452620_at	Pck2	4.113798961	0.009599886
1419116_at	5430428G01Rik	4.127933982	0.009470413
1451902_at	BC021442	4.164797416	0.009142157
AFFX-18SRNAMur/X00686_M_at		4.172558479	0.009074738
1449838_at	Crisp3	4.181403551	0.008998604
1450430_at	Mrc1	4.194900667	0.008883849
1431893_a_at	Tprt	4.199779855	0.008842784
1453360_a_at	Tex9	4.212874201	0.008733659
1418417_at	Msc	4.222435464	0.008654964

1456225_x_at	Trib3	4.225132709	0.008632913
1427649_at	Wdr22	4.23098011	0.008585331
1452502_at	Serf1	4.234174345	0.008559468
1452070_at	Deddd2	4.27019116	0.008274009
1448457_at	Krt2-6g	4.278133662	0.008212555
1416268_at	Ets2	4.278591968	0.008209025
1425831_at	Zfp101	4.280752877	0.008192405
1449693_at	Map3k7	4.284119202	0.008166592
1449229_a_at	Cdkl2	4.286647093	0.008147271
1452142_at	Slc6a1	4.305051867	0.008008185
1430634_a_at	Pfkip	4.316779598	0.007920996
1428060_at	Cd3z	4.323698776	0.007870073
1434326_x_at	Coro2b	4.329579399	0.007827092
1424843_a_at	Gas5	4.357574401	0.007626183
1421279_at	Lamc2	4.404512781	0.007302618
1421496_at	2410116105Rik	4.40646557	0.007289506
1419921_s_at	Usp7	4.409499533	0.007269189
1448348_at	Gpiap1	4.411257474	0.007257448
1423809_at	Tcf19	4.516818863	0.006591172
1429427_s_at	Tcf7l2	4.555365814	0.006365827
1431117_x_at	1810029B16Rik	4.593837426	0.006149825
1450791_at	Nppb	4.610263947	0.006060208
1451524_at	Fbxw2	4.619741472	0.006009196
1426458_at		4.621344819	0.006000616
1431716_at	Herc4	4.621895613	0.005997671
1421257_at	Pigb	4.628246541	0.005963844
1425285_a_at	Rab27a	4.63066177	0.005951038
1425709_at	Rnf17	4.684098899	0.005675746
1418460_at	Sh3d19	4.750024695	0.005356347
1434674_at	Lyst	4.776236981	0.005235221
1422567_at	Niban	4.818450492	0.005046817
1423154_at	BC005537	4.830514081	0.00499444
1425837_a_at	Ccrn4l	4.886163806	0.004760904
1419929_at		4.934007639	0.004570291
1427285_s_at	2210401K01Rik	4.971430915	0.004427403
1424841_s_at	Rbks	4.999229746	0.004324644
1456511_x_at	Eras	5.002953677	0.004311091
1460464_at	2700089E24Rik	5.011990225	0.004278412
1435106_at	3732412D22Rik	5.067838157	0.004082764
1420605_at	Mtag2	5.106047884	0.003954942
1438403_s_at	Ramp2	5.1302574	0.003876383
1438824_at	Slc20a1	5.153244671	0.003803479
1422986_at	Esrrb	5.181240688	0.003716852
1437867_at		5.202674306	0.003652093
1451416_a_at	Tgm1	5.218518597	0.003605072
1455930_at		5.274806147	0.003443681
1422903_at	Ly86	5.294879889	0.003388188
1420947_at	Atrx	5.29861609	0.003377976
1426267_at	Zbtb8os	5.321555804	0.003316062
1420946_at	Atrx	5.339806851	0.003267753
1443949_at	Ppp2r5e	5.375119299	0.003176613
1418189_s_at		5.379928117	0.003164434
1427408_a_at	Thrap3	5.479919443	0.002923217
1418188_a_at		5.510099766	0.002854707
1416325_at	Crisp1	5.528967926	0.002812835
1423411_at	BC013481	5.560493925	0.002744476
1449167_at	Epb4.114a	5.632515885	0.002595515
1417755_at	Topors	5.656705105	0.002547636
1424786_s_at	Wdr45	5.706240554	0.002452797
1417548_at	Sart3	5.718574747	0.002429833
1420781_at	Etos1	5.757749264	0.002358561
1425019_at	Ubxtd4	5.830010327	0.002233456
1420909_at	Vegfa	5.932031625	0.002069901
1450051_at	Atrx	5.978799312	0.001999675
1420169_at		6.10858383	0.001819049
1447984_at	Gpatc2	6.132886435	0.001787407
1422259_a_at	Ccr5	6.259509495	0.001632696
1442566_at	Jarid2	6.267516862	0.001623457
1437534_at		6.415989061	0.001462848
1428786_at	4930568P13Rik	6.897342617	0.001057372

1418350_at	Hbegf	8.389351415	0.00043149
1449898_at	1-Sep	9.133148915	0.00029058

Top 200 differentially expressed genes in incorporating stem cells relative to non-incorporating stem cells.

Table 4 Top gene ontology (proliferation)

Categories	Diseases or functions annotation	P value	No. of molecules
Cellular growth and proliferation	Proliferation of cells	1.08E-08	114
Cellular development, cellular growth and proliferation	Proliferation of stem cells	5.45E-05	11
Cellular development, cellular growth and proliferation, embryonic development, development	Proliferation of embryonic cells	4.41E-04	13

The category and sub-categories annotated as “proliferation”. Shown are the top gene ontology results ($P < 10E-4$, number of molecules ≥ 5) of analysis performed on the 391 genes found to be significantly different between groups ($P < 0.02$). The analysis was performed with IPA (<http://www.ingenuity.com/products/ipa>, October 2014).

categories that passed our significance threshold were “proliferation of stem cells” at $P = 5.45E-5$ and “proliferation of embryonic cells” at $P = 1.41E-4$ (Table 4). These two, more specific, categories further connect the results of our gene ontology analysis to the function of embryonic stem cells. These two candidate genes (*ESRRB* and *ERAS*), as well as the others we highlight (*KLF5* and *MYC*) are found in the significant “proliferation” sub-categories. Only 18 distinct genes are found in these two sets. Estrogen-Related Receptor Beta (*Esrrb*), *Eras* and *myc* are found in “proliferation of embryonic cells” (Table 4). The other significant sub-category of proliferation, “proliferation of stem cells”, contained the genes *Eras*, *Kruppel-Like Factor (Klf-5)*, and *myc* (Table 4).

Thus, we turned our attention to a particular set of genes that were differentially higher in naive stem cells. In particular, *Klf-5* (5 1451021_a_at, 1451739_at), *c-myc* (1424942_a_at), *Rnf-17* (1425709_at), *Esrrb* (1422986_at), *Eras* (ES Cell Expressed Ras 1456511_x_at) have been implicated in stem cell growth and pluripotency. It is important to note that there were several genes that were upregulated in the primed, that are implicated in differentiation, such as bone morphogenetic protein 7 (*Bmp-7*) and paired box 6 (*Pax-6*). Microarray results were validated using qRT-PCR (Figure 2).

Manipulation of primed cells and in-vivo incorporation

Primed cells were transduced with GFP containing vectors expressing either *Esrrb* or *Eras*. In addition to these two genes, cells were transduced with *c-myc* and *Klf-4*. Gene expression was assessed with RT-PCR (Figure 2, only *ESRRB* + *c-myc* + *Klf-4* transduced

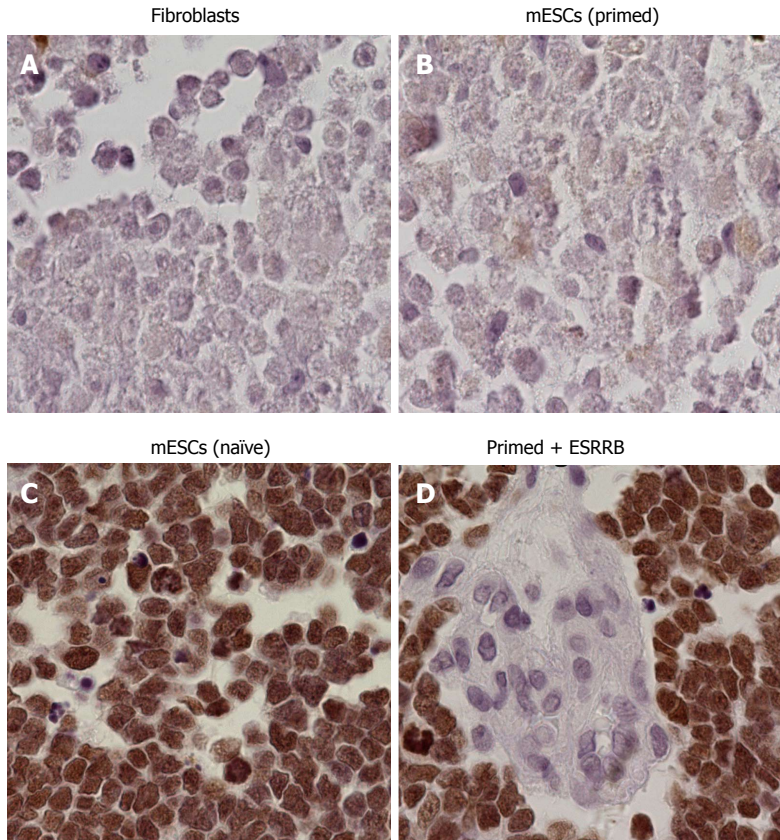


Figure 4 Sample tissue of mouse embryos after blastocyst injections of embryonic stem cells (100 \times). Five days old embryos that were produced with GFP labeled cells were sectioned and stained for GFP (brown color). Sample tissues are shown here. Cells that were reprogrammed with *Esrrb* + *Klf-4* + *c-myc* integrated into the embryo (D), as well as positive control naïve ESCs (C). No incorporation was observed in the primed state (B) or in control embryos injected with fibroblasts (A). Sample size was set at $n = 4$ mice per cell type. ESCs: Embryonic stem cells; GFP: Green fluorescent protein.

cells shown). Embryos injected with primed transduced cells over-expressing *ESRRB* were able to incorporate into mouse embryos, whereas those same cell controls were not. When *ESRRB*, *c-myc* and *Klf-4* were expressed in the same primed ESC, cells incorporated into 5 out of 6 of the embryos (Figure 4). However, cells overexpressing *Eras* alone, or *Eras* with *Klf-4* and *c-myc*, were not able to incorporate, with the exception of one sample containing all three (1 out of 6). Cells transduced with *c-myc* and *Klf-4* only did not incorporate. Cells overexpressed with *Nanog* only did not incorporate, demonstrating that the effect is *Esrrb* dependent. Positive control groups (naïve ESCs, Figure 4C) and negative control groups (fibroblasts, Figure 4A) showed the expected results.

Given the results, we performed expression profiles on *Esrrb* levels of primed ESCs. The data shows that all of them expressed significantly less *Esrrb* than their naïve counterparts.

DISCUSSION

Establishing mouse ESC cell lines from blastocysts or after gene targeting experiments can be a laborious endeavor, which may produce naïve or primed ESCs. Here we report that, although there are no significant differences in morphology, proliferation, telomerase

activity, there are however some significant differences in the expression level of key genes. Upregulation of key genes is observed in primed cells that indicate differentiation, such as *Bmp-7* and *Pax-6*. *Bmp-7* is a bone morphogenetic protein has been shown to be important in development, particularly, bone formation^[2,22] and embryogenesis^[23]. *Pax-6* is a transcription factor that is implicated in embryonic development, particularly the brain and eye^[24], ensuring proper tissue formation. Although further studies are necessary, overexpression of these factors, relative to a base ESC range, could provide an early marker to determine if the cell clones are naïve or primed.

Our attention focused on genes that were down-regulated in primed ESCs. Ingenuity Pathway Analysis showed that the top gene ontology category was proliferation. Interestingly, there was no significant difference in proliferation rates, when measured by MTT (Figure 3A). However, some of these genes have also been implicated in pluripotency and stem cell self-renewal. This may indicate that either pluripotency genes are the driving force, or that diminishing proliferation rates may be small and biologically significant or may be observed in further cell passages. In any case, downregulation of these genes may serve a similar diagnostic purpose as the upregulated ones.

Specifically we examined several genes that were

downregulated in primed cells; namely *Eras*, *Esrrb*, *c-myc*, *Klf-5*, *Atrx*, and *Rnf-17*. All of these genes were shown to be significantly downregulated relative to functional ESCs (Figure 2). *Eras* produces a constitutively active product that stimulates ESC proliferation^[25], while *Esrrb* has been shown to have an essential role in placental development and has recently been used as a marker for iPSC reprogramming and substitute for *Sox-2*^[26,27]. *Eras* has been identified to provoke tumorigenic growth, expressed only in stem cells and silenced in somatic cells due to epigenetic changes. Adding *Eras* exogenously in a constitutively expressed promoter would overcome this limitation. Besides from *Sox-2*, *Esrrb* has also been identified as prominent transcription factor that targets *Nanog*^[28]. However, interestingly, when overexpressing *Nanog* only in primed cells, they did not acquire a naïve phenotype, showing that *ESRRB*'s role spans beyond only *NANOG*. In fact, *ESRRB*'s role interacting with key stem cell master factors, made it a prime candidate to study not only as a diagnostic indicator, but also as a potential reprogramming factor^[29]. In addition *ESRRB* has been implicated as key downstream regulator of self-renewal, downstream of *GSK-3*^[27]. Inhibition of *GSK-3* has been implicated in supporting mESC state.

Low induction of endogenous *Klf-5* may be due to the redundancy of the *Klf* family^[30], or a lineage specific difference of mammals. It has been shown that the *Klf* family preferentially regulates genes involved in cell adhesion, either activating or inhibiting adhesion, and that cell adhesion can inhibit proliferation^[31]. *Myc*, in particular *c-myc*, is known to induce proliferation, by repressing growth arresting genes^[32]. This makes it a key contributor in inducing the self-renewal state of the cell. Recently, other factors that are less oncogenic have been shown to be suitable substitutes for *c-myc*, such as *Glis1*^[14]. However, *Glis1* is not differentially regulated between the naïve and primed cell types. Although we were not able to produce *Atrx* and *Rnf-17* vectors, they do serve as key indicators. *Atrx* [alpha thalassemia/mental retardation syndrome X-linked homolog (human)] is known for its role in mental retardation, but it has recently been shown that it is a key element in maintaining telomere integrity in pluripotent stem cells^[33]. Three different times this gene (*1420947_at* *1420946_at* and *1450051_at*) is in the top 20 genes downregulated, and differences in expression level were significant (Figure 2 and Table 4). Future studies will look at this particular gene and its novel function. *Rnf-17* is involved in early stages of germ cells, such as PGCs^[34,35]. It is also known that *Rnf-17* enhances *c-myc* function, through interaction with all four known Mad proteins^[36]. Although germline transmission is beyond the scope of this paper, primed cells do not possess this quality. We encourage others to examine the differentially expressed genes to further elucidate important mechanisms in the maintenance and plasticity of ESCs (Table 3).

It is interesting to note that key stem cell "master

factor" genes, such as *Oct-4*, *Sox-2* and *Nanog*^[37], are not differentially expressed in cells whose *in-vivo* function is limited. These results may therefore yield insights into proper reprogramming of iPSCs, as all of these genes may be upregulated, but other key co-regulators may be lagging.

Another important question in our project was to determine if we could restore the fully functional naïve phenotype, by overexpressing some of these key genes. Here we show at least one combination of transfections (*Esrrb* + *Klf-4* + *c-myc*) in primed cells was able to alter the expression profile and establish functionality as determined by the degree of incorporation of ESCs into embryos (Figure 4). Also, in two cases, *Esrrb* was sufficient to establish pluripotency in primed stem cells. We do not claim that these vectors will work for every case, but do demonstrate the principle that these cells can be reprogrammed into a naïve state, without the need for the OSCK cassette^[20]. This suggests that, through genetic manipulation, it is possible to restore the functional naïve state of a primed mESC. The results may be a translational gateway into reprogramming human ESCs, into a naïve state with full ESC features and function.

Although there were strain differences observed in terms of gene expressions, all of the genes utilized in our experiments were differentially expressed in both C57BL/6 and 129SV derived ESCs. Further studies are needed to assess if there are significant strain differences, and what their implications are.

Our study shows that there is a significant set of genes that are differentially expressed between naïve and primed mESCs. These genes tend to be implicated in proliferation and pluripotency. Overexpression of at least one set of genes restores the functional naïve phenotype in the primed cells. Taken together, primed cells can be identified at early stages, allowing the researcher to disregard this cell type or attempt to change it into a naïve state. Future studies into other genes, such as *ATRX*, should yield further insight into the nature of ESC functionality and phenotypes, providing a platform to study the ESC ground state and iPSC reprogramming fate.

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COMMENTS

Background

Derivation of mouse embryonic stem cells (ESCs) or gene targeting of ESCs is a lengthy process that sometimes produces cell lines that have all of the features inherent in ESCs, but fail to incorporate into the germline. Identifying this limitation takes many months, from blastocyst injection of ESCs to testing chimeric males for germline transmission of the ESC genome.

Research frontiers

Cell plasticity, reprogramming, and maintenance of stem cells are all inherent topics in this research.

Innovations and breakthroughs

No study, that the authors are aware of, had looked at the differences between two phenotypically identical stem cells, and determined the features that make them behave differently. In addition, here the authors demonstrated that the incorporating/pluripotent feature can be induced in these stem cells as well as potentially controlled.

Applications

Researchers will be able to detect within days if the stem cells they are working with have the capacity to be functional, *i.e.*, generate germline transmitting chimeras, or not. This is a key feature that will save time, money, and effort.

Terminology

Several proteins and gene products are discussed in this paper. Importantly, *ESRRB* is an estrogen related receptor beta that has been implicated as a downstream regulator of self-renewal and embryonic stem cell expressed RAS, has been implied with tumorigenic growth in stem cells.

Peer-review

The paper is well written and addresses an important issue of the ESC functionality. Authors performed transcriptom analysis of functional and non-functional ESC lines and found some differences in gene expression signature. Overexpression of the downregulated *ESRRB* gene along with Klf-5 and c-myc provided better chimera formation.

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Renal progenitors: Roles in kidney disease and regeneration

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millions of people worldwide, and its prevalence is predicted to significantly increase. The kidney is a complex organ encompassing many diverse cell types organized in an elaborate tissue architecture, making regeneration a challenging feat. In recent years, there has been a surge in the field of stem cell research to develop regenerative therapies for various organ systems. Here, we review some recent progressions in characterizing the role of renal progenitors in development, regeneration, and kidney disease in mammals. We also discuss how the zebrafish provides a unique experimental animal model that can provide a greater molecular and genetic understanding of renal progenitors, which may contribute to the development of potential regenerative therapies for human renal afflictions.

Key words: Kidney; Renal progenitor; Nephrogenesis; Development; Nephron; Regeneration; Zebrafish; Parietal epithelial cell; Tubular progenitor cell

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Core tip: The kidney is a complex organ comprised of many diverse cell types. Damage of renal cells leads to devastating kidney diseases because humans have limited abilities to regenerate these cells. Here, we explore recent research that has sought to better characterize renal progenitors during development, to identify whether renal stem cells exist in the adult kidney, and to understand the enigmatic properties of renal progenitors across diverse vertebrate species such as fish.

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Abstract

Kidney disease is a devastating condition that affects

INTRODUCTION

The kidney is a vital organ comprised of many specific

cell types that work in conjunction to maintain body fluid homeostasis^[1]. Notably, this organ is responsible for regulating pH, secreting hormones, maintaining blood pressure, and controlling red blood cell numbers^[2]. Each human kidney contains up to 2 million functional units called nephrons that are divided into distinct epithelial segments^[3]. Nephrons are organized within an intricate tissue architecture, where they are joined to a centralized collecting duct (CD) network for waste excretion^[4]. Due to the complexity of the kidney, the coordination of developmental events that create nephrons and their surrounding interstitial populations from embryonic progenitor cells remains a key question in the biomedical field.

Previous studies using the murine animal model have shown that the mammalian kidney is derived from *Osr1*⁺ cells of the intermediate mesoderm (IM) (Figure 1)^[5]. The *Osr1*⁺ cells give rise to the metanephric mesenchyme (MM), which condenses to form the cap mesenchyme (CM) (Figure 1)^[6]. The CM is a self-renewing renal stem cell population from which nephrons are crafted through a reiterative, coordinated process that involves inducing cohorts of CM cells to undergo a simultaneous mesenchymal-to-epithelial transition (MET) upon receiving differentiation signals from the adjacent ureteric bud (UB) (Figure 1)^[7]. A pre-tubular aggregate arises from each cohort of these induced renal progenitors, which ultimately becomes an epithelialized renal vesicle (RV) (Figure 1). The activated RVs signal reciprocally to the UB to undergo branching morphogenesis, eventually forming an elaborate, arborized CD network^[8]. Meanwhile, the RV structures undergo proliferation and morphogenesis, changing to form a comma-shaped body (CSB) followed by an S-shaped body (SSB) (Figure 1)^[9]. The SSBs undergo further elongation and maturation, becoming an intricately segmented nephron (N) structure that connects to the CD system (Figure 1), and contains discrete glomerular, proximal, and distal regions^[10].

During development, nephrogenesis involves a synchronized sequence of dynamic cellular events reliant upon the replenishment of the self-renewing CM and the subsequent patterning of the renal progenitors. Interestingly, nephrogenesis in mammalian and avian species ceases either at the end of gestation or shortly after birth, while in other vertebrates such as fish, reptiles, and amphibians, nephrogenesis has been documented to occur throughout the animal's lifetime^[11,12]. Further, the mammalian kidney is believed to be an organ with relatively limited regenerative potential compared to structures such as the liver^[12]. This is problematic, as kidney disease is an escalating global health issue in today's society. Upon acute injury, however, the mammalian kidney has been observed to undergo considerable structural remodeling and repair^[13-15]. Whether an endogenous adult stem cell population contributes to this process of epithelial regeneration remains controversial.

RENAL PROGENITORS DURING MAMMALIAN DEVELOPMENT

In 2008, Kobayashi *et al.*^[16] provided the first evidence of multipotent renal progenitors in the developing mammalian kidney. The investigators' approach consisted of using transgenic mice to perform lineage tracing of *Six2*⁺ cells. It had been previously shown that the transcription factor *Six2* is necessary for nephrogenesis during murine development^[17]. During early stages of nephron induction, *Six2*⁺ labeled cells were observed in the CM surrounding the UB epithelium^[16]. The CM progenitors receive signals from the UB to either self-renew, thus exhibiting a key stem cell attribute, or undergo MET and differentiate into distinct epithelial segments of the nephron^[6]. Under the correct signals, these *Six2*⁺ cells form RV, and subsequently the SSB. This RV progenitor pool eventually gives rise to multiple epithelial cell types that comprise the nephron including proximal tubular cells, distal tubular cells, connecting tubular cells, and podocytes. Interestingly, the *Six2*⁺ progenitors did not contribute to the CD, vasculature, or interstitium. The transcriptional regulator, *Osr1*, is broadly expressed in the IM, and was found to be required for the formation of the *Six2*⁺ progenitor population^[5]. In a separate study by Boyle *et al.*^[18] (2008) a transgenic strategy was employed to trace a renal progenitor pool expressing *Cited1*. Similar to the previously mentioned study^[16], the *Cited1*⁺ progenitors are induced in the MM and continually contribute to nephron formation during kidney organogenesis. Over time, the self-renewing CM stem cells cease to self-renew and found a final wave of nephrons at the cortex of the metanephros^[9].

In the following sections, we discuss how the maintenance of renal stem or progenitor cells in the adult kidney has been debated extensively based on a series of conflicting experimental observations. The existence of renal stem/progenitor cells has been proposed as an explanation for the observation that injuries to nephron epithelial cells can be healed through replenishment with newly proliferative cells (Figure 2). At present, however, it remains an unsettled controversy as to whether the adult mammalian kidney contains self-renewing renal progenitors or can be induced to form cells that exhibit stem cell-like behaviors in the context of renal injuries and other disease conditions.

EARLY EVIDENCE FOR ADULT RENAL STEM CELLS IN MAMMALS

In 2003, Maeshima *et al.*^[19] identified progenitor-like cells scattered throughout the tubules of the adult rat kidney by utilizing *in vivo* BrdU labeling. Upon ischemic injury, the label-retaining cells underwent multiple cell divisions, becoming positive for proliferating cell nuclear antigen^[19]. The progeny of the BrdU⁺ cells first expressed vimentin,

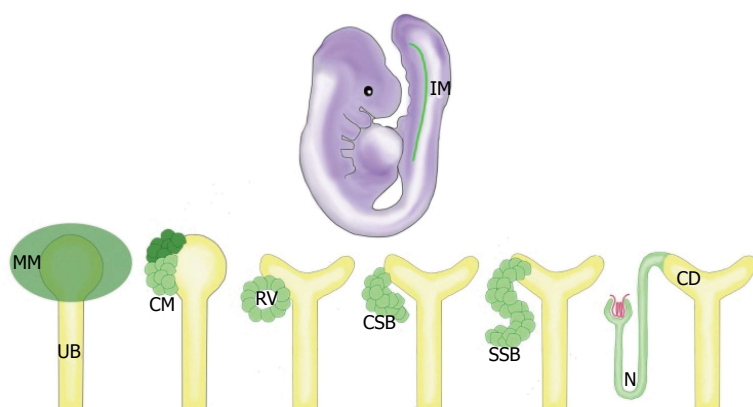


Figure 1 The progression of kidney organogenesis in mammals. The MM is derived from the IM. The MM condenses to form the CM, a renal progenitor population. These progenitors receive signals to self renew (dark green) or differentiate (light green). Cells receiving differentiation signals are organized into an epithelialized RV. Upon further maturation, these cells form a CSB, then an SSB, and finally the N. IM: Intermediate mesoderm; MM: Metanephric mesenchyme; UB: Ureteric bud; CM: Cap mesenchyme; RV: Renal vesicle; CSB: Comma-shaped body; SSB: S-shaped body; N: Nephron; CD: Collecting duct.

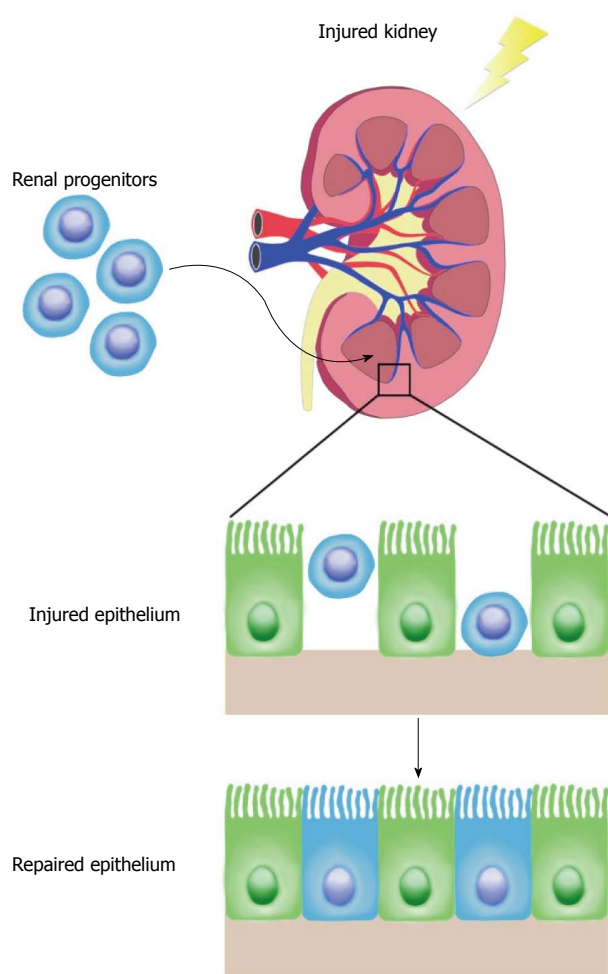


Figure 2 Regenerative capacity of adult renal progenitors. Proposed adult renal stem cells were isolated and transplanted into hosts with injured kidneys. These progenitors homed to the site of injury and repopulated the renal tubular epithelium robustly. This supports a model in which an adult stem cell population contributes to kidney regeneration after acute injury.

a mesenchymal marker, but later began to express E-cadherin, an epithelial cell marker^[19]. Collectively, results from this study suggest that label-retaining cells are renal progenitors that contribute to regeneration of the rat kidney.

In a follow-up study, Kitamura *et al.*^[20] (2005) dissected a single nephron from an adult rat kidney and isolated

a cell line (rKS56) with high proliferative potential. Upon genetic analysis, the rKS56 cells expressed both developmental markers and mature tubular markers^[20]. When these cells were transplanted into injured rat kidneys, they readily engrafted, restored tubules, and improved renal function^[20]. These rat studies support the existence of renal adult stem cells that possess the capability to repair tissue and self-renew.

In the same year, Bussolati *et al.*^[21] (2005) discovered CD133⁺ progenitor cells derived from the adult human kidney. These cells expressed Pax2, which is an embryonic kidney marker, and were capable of expansion and self-renewal *in vitro*^[21]. Interestingly, when these cells were implanted subcutaneously into immunocompromised mice, they formed tubules expressing renal epithelial markers^[21]. Upon intravenous injection of CD133⁺ cells into mice with acute tubular injury, they homed to the kidney and assimilated into the proximal and distal tubules^[21]. These data support that an adult stem cell population exists in the adult kidney and may participate in regeneration after injury.

In 2006, Dekel *et al.*^[22] isolated Sca1⁺Lin⁻ multipotent progenitors that were distinct from hematopoietic stem cells from the adult mouse kidney by fluorescence-activated cell sorting. Upon transplantation of this population into mice with ischemic injured kidneys, the cells engrafted into the interstitial space and repopulated the renal tubule^[22]. Because the Sca1⁺Lin⁻ progenitors were able to contribute to tubule repair, this provides further evidence that may suggest the existence of resident adult renal stem/progenitor cells in mammals.

PARIETAL EPITHELIAL CELLS AS RENAL PROGENITORS

Previous findings suggest that renal progenitor cells (RPCs) are present in humans and may be the origin of podocyte replacement (Figure 3)^[21,23,24]. In humans, these RPCs are a subset of parietal epithelial cells (PECs) located in Bowman's capsule that coexpress species-specific surface markers CD133 and CD24. Under correct culture conditions, CD133⁺CD24⁺ PECs have the potential to differentiate into podocytes or tubular epithelium^[24]. However, in some cases activation of RPCs can be harmful,

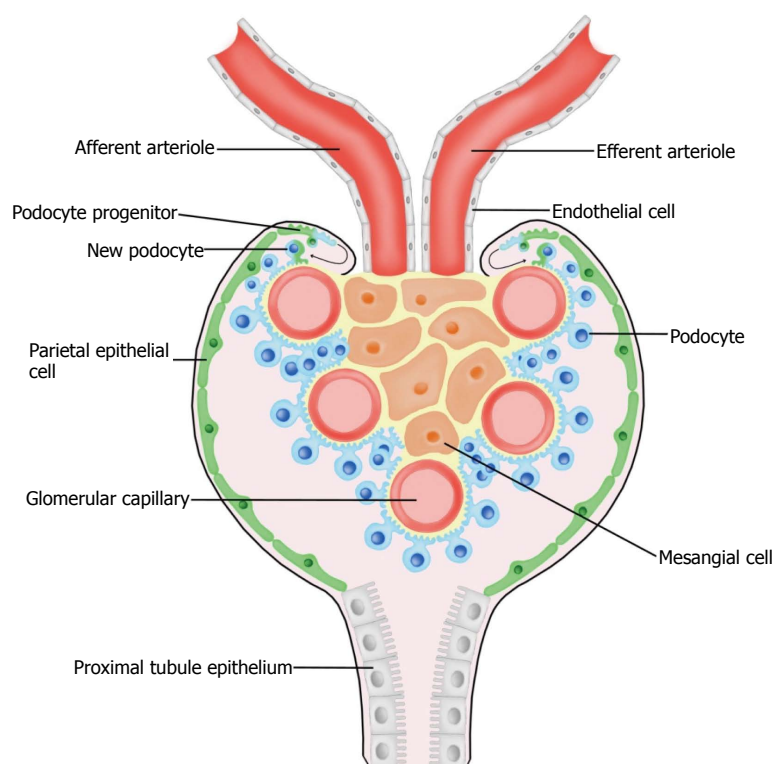


Figure 3 A model of podocyte maintenance and re-generation. Parietal epithelial cells (green) line the inside of Bowman's capsule, and are suggested to serve as a renal progenitor population. Upon acute injury, parietal epithelial cells have been observed to give rise to fully differentiated podocytes (blue).

as they have been shown to contribute to hyperplastic lesions within the glomerulus leading to degenerative disease^[25].

Wanner *et al.*^[26] (2014) investigated the regenerative role of RPCs during glomerular aging and injury. The researchers further characterized the function of PECs during kidney development by using a transgenic mouse system where upon administration of doxycycline, PECs become genetically labeled with membrane-tagged green fluorescent protein. Upon further analysis, mice exposed to doxycycline from days E8.5 to P28 exhibited mG-labeled cells with foot processes, indicating that PECs can give rise to fully differentiated podocytes. Then, the researchers induced acute podocyte loss in an mT/mG reporter strain of mice by utilizing an inducible diphtheria toxin receptor system. In this context, only podocytes coexpressing mG and the diphtheria toxin receptor are ablated. Upon flow cytometric analysis of kidneys four weeks after ablation, it was observed that there was a significant increase in the numbers of resident mT-labeled podocytes. This data illustrates how podocytes possess regenerative capacity after acute injury. Alternatively, in a unilateral nephrectomy damage context, podocyte turnover was not detected. In addition, it was observed that during aging, podocyte renewal does not occur. Taken together, these results suggest that podocyte regeneration seems to be limited to developmental and acute injury contexts. This study was the first to report that PECs can form fully differentiated podocytes, however their model does not identify the source of the new podocyte population after acute injury.

In a recent study conducted by Lasagni *et al.*^[27] (2015) the regenerative potential of these RPCs in response

to podocyte injury was studied in mice. In order to examine if the generation of new podocytes influences disease outcome, an inducible transgenic mouse model (NPHS2.iCreER^{T2}; mT/mG) was used. Upon tamoxifen administration, podocytes were genetically labeled with GFP, while all other kidney cells were labeled with TomRed. Although, after tamoxifen withdrawal, newly generated podocytes are labeled with TomRed. Mice were injected with doxorubicin to induce Adriamycin nephropathy and later biopsied, where the numbers of GFP⁺/Syn⁺ cells (pre-existing podocytes) and TomRed⁺/Syn⁺ cells (newly generated podocytes) were counted. It was found that a significant increase of newly generated podocytes occurred after injury. In addition, it was determined that remission of proteinuria in these mice is associated with the generation of new podocytes. These data suggest that RPCs may play a role in the remission of glomerular disease in mice.

Further, a model for RPC lineage tracing was established by Lasagni *et al.*^[27] (2015) using an inducible transgenic mouse line where green, yellow, cyan, or red are randomly expressed under the control of the Pax2 promoter. It was observed that Pax2⁺ cells localized in the parietal epithelium of the glomerulus are progenitors that give rise to podocytes during postnatal kidney development. Interestingly, nascent podocytes were labeled with different colors, indicating that these cells did not arise due to clonal division of a single progenitor. These Pax2⁺ RPCs were found to be responsible for podocyte regeneration in the Adriamycin nephropathy disease context. Mice with proteinuria remission exhibited abundant intraglomerular Pax2⁺ cells surrounding capillaries. Conversely, mice with persistent

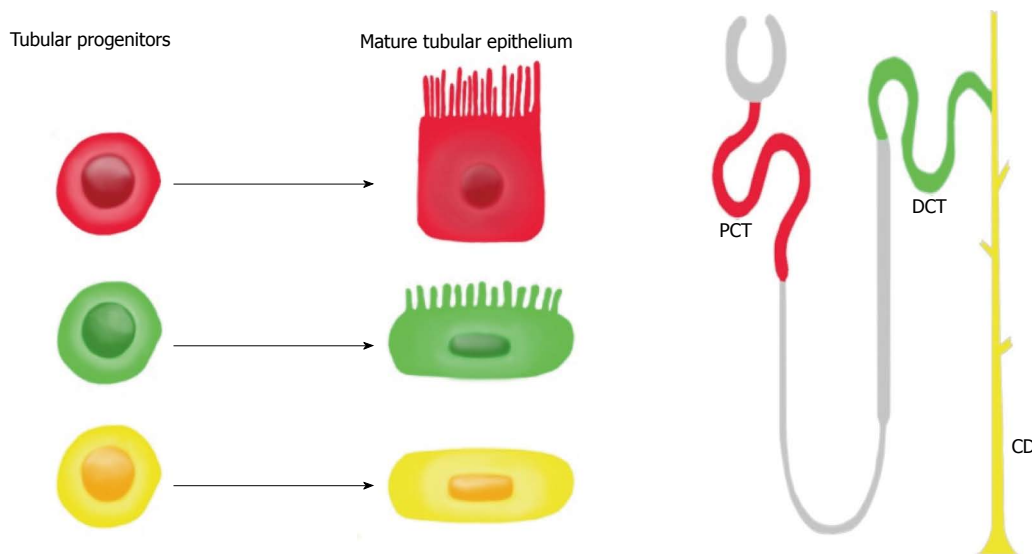


Figure 4 The fate of tubular progenitors. Tubular progenitors are believed to be involved in renal tissue maintenance and regeneration. This stem cell population is lineage-restricted to a specific segment of the nephron. For example, the red progenitor is predestined to become PCT, the green progenitor is predestined to become DCT, and the yellow progenitor is predestined to become CD. PCT: Proximal convoluted tubule; DCT: Distal convoluted tubule; CD: Collecting duct.

proteinuria exhibited virtually no intraglomerular Pax2⁺ cells. Furthermore, treating diseased animals with the GSK3 inhibitor BIO significantly increased the number of Pax2⁺Syn⁺ cells. All eight BIO-treated mice underwent proteinuria remission, where only two DMSO-treated mice exhibited proteinuria remission. Significantly, the differentiation of RPCs into podocytes can be pharmacologically driven in order to reverse glomerular disease.

RESIDENT TUBULAR PROGENITOR CELLS

In 2011, Lindgren *et al.*^[28] provided the first evidence for the existence of tubular progenitor cells in humans (Figure 4). In this study, progenitor cells were isolated from renal tissue by cell sorting for high ALDH enzymatic activity. It was observed that these progenitors were scattered throughout the proximal tubules and displayed stem cell properties such as sphere formation and anchorage-independent growth^[28]. Human tubular progenitors are localized in the proximal tubule and distal convoluted tubule and possess the following expression profile: CD133⁺CD24⁺CD106⁻. Upon injection of these progenitors into SCID mice with acute kidney injury, these cells were able to engraft, form new tubule cells, and improve renal function^[29].

Recently, Rinkevich *et al.*^[30] (2014) sought to further characterize renal tubular progenitor involvement in development, maintenance, and regeneration. The investigators crossed Actin CrER mice with "Rainbow" mice in order to genetically trace individual epithelial cells within the adult kidney. Offspring were injected with tamoxifen at 12 wk old, and were sacrificed at varying time points for clonal analysis. After 1 mo, 2-3 cell clones were scattered throughout the renal cortex, medulla, and papillae. These singly colored clones later grew in size

and contributed to existing tubules. The composition of these clones was further examined by immunostaining for segment-specific markers, where it was determined that they did not expand into different segments. These results suggest a model in which tissue-restricted progenitor cells are responsible for kidney maintenance.

In addition, the researchers performed similar clonal analysis during kidney development, where they traced embryonic renal progenitors from E13.5 to P1^[30]. Resulting tubules were observed to be polyclonal, indicating several progenitor cells are present during organogenesis. Immunostaining for segment-specific markers revealed clones separately composed of proximal tubule, distal tubule, or CD fates. These results support that renal progenitors during development are lineage-restricted to a specific tubule type. Furthermore, the clonal response to acute injury was studied by performing unilateral ischemia/reperfusion to the left kidneys of adult animals. After 2 mo, single colored clones appeared restricted to specific tubule segments. In damaged areas, significant tubule regeneration was observed where clones contributed circumferentially to the entire tubule. The clones expanded longitudinally and perpendicularly within the same tubule, however they did not extend into adjacent segments of the nephron or invade into neighboring nephrons. Upon long-term fate analysis, clones maintained the identity of a single epithelial lineage. These adult renal clones were found to originate from Wnt-responsive precursors that form segment specific tubules. Harvested kidneys from transgenic animals were dissociated into single cells and cultured in Matrigel to form organoids. Each monoclonal renal sphere was comprised of a distinct epithelial cell type. Collectively, data from this study supports the existence of fate-restricted progenitors that function in

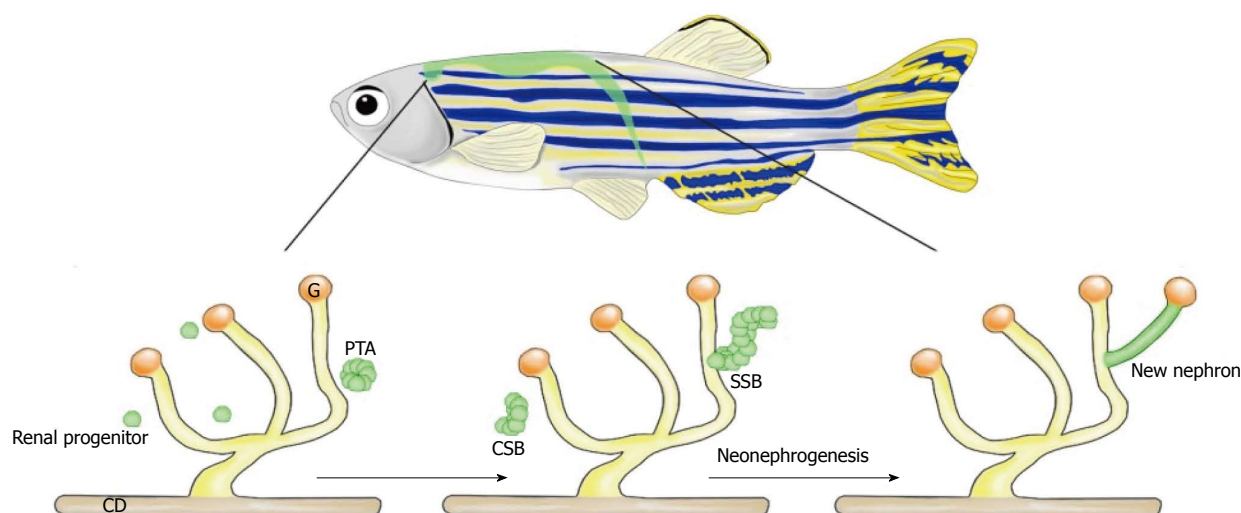


Figure 5 Neonephrogenesis in the adult zebrafish. Adult zebrafish possess the unique ability to generate new nephrons during adulthood. Neonephrogenesis in zebrafish mimics the cellular dynamics of nephrogenesis during mammalian kidney development. Renal progenitors cluster to create a PTA. This aggregate changes morphology as it first forms a comma-shape followed by an S-shape. The SSB differentiates into specific cell types that comprise the blood filter and tubules of a mature nephron. CD: Collecting duct; G: Glomerulus; PTA: Pre-tubular aggregate; CSB: Comma-shaped body; SSB: S-shaped body.

maintaining and regenerating the mammalian kidney^[30].

FISH AS A MODEL TO STUDY RENAL PROGENITORS AND REGENERATION

Renal progenitors exist in the adult kidney across many different vertebrate species, such as fish (Figure 5)^[11]. In lower vertebrates, renal regeneration and structural remodeling occurs in response to injury due to the presence of potent renal progenitors. Interestingly, the presence of these progenitors can result in the formation of new nephrons during adult growth as well as during regeneration, in a process termed neonephrogenesis^[4]. In stark contrast, mammals cease the generation of new nephrons at birth or shortly after^[9]. While we have previously discussed observations that have led the hypothesis that renal progenitors may exist in the adult mammalian kidney, there are alternative views including the generation of scattered progenitors in response to injury^[31]. Despite such controversies, it is well accepted that the mammalian kidney responds to resection with compensatory glomerular and tubular hypertrophy^[32].

To date, the existence of renal progenitors capable of neonephrogenesis has been most extensively documented in a number of fish species including skate, zebrafish, dogfish, rainbow trout, catfish, goldfish, toadfish, and tilapia^[33-44]. A deeper understanding of the molecular mechanisms driving neonephrogenesis in these fish may provide novel insights in the pathogenesis of human kidney diseases and potential regenerative therapeutics.

In a study by Elger *et al.*^[45] (2003) partial nephrectomy was performed to characterize kidney regeneration in *Leucoraja erinacea*, a species of skate^[46]. Interestingly, upon resection a neonephrogenic zone was identified that resembles the mammalian embryonic metanephric kidney. This zone encompassed stem cell-

like mesenchymal cells that were observed to aggregate around the CD tips. These cells proceeded to epithelialize and form cysts of varying morphologies, which appeared similar to mammalian metanephric structures such as RVs, comma-shaped bodies, and S-shaped bodies. The cysts progressively differentiated into distinct nephron segments, and vascularization of the glomerulus occurred. Neonephrogenesis not only occurred within the injured kidney, but also occurred within the uninjured contralateral kidney. Because neonephrogenesis in fish proceeds through similar stages as mammals, this suggests that genes regulating these events are conserved^[38]. This study presents a possible model where renal stem cells persist in the adult kidney of skates and lower vertebrates^[45].

In 2011, Diep *et al.*^[41] performed the first extensive molecular analysis of adult nephron progenitors in the adult zebrafish kidney and assessed their self-renewal capacity through transplantation studies as well. First, the researchers isolated whole-kidney marrow (WKM) cells from transgenic animals that express fluorescent reporters in the distal nephron. Upon transplant of these cells into immunocompromised, gentamicin treated recipients, many donor-derived nephrons were observed. The donor-derived nephrons were capable of blood filtration, indicating integration of the new structures in the recipient's tissue. These results support that renal progenitors are present in the adult zebrafish kidney and are able to engraft and give rise to new nephrons after transplantation. When a mix of EGFP⁺ and mCherry⁺ WKM cells was transplanted into conditioned recipients, mosaic nephrons resulted. This indicates that multiple progenitors can contribute to an individual nephron, consistent with similar data from mouse studies^[16]. In addition, serial transplantation of WKM revealed that nephron progenitors are a self-renewing population possessing substantial proliferative

potential. It was determined that *lhx1a*⁺ cellular aggregates are comprised of renal progenitors, and when these aggregates are ablated, nephrogenesis is terminated. Transplantation of a single *lhx1a*⁺ cellular aggregate was sufficient to form multiple nephrons. This study illustrates *lhx1a*⁺ progenitors in adult zebrafish act comparably to the Six2⁺ CM cells during mammalian development. Although zebrafish *lhx1a*⁺ progenitors and mouse Six2⁺ progenitors possess distinct global gene expression profiles, several factors associated with renal development and stem cell potential were found to be conserved between the two cell populations. Using zebrafish as a model to elucidate molecular pathways regulating renal progenitors may be translatable in the establishment of novel stem cell therapies to treat human kidney diseases.

DISCUSSION

Chronic kidney disease (CKD) continues to be a problem that plagues our society, as it affects millions of individuals worldwide^[47]. CKD can progress to end stage renal disease, which is ultimately an irreversible condition. The only treatment options for patients with end stage renal disease are organ transplant or dialysis^[48]. This poses a serious problem, as the availability of donor organs is low and dialysis is not a permanent cure. In addition to CKD, a variety of developmental disorders affecting the renal and urinary tract exist^[49]. Although these congenital conditions are rare, they involve severe kidney malformations that give rise to many health complications. Achieving a greater understanding of the dynamic biological mechanisms governing kidney development will unravel the mysteries of disease pathogenesis and lead to the discovery of innovative regenerative therapies.

The identification and characterization of adult renal progenitors paves the way for potential stem-cell therapies. Stem cell populations, like renal progenitors, are ideal targets for gene therapy, cell transplantation, and tissue engineering^[50]. For example, it has been shown in various studies that the transplant of renal progenitors into injured rodents drives tissue repair and improves kidney functionality^[20-22,27,29].

In addition to mice, zebrafish provide a unique model system to study kidney development and regeneration^[51-53]. Zebrafish are incredible animals, as they are experts of kidney regeneration due to their extraordinary ability to undergo neonephrogenesis throughout their adult life, which can be induced further with well-established injury models^[54-59]. Although vertebrates possess kidneys of varying organization and complexity, the genetic pathways that regulate organogenesis are highly conserved^[60]. The diverse cell types that comprise the nephron are conserved across species, contributing to a growing appreciation of zebrafish as a relevant model system to study kidney development and regeneration. Furthermore, zebrafish may help identify novel genes regulating renal progenitors, neonephrogenesis, and regeneration. Future studies could determine factors essential for activating

renal progenitors in adult zebrafish, which could potentially be translated to humans in order to induce these cells to facilitate tissue repair in disease contexts. The discovery of molecular mechanisms directing renal progenitor cell-fate decisions during development and regeneration holds great promise in advancing the fields of tissue engineering and stem-cell therapy.

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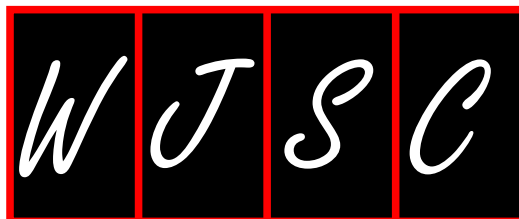
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Mesenchymal stem cell therapy in retinal and optic nerve diseases: An update of clinical trials

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Abstract

Retinal and optic nerve diseases are degenerative ocular pathologies which lead to irreversible visual loss. Since the advanced therapies availability, cell-based therapies offer a new all-encompassing approach. Advances in the knowledge of neuroprotection, immunomodulation and regenerative properties of mesenchymal stem cells (MSCs) have been obtained by several preclinical studies of various neurodegenerative diseases. It has provided the opportunity to perform the translation of this knowledge to prospective treatment approaches for clinical practice. Since 2008, several first steps projecting new treatment approaches, have been taken regarding the use of cell therapy in patients with neurodegenerative pathologies of optic nerve and retina. Most of the clinical trials using MSCs are in I / II phase, recruiting patients or ongoing, and they have as main objective the safety assessment of MSCs using various routes of administration. However, it is important to recognize that, there is still a long way to go to reach clinical trials phase III-IV. Hence, it is necessary to continue preclinical and clinical studies to improve this new therapeutic tool. This paper reviews the latest progress of MSCs in human clinical trials for retinal and

optic nerve diseases.

Key words: Mesenchymal stem cells; Cell therapy; Optic nerve diseases; Clinical trials; Retinal diseases

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Core tip: Advances in the knowledge of neuroprotection, immunomodulation and regenerative properties of mesenchymal stem cells (MSCs) are contributed by several preclinical studies of various neurodegenerative diseases. It has provided opportunity to perform the translation of treatment approach to the clinical practice. Several clinical trials in patients with retinal and optic nerve diseases have been developed since 2008. Most of them using MSCs are in I / II phase. However, there is still a long way to go to reach clinical trials Phase III-IV. Hence, it is necessary to continue with preclinical and clinical studies to improve this new therapeutic tool.

Labrador-Velandia S, Alonso-Alonso ML, Alvarez-Sanchez S, González-Zamora J, Carretero-Barrio I, Pastor JC, Fernandez-Bueno I, Srivastava GK. Mesenchymal stem cell therapy in retinal and optic nerve diseases: An update of clinical trials. *World J Stem Cells* 2016; 8(11): 376-383 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v8/i11/376.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v8.i11.376>

INTRODUCTION

Retinal dystrophies, diabetic retinopathy, age related macular degeneration and optic nerve diseases are chronic and degenerative ocular pathologies which lead to irreversible visual loss. Retinal degeneration is a leading cause of incurable low vision and blindness worldwide^[1]. Most retinal and optic nerve diseases are caused by irreversible apoptosis of retinal neural cells or adjacent supporting tissue. Because there is no curative treatment for these degenerative diseases, current therapies mainly focus on the aetiology cause or at specific situations, such as late complications. However, most of them have low efficacy. Since the advanced therapies availability, cell-based therapies offer a new all-encompassing approach^[2].

Mesenchymal stem cells (MSCs) are multipotent and self-renewing stem cells derived from bone marrow, adipose tissue, and other mesenchymal tissues, which can be induced to differentiate into bone marrow, cartilage, muscle, lipid, myocardial cells, glial cells and neurons^[3,4]. MSCs have some features that make them useful in cell therapy research. These are easy to isolate and expand rapidly after a short period of dormancy^[5]. They are free of ethical issues associated with the harvesting of embryonic stem cells^[6]. Also, it is considered that MSCs are "immunoprivileged" because they do not express Major Histocompatibility Complex class II (MHC-II) on

their surface, associated with transplant rejections^[7], and this advantage allows its use as an autologous or allogenic form^[8]. Furthermore, MSCs produce several growth factors with paracrine actions that are believed to modulate the microenvironment of diseased tissues, promote survival and activate endogenous repair mechanisms^[9].

Due to this features MSCs have been used in several preclinical studies of retinal and optic nerve diseases, where they have demonstrated their properties of immunomodulation, neuroprotection and tissue repair^[10-13]. These properties support the clinical use of MSCs as an opportunity for tissue repair and regeneration in several neurodegenerative disorders. To remember, the stages of clinical trials for drugs in development can be divided into four phases. The main purpose of the first clinical stage, phase I, is to observe the tolerance and pharmacokinetic characteristics of the drug in the human body and to provide evidence to establish the phase II administration protocol. The purpose of phase II clinical trials is to evaluate the efficacy and safety of the drug in patients with the target indication. In phase III, the efficacy and safety of the drug in patients with the target indication is further validated, providing the basis of the evidence used for review during the drug registration and application process. The phase IV clinical trial, which takes place during the post marketing period, provides further evidence regarding the drug's efficacy and any emerging adverse reactions under conditions of real-life use in large numbers of patients^[14].

In this review, we summarize the latest progress of MSCs in human clinical trials for retinal and optic nerve diseases.

TISSUE SOURCES OF MSCS

Bone marrow is the first isolation source of MSCs following by umbilical cord and adipose tissue^[15]. Although bone marrow is the best source of obtaining MSCs, there are some aspects that reduced their use: Limited growth rate, differentiation capability depending on the donor age, and risk inherited to sample collection^[15]. Regarding to umbilical cord source to obtain MSCs, it is required an optimal protocol such as, time of recollection and process less than 16 h, as well as, volume collection higher than 30 mL to get a success culture^[16]. MSCs obtaining by adipose tissue source have a similar morphology and phenotype to the bone marrow source, but these cells have a higher capability of proliferation and adipose tissue samples are easier to collect from liposuction procedures^[17].

CRYOPRESERVATION OF MSCS

Cryopreservation consists on the interruption of cellular metabolism regulated by processes of freezing and thawing, maintaining a good functional and structural cellular state. To preserve a biological sample as long as possible, without losing their properties, cells are immersed in liquid nitrogen at extremely low temperature

(-196 °C), stopping the metabolic activity of the cells^[18].

Cryopreservation has been performed primarily for the purpose of preserving the hematopoietic stem cell populations for transplantation. Currently, the use of this procedure has been extended, allowing the preservation of the biological potential, and to retain the biological age at time of cryopreservation. In autologous patients, MSCs are collected and cryopreserved for later clinical use. In allogeneic patients, cryopreservation permits banking of cells for human leukocyte antigen typing and matching, facilitating the logistical transport of cellular products to transplant centers, and allowing enough time for the screening of transmissible diseases in the donated cells before transplantation^[19].

CLINICAL TRIALS USING MSCS

Today, there are ongoing clinical trials of advanced therapies' using MSCs in various retinal and optic nerve diseases. In these clinical trials the main route of administration is the intravitreal injection following by subretinal implant and then intravenous route. In all these studies it is used autologous stem cells from bone marrow or adipose tissue. On Table 1 it is shown all clinical trials finished and ongoing registered in clinicaltrials.gov and the International Clinical Trials Registry Platform, until today (Last search performed on 18 May 2016).

Clinical trials in retinal dystrophies: Retinitis pigmentosa and stargardt's disease

Retinitis pigmentosa (RP) includes some inherited diseases which are 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 due to the severe loss of rod and cone photoreceptors^[20]. The RP is one of the leading hereditary degenerative retinal diseases, affecting 1 in 4000 individuals^[20]. RP is characterized by the classic triad of decreased arteriolar diameter, pigment spicules deposits in the mid periphery of the retina and pallor of the papilla^[20].

Stargardt's disease (SD) is the most common form of inherited juvenile macular degenerations. Its prevalence worldwide is estimated to be 1 in 10000 individuals^[21]. Patients initially present with reduced central vision. The pathology is defined by the accumulation of lipofuscin in the apical zone of the RPE cells. The patients present decreased vision to legal blindness and secondary choroidal neovascularization, with bilateral gradual involvement of vision^[21].

There are nine clinical trials that use MSCs to treat this kind retinal dystrophies (6 for RP, 2 for SD and RP and 1 for RP and other diseases) (Table 1). Although most clinical trials are in recruitment phase, there are two completed to treat retinitis pigmentosa, both were held at Hospital das Clinicas (Medical school Ribeirao Preto, Sao Paulo) - (NCT01068561 phase I , NCT01560715 phase II). The cells used were autologous bone marrow-derived

MSCs, which were administered through intravitreal injection containing 10×10^6 cells/0.1 mL. The MSCs were obtained through aspiration of 10 mL bone marrow tissue from the posterior iliac crest and were separated by Ficoll-Hypaque gradient centrifugation. Regarding to the clinical trial NCT01068561 (phase I), there is a case reported^[22]. The case is about one recruited patient of this study, who had macular oedema associated with RP, which showed complete resolution of the oedema 7 d after injection, and the effect remained for one month of follow-up with optical coherence tomography. They concluded that the adult stem cells can restore the blood ocular barrier due their paracrine effects or by osmotic gradient allowing the absorption of macular oedema^[22]. The trial NCT01560715 (phase II) is completed and also have published results^[23], they concluded that the therapy with intravitreal use MSC can improve the quality of life of patients with RP, although the improvement is lost with time. Patient's improvement has been evaluated with vision-related quality of life test (NEI VFG-25) before therapy and 3 and 12 mo later. There was a statistically significant improvement 3 mo after treatment, whereas by 12th month there was no significant difference from baseline^[23].

At the hospital Virgen de la Arrixaca, Murcia (Spain), it is being carried out a phase I clinical trial with autologous bone marrow stem cells in patients with RP. This clinical trial continues recruiting patients. Regarding to the other clinical trials for RP and Stargardt's disease (NCT01531348, NCT017336059, NCT01914913, NCT02280135, NCT02709876 and NCT01518127), they are on phase I or I / II , and they are recruiting patients (Table 1).

Clinical trials in diabetic retinopathy and age macular degeneration

Diabetic retinopathy (DR) is a prevalent microvascular complication of diabetes, and remains the leading cause of preventable blindness in working-aged people (20-74 years)^[24]. About 30% all diabetics have signs of diabetic retinopathy, and 30% of these might have vision-threatening retinopathy, defined as severe retinopathy or macular edema^[25]. The current standard treatment for management of these disorders relies mainly on laser therapy, which is inherently destructive, or antiangiogenic therapy, both associated with unavoidable ocular/systemic side-effects^[25].

Age-related macular degeneration (AMD) is a progressive chronic disease of the central retina and a leading cause of vision loss worldwide, it accounts for 8% of all blindness worldwide and is the most common cause of blindness in developed countries^[26], particularly in people older than 60 years. Its prevalence is likely to increase as a consequence of exponential population ageing. There have been significant advances in the management of exudative AMD with the introduction of anti-angiogenesis therapy, and patients now have effective treatment options that can prevent blindness and, in many cases, restore vision^[27]. However antiangiogenic treatment doesn't stop the progression nor serves to treat dry AMD.

Table 1 Clinical trials for retinal and optic nerve diseases

Clinical trial	Condition	Cells	Route of administration	Dose	Estimated enrollment	Recruitment status	Study phase	Country	Start date
NCT01068561 ¹	Retinosis pigmentaria	ABMSC	Intravitreal injection	10 × 10 ⁶ cells/0.1 mL	5	Completed	I	Brazil	2010
NCT01531348	Retinosis pigmentaria	ABMMSC	Intravitreal injection	1 × 10 ⁶ cells/0.1 mL	10	Enrolling by invitation	I	Tailandia	2012
NCT01560715 ²	Retinosis pigmentaria	ABMSC	Intravitreal injection	10 × 10 ⁶ cells/0.1 mL	50	Recruiting	II	Brasil	2012
NCT01736059 ³	Retinosis pigmentaria, AMD, DR, VO	ABMSC	Intravitreal injection	3.4 × 10 ⁶ cells/0.1 mL	15	Enrolling by invitation	I	EEUU	2012
NCT01914913	Retinosis pigmentaria	ABMSC	-	-	15	Recruiting	I / II	India	2014
NCT02280135	Retinosis pigmentaria	ABMSC	Intravitreal injection	30 × 10 ⁶ cells/0.1 mL	10	Recruiting	I	Spain	2014
NCT02709876	Retinosis pigmentaria	ABMSC	Intravitreal injection	-	50	Recruiting	I / II	Arabia	2014
NCT01518127	Stargardt's disease and AMD	ABMSC	Intravitreal injection	10 × 10 ⁶ cells/0.1 mL	10	Recruiting	I / II	Brazil	2011
NCT01736059 ³	Stargardt's disease, AMD, DR, VO, RP	ABMSC	Intravitreal injection	3.4 × 10 ⁶ cells/0.1 mL	15	Recruiting	I	EEUU	2012
Carta al editor Act. Ophth ⁴	Diabetic retinopathy	ABMSC	Intravitreal injection	18 × 10 ⁷ cells/0.5 mL	1	Completed	I	Germany	2008
NCT01518842	Diabetic retinopathy	ABMSC	Intravitreal injection	2 × 10 ⁴ cells/0.1 mL	30	Unknown	I / II	Brasil	2011
IRCT201111291414N29	Diabetic retinopathy	ABMMSC	Intravenous	2 × 10 ⁶ cells/kg	20	Ongoing	I / II	Iran	2011
NCT01736059 ³	Diabetic retinopathy, VO, HRD	ABMSC	Intravitreal injection	3.4 × 10 ⁶ cells/0.1 mL	15	Recruiting	I	EEUU	2012
ChiCTR-ONC-16008055	Diabetic retinopathy	ASMSC	-	-	30	Recruiting	I / II	China	2013
NCT01518127	AMD, Stargardt's disease	ABMSC	Intravitreal injection	10 × 10 ⁶ cells/0.1 mL	10	Recruiting	I / II	Brasil	2011
NCT01736059 ³	AMD, DR, VO, HRD	ABMSC	Intravitreal injection	3.4 × 10 ⁶ cells/0.1 mL	15	Recruiting	I	EEUU	2012
NCT02016508	AMD	ABMSC	Intravitreal injection	-	1	Unknown	I / II	Egypt	2013
NCT02024269	AMD	AASC	Intravitreal injection	-	-	Withdrawn	I	EEUU	2013
NCT00787722	Neuromielitis óptica	AHSC	Intravenous	-	10	Recruiting	I	EEUU	2008
NCT01364246	Neuromielitis óptica	UC-MSC	Intravenous	-	20	Unknown	I / II	China	2010
NCT01339455	Neuromielitis óptica	AHSC	Intravenous	-	3	Ongoing	I / II	Canada	2011
NCT02249676	Neuromielitis óptica	ABMMSC	Intravenous	2 × 10 ⁶ cells/kg	15	Recruiting	II	China	2014
NCT02638714	Optic nerve atrophy	AHSC	-	-	100	Ongoing	I / II	Jordania	2013
NCT01834079	Optic nerve atrophy	ABMSC	Intrathecal	10 × 10 ⁷ cells/dose	24	Recruiting	I / II	India	2014
ChiCTR-TRC-14005093	Traumatic optic neuropathy	UC-MSC	Endonasal	-	70	Recruiting	I / II	China	2014
NCT02330978	Glaucoma	ABMMSC	Intravitreal injection	1 × 10 ⁶ cells/0.1 mL	10	Recruiting	I	Brasil	2014
NCT02144103	Glaucoma	AASC	Subtenon injection	0.5 mL	16	Enrolling by invitation	I	Russia	2014
NCT01920867 ⁵	Retinal diseases, Macular degeneration, HRD, OND, glaucoma	ABMSC	Retrobulbar, subtenon, intravenous, intravitreal and intraocular injection	1.2 × 10 ¹² cells/15 mL	300	Recruiting	I	Estados Unidos	2013

Last search performed in Clinicaltrials.gov and the International Clinical Trials Registry Platform, 18 May 2016. ¹Case reported^[22], ²Case reported^[23], ³Case reported^[30], ⁴Case reported^[28], ⁵Case reported^[36,37]. ABMSC: Autologous bone-marrow stem cells; ABMMSC: Autologous bone-marrow mesenchymal stem cells; ASMSC: Autologous stromal mesenchymal stem cells; AASC: Autologous adipose stem cells; AHSC: Autologous hematopoietic stem cells; UC-MSC: Umbilical cord mesenchymal stem cells; AMD: Age-related macular degeneration; DR: Diabetic retinopathy; HRD: Hereditary retinal diseases; OND: Optic nerve diseases; RP: Retinitis pigmentosa; VO: Vein occlusions.

Thus, new approaches like stem cell therapy are needed.

The use of bone marrow derived stem cells (BMDSC) therapy for the DR has been evaluated^[28,29] and there are five ongoing clinical trials (NCT01518842,

IRCT 201111291414N29, NCT01736059, ChiCTR-ONC-16008055 and NCT01920867) (Table 1). In relation to this therapy for the AMD, it has been evaluated in four (4) ongoing clinical trials (NCT02016508, NCT01

920867, NCT01736059 y NCT01518127). One of them (NCT01736059) has published results in the AMD patients^[30]. Bone marrow stem cells used in these clinical trials was harvested from the patient's own iliac crest (autologous use) with an average final volume of 50 mL (20-100 mL). Then, mononuclear cells were separated by Ficoll-gradient centrifugation. The dose of cells is between 2×10^4 - 1.8×10^8 suspended in 0.1 mL buffered saline solution. A trial using adipose derived stems cells (ADSC) has been withdrawn prior to enrollment (NCT02024269), however they don't explain the reasons.

Results of stem cell-treatment for the DR are limited to the report on two patients. A 43-year-old patient with very advanced atrophy of the retina and optic nerve caused by the DR and vision limited to defective light perception, after cell treatment patient have improvement but no signs of any side-effects, such as inflammation or infection^[28]. The other reports a patient with macular oedema associated with macular ischemia, and describe the decrease of macular oedema and the improvement of retinal function after intravitreal injection of BMDSC^[29].

Moreover, the only clinical results of MSCs therapy for the AMD^[30] describes two patients who start from a visual acuity (VA) of 20/200. After intravitreal injection, they had an improvement with its new VA of 20/80 and 20/160. The patient with VA 20/80 kept it during first six months and the other patient with VA 20/160 worsened to its initial state of 20/200. A slight growth of extrafoveal geographic atrophy in both eyes of both patients was detected by fluorescein angiography. The results of electroretinography showed a slight worsening of the macular function of both eyes that could be attributed to the disease progression. In analysis by OCT hyperdense deposits were evident within the retinal layers after a month of therapy that correspond in size with CD34⁺ cells, however, more studies are needed to prove whether it corresponds to intraretinal incorporation of CD34⁺ cells. The results suggest that this cell therapy in patients with the AMD, especially in advanced stages, would not stop the progression^[30].

Clinical trials of MSCs for optic neuropathies

Optic neuropathies are characterized by damage to the optic nerve and they can be due to various causes, such as glaucoma, autoimmune diseases, inflammation, infections, traumas, ischemia or compression. Glaucoma is the most common cause of optic nerve-related visual loss in adults, followed by nonarteritic anterior ischaemic optic neuropathy (NAION)^[31]. The treatment for glaucoma is based on drugs and surgery that reduce intraocular pressure, whereas there is no treatment for NAION, nor to reverse the process nor for its recurrence^[32]. Traumatic optic neuropathy is a cause of severe visual loss and it has no reliable treatment^[33]. Neuromyelitis optica, also known as Devic's disease, is an autoimmune, demyelinating disorder which causes optic neuritis. Its prevalence is about 1-3/100000^[34]. Nowadays neuromyelitis optica treatment is based in corticosteroids and plasma exchange

for the acute attacks and immunosuppressant drugs for the maintenance therapy^[35].

Currently, there are two clinical trials at phase I using MSCs to treat glaucoma (NCT02330978 and NCT02144103), both of them are recruiting patients at the moment. One of them is being held at Medical School Ribeirao Preto, University of São Paulo, Brazil (NCT02330978), and the other one in Burnasyan Federal Medical Biophysical Center, Russia (NCT02144103). The Brazilian one uses an intravitreal injection of 10^6 autologous bone marrow derived mesenchymal stem cells (BMMSCs) to assess the safety of the procedure and how it improves visual field and visual acuity. The Russian one uses a sub Tenon administration of autologous adipose-derived regenerative cells that have been extracted from the patient's front abdominal wall. There are still no published results of these studies.

In the SCOTS clinical trial (NCT01920867), held at the Johns Hopkins Hospital, United States, there is one case reported of autoimmune optic neuropathy^[36]. They made a vitrectomy and intra-optic injection of autologous bone marrow stem cells (BMSCs) in one patient's eye and retrobulbar, sub Tenon and intravitreal injection in the other eye, improving the visual acuity, macular thickness and fast retinal nerve fiber layer thickness. In this clinical trial there is also a case reported of idiopathic bilateral optic neuritis^[37]. The patient received a retrobulbar injection, sub Tenon injection and intravitreal injection of autologous BMSCs for the right eye (OD), and vitrectomy and direct intra-optic nerve injection of autologous BMSCs for the left eye (OS), followed by intravenous infusion. After this procedure, there was an improvement in visual acuity in both eyes and remained stable at the 12 mo post-operative^[37].

For neuromyelitis optica there is one active clinical trial at Foothills Medical Centre, University of Calgary, Canada (NCT01339455), two recruiting patients at Northwestern University, United States (NCT00787722), one ongoing clinical trial in Tianjin Medical University General Hospital, China (NCT02249676), and one with unknown status at Nanjing University Medical College Affiliated Drum Tower Hospital, China (NCT01364246). Most of them, active and recruiting clinical trials, use immunosuppressive treatment followed by an autologous hematopoietic stem cells transplantation. While the Nanjing University uses human umbilical cord mesenchymal stem cells transplantation. In this clinical trial (NCT01364246), 5 patients were followed for 18 mo including evaluation of Expanded Disability Status Scale (EDSS) levels, clinical course, magnetic resonance imaging (MRI) characteristics and adverse events. and they reported an improvement in the symptoms and signs of neuromyelitis optica in four out of five patients treated^[38]. There is another clinical trial for secondary progressive multiple sclerosis with evidence of optic nerve involvement (NCT00395200), in which patients were treated with autologous bone marrow stem cells transplantation and that resulted in an increase in visual acuity, visual evoked response latency, and optic nerve

area^[39]. Some individual cases with neuromyelitis optica treated with allogeneic hematopoietic stem cells have been reported^[40].

Traumatic optic neuropathy is being studied in a clinical trial in China, by the Cell Biotherapy Center, Daping Hospital, Third Military Medical University (ChiCTR-TRC-14005093). Currently, they are recruiting patients and will use human umbilical cord derived mesenchymal stem cells transplantation. There are still no results.

There are also clinical trials for optic neuropathies, without considering what caused it. One of them is currently active (NCT02638714) and is held by Stem Cells of Arabia, Jordan. The patients will be treated with a transplantation of purified adult autologous bone marrow derived CD34⁺, CD133⁺, and CD271⁺ stem cells due to their diverse potentialities to differentiate into specific functional cell types to regenerate damaged optic nerves, supporting tissues and vasculature. They will use clinical-grade purification system (CliniMACS) and Microbeads to purify the target cell populations. There is another clinical trial on optic atrophy, currently recruiting patients (NCT01834079) in Chaitanya Hospital in Pune, India. Patients will receive three intrathecal injections of 100 million autologous bone marrow derived mononuclear cells per dose at intervals of 7 d. There are no results posted yet of these studies.

DISCUSSION

Advances in the knowledge of neuroprotective, immunomodulative and regenerative properties of MSCs are continuously generated by several preclinical studies *in vitro* and *in vivo* in animal models of various neurodegenerative diseases, including optic nerve and retinal diseases. It has given the opportunity to perform the translation of treatment approaches to the clinical practice. Since 2008, several first steps, projecting new treatment approaches, have been taken regarding the use of cell therapy in patients with neurodegenerative pathologies of optic nerve and retina. It is about Phase I or I / II clinical trials, which have as main objective the safety assessment of MSCs using various routes of administration, where the main route used is the intravitreal injection.

Nevertheless, of the 24 clinical trials registered on clinicaltrials.gov, there are only 2 clinical trials finished, 3 are ongoing, 15 are in recruiting patients phase, 3 are in unknown state and 1 clinical trial has been withdrawn without knowing the reasons for this decision. Most of the results published to date, are reduced to 6 cases reported in various retinal/optic nerve pathologies, their number of patients is very low, and these are exceptional cases, so, there is not enough evidence to get any valid and scientific conclusion.

Furthermore, most of these clinical trials use autologous cells, obtaining by bone marrow aspirates, so the final content to be administered is a concentrate of mononuclear cells, containing a very small percentage of MSCs (0.1%)^[15], only four clinical trials use a specific concentration of MSCs without added another cell

type. It is surprising that, although MSCs derived from adipose tissue are easier to obtain and in a higher concentration^[17], there are only 2 clinical trials using this cell type, and one of them has been withdrawn without explanation. Regarding the use of allogenic MSCs, is limited to 2 clinical trials, which use MSCs derived from umbilical cord, however, it is not known whether their patients will receive immunosuppressive therapy.

Regarding to cell dose used in various clinical trials, there is a great variation from one to another. There is no consensus regarding the calculation of cell dose for the use of these cells through intravitreal injection. The clinical trials which use mononuclear cells aspirate, the doses are usually high (between 3×10^6 cells/0.1 mL and 30×10^6 cells/0.1 mL), whereas clinical trials using a concentrated purified of MSCs, doses are lower (1×10^6 cells/0.1 mL). However, the information collected by clinicaltrials.gov and the International Clinical Trials Registry Platform not specify the cell dose calculation or the cell production process.

CONCLUSION

It is important to know the development of cell therapy in relation to its use in the clinical practice. However, it is also important to recognize that, there is still a long way to go to reach clinical trials phase III-IV. One of the factors necessary to move forward is to establish unified criteria for the dose to be used, another important factor is the use of only MSCs without another cells added, because MSCs are immunoprivileged cells, and do not produce rejection. It is also important to use more frequently allogeneic MSC associated with cryopreservation processes. It can be the key to a better bioavailability of these cells, getting greater advantages of MSCs derived from adipose tissue, which are easier in obtaining and production. Therefore, it is necessary to continue preclinical and clinical studies to improve this new therapeutic tool.

Limitations

Most of the clinical trials using MSCs are in I / II phase, recruiting patients or ongoing. The information available in clinicaltrials.gov about the procedure obtaining cells or the dose used in each clinical trial is not described in all cases. Hence, there are not enough published results to have scientific evidence about the use of these cells in retinal and optic nerve diseases.

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Basic Study

Gene expression and pathway analysis of *CTNNB1* in cancer and stem cells

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Informed consent statement: Written informed consent from each patient were obtained.

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Data sharing statement: The microarray data for mesenchymal stem cells and diffuse-type gastric cancer are available to the public in NCBI's Gene Expression Omnibus (GEO) database and are accessible via GEO Series accession number GSE7888 and GSE42252, respectively.

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Abstract

AIM

To investigate β -catenin (CTNNB1) signaling in cancer and stem cells, the gene expression and pathway were analyzed using bioinformatics.

METHODS

The expression of the catenin β 1 (CTNNB1) gene, which codes for β -catenin, was analyzed in mesenchymal stem cells (MSCs) and gastric cancer (GC) cells. Beta-catenin signaling and the mutation of related proteins were also analyzed using the cBioPortal for Cancer Genomics and HOMology modeling of Complex Structure (HOMCOS) databases.

RESULTS

The expression of the CTNNB1 gene was up-regulated in GC cells compared to MSCs. The expression of EPH receptor A8 (EPHA8), synovial sarcoma translocation chromosome 18 (SS18), interactor of little elongation

complex ELL subunit 1 (ICE1), patched 1 (PTCH1), mutS homolog 3 (MSH3) and caspase recruitment domain family member 11 (CARD11) were also shown to be altered in GC cells in the cBioPortal for Cancer Genomics analysis. 3D complex structures were reported for E-cadherin 1 (CDH1), lymphoid enhancer binding factor 1 (LEF1), transcription factor 7 like 2 (TCF7L2) and adenomatous polyposis coli protein (APC) with β -catenin.

CONCLUSION

The results indicate that the epithelial-mesenchymal transition (EMT)-related gene *CTNNB1* plays an important role in the regulation of stem cell pluripotency and cancer signaling.

Key words: β -catenin; *CTNNB1*; Epithelial-mesenchymal transition; Mesenchymal stem cell; Stem cell

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Core tip: β -catenin signaling consists of several pathway cascades, such as those that are involved in pluripotent stem cell generation and cancer. Several genes, including *EPHA8*, *SS18*, *ICE1*, *PTCH1*, *MSH3* and *CARD11*, are mutated along with *CTNNB1*. The expression of the *CTNNB1*, *CDH1*, *MYC*, *LEF1* and *TCF7L2* genes, which are related to the *CTNNB1* network, is up-regulated in diffuse-type GC cells compared to MSCs. 3D complex structures for β -catenin (CTNB1_HUMAN) with LEF_MOUSE and TCF7L2_HUMAN were found using the HOMCOS database. The EMT-related gene *CTNNB1* plays an important role in pluripotent stem cell signaling and cancer signaling.

Tanabe S, Kawabata T, Aoyagi K, Yokozaki H, Sasaki H. Gene expression and pathway analysis of *CTNNB1* in cancer and stem cells. *World J Stem Cells* 2016; 8(11): 384-395 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v8/i11/384.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v8.i11.384>

INTRODUCTION

Changes in the phenotypes of cancer and stem cells are related to changes in gene expression and protein signaling. This study aims to reveal the β -catenin (*CTNNB1*) regulation in diffuse-type gastric cancer (GC) cells and mesenchymal stem cells (MSCs). Wnt/ β -catenin signaling is necessary for epithelial-mesenchymal transitions (EMT)^[1]. Stem cell division is strongly correlated with cancer risk, and this highlights the importance of molecular signaling in stem cells and cancer cells^[2]. Epigenetics and stem cell functions are regulated by several exogenous stimuli, including cell-cell and cell-matrix interactions^[3]. To ensure the safety of therapeutic stem cell applications in terms of stem cell modification, an understanding of the regulation of the stem cells and their niche is necessary^[4]. In the case of bone metastasis, the tissue-specific stromal

response for prostate cancer can be identified by a molecular signature for which a novel mechanism has been revealed in hematopoietic and prostate epithelial stem cell niches^[5].

Cancer stem cell (CSC) maintenance requires hypoxia-inducible factor (HIF)- α transcription factors and the inhibitor of DNA binding 2 (ID2)^[6]. The down-regulated expression of ID2 is associated with a poor prognosis in hepatocellular carcinoma^[7].

Because the compendium of gene expression, chromosomal copy number and sequencing data from human cancer cell lines, which is called the Cancer Cell Line Encyclopedia (CCLE), has revealed that genomic data are capable of predicting anti-cancer drug sensitivity, molecular and network analyses should be carried out^[8]. It has been reported that cadherin 1 (*CDH1*) is up-regulated in diffuse-type GC cells compared to MSCs^[9]. However, *CDH2* was down-regulated in diffuse-type GC cells compared to MSCs; this provides a useful indicator - the ratio of *CDH2* to *CDH1* expression - to distinguish the mesenchymal and epithelial phenotypes of the cells^[9]. It has been reported that catenin β 1 (*CTNNB1*) is mutated in hepatocellular carcinoma^[10,11]. To further elucidate the EMT phenotype and the molecules that are involved in β -catenin signaling in cancer, the *CTNNB1* network and the β -catenin binding partners have been investigated in this report using bioinformatics tools such as microarray analysis and databases.

MATERIALS AND METHODS

Gene expression analysis of MSCs and diffuse-type GC cells

Gene expression in MSCs ($n = 12$) and diffuse-type GC cells ($n = 5$) was analyzed using Human Genome U133 Plus 2.0 microarrays, as previously described^[9,12]. In brief, total RNA was purified from the cells, biotinylated and hybridized to microarrays. The signal intensity of each gene transcript was analyzed and compared between MSCs and diffuse-type GC cells. The microarray data for MSCs and diffuse-type GC cells are available to the public in NCBI's Gene Expression Omnibus (GEO) database and are accessible via GEO Series accession number GSE7888 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE7888>) and GSE42252 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42252>), respectively^[9,12].

Diffuse-type GC tissues

Diffuse-type GC tissues were originally provided by the National Cancer Center Hospital after obtaining written informed consent from each patient and approval by National Cancer Center Institutional Review Board. All cancer specimens were reviewed and classified histopathologically according to the Japanese Classification of Gastric Cancer. Tissue specimens were immediately frozen with liquid nitrogen after surgical extraction, and stored at -80°C until microarray analysis^[9,13]. The existing data

Table 1 3D complex structures of β -catenin (CTNNB1) and interacting proteins

pdb_id	β -catenin (CTNNB1)				Proteins that interact with β -catenin				
	ChainID	Length	UniProtID	Molecule	ChainID	Length	UniProtID	Contact protein name	Regulation of gene expression in GC cells compared to MSCs
1th1	B	513	CTNB1_HUMAN	APC	D	54	APC_HUMAN	Adenomatous polyposis coli protein	Not changed/-
1qz7	A	524	CTNB1_HUMAN	AXIN1	B	17	AXN_XENLA	Axin-1	-
3sl9	B	165	CTNB1_HUMAN	BCL9	D	23	BCL9_HUMAN	B-cell CLL/lymphoma 9 protein	-
1i7w	C	509	CTNB1_MOUSE	CDH1	D	60	CADH1_MOUSE	Cadherin-1	Up-regulated
1m1e	A	512	CTNB1_MOUSE	CTNNBIP1	B	65	CNBP1_HUMAN	Beta-catenin-interacting protein 1	-
3oux	A	503	CTNB1_MOUSE	LEF1	B	47	LEF1_MOUSE	Lymphoid enhancer-binding factor 1	Up-regulated
3tx7	A	504	CTNB1_HUMAN	NR5A2	B	218	NR5A2_HUMAN	Nuclear receptor subfamily 5 group A member 2	-
1g3j	A	439	CTNB1_HUMAN	TCF7L1	B	34	T7L1A_XENLA	Transcription factor 7-like 1-A	-
1jdh	A	508	CTNB1_HUMAN	TCF7L2	B	38	TF7L2_HUMAN	Transcription factor 7-like 2	Up-regulated
1dow	B	32	CTNB1_MOUSE	CTNNA1	A	205	CTNA1_MOUSE	Catenin alpha-1	Not changed/-
4ons	D	56	CTNB1_MOUSE	CTNNA2	C	230	CTNA2_MOUSE	Catenin alpha-2	-

already available to the public were analyzed in the article.

Analysis of cancer genomics using cBioPortal

The cancer genomics data analysis was performed relative to *CTNNB1* using the cBioPortal for Cancer Genomics (<http://www.cbioportal.org>)^[14,15]. The term "CTNNB1" was searched in the cBioPortal for Cancer Genomics database, and a cross-cancer alteration summary was obtained for *CTNNB1*. A study on stomach adenocarcinoma was further analyzed for enrichments^[16]. Genes with mutations that were enriched in samples that contained altered *CTNNB1* were selected in the cBioPortal for cancer genomics for further study.

3D complex structures

3D complex structures were searched in the HOMology modeling of COMplex Structure (HOMCOS) database (<http://homcos.pdbj.org>) using the search engine that was provided by the VaProS server (<http://pford.info/vapros>)^[17]. The UniProtID "CTNB_HUMAN" was input as the query for the "searching contact molecule" field of the HOMCOS. Only close homologues (sequence identity > 95%) were selected. The complex structures that were found were superimposed using the MATRAS program^[18].

Statistical analysis

The data were expressed as the mean \pm SE. For the statistics, Student's *t* test was used. *P* < 0.01 was considered as statistically significant.

RESULTS

Expression of EMT-related genes in MSCs and diffuse-type GC cells

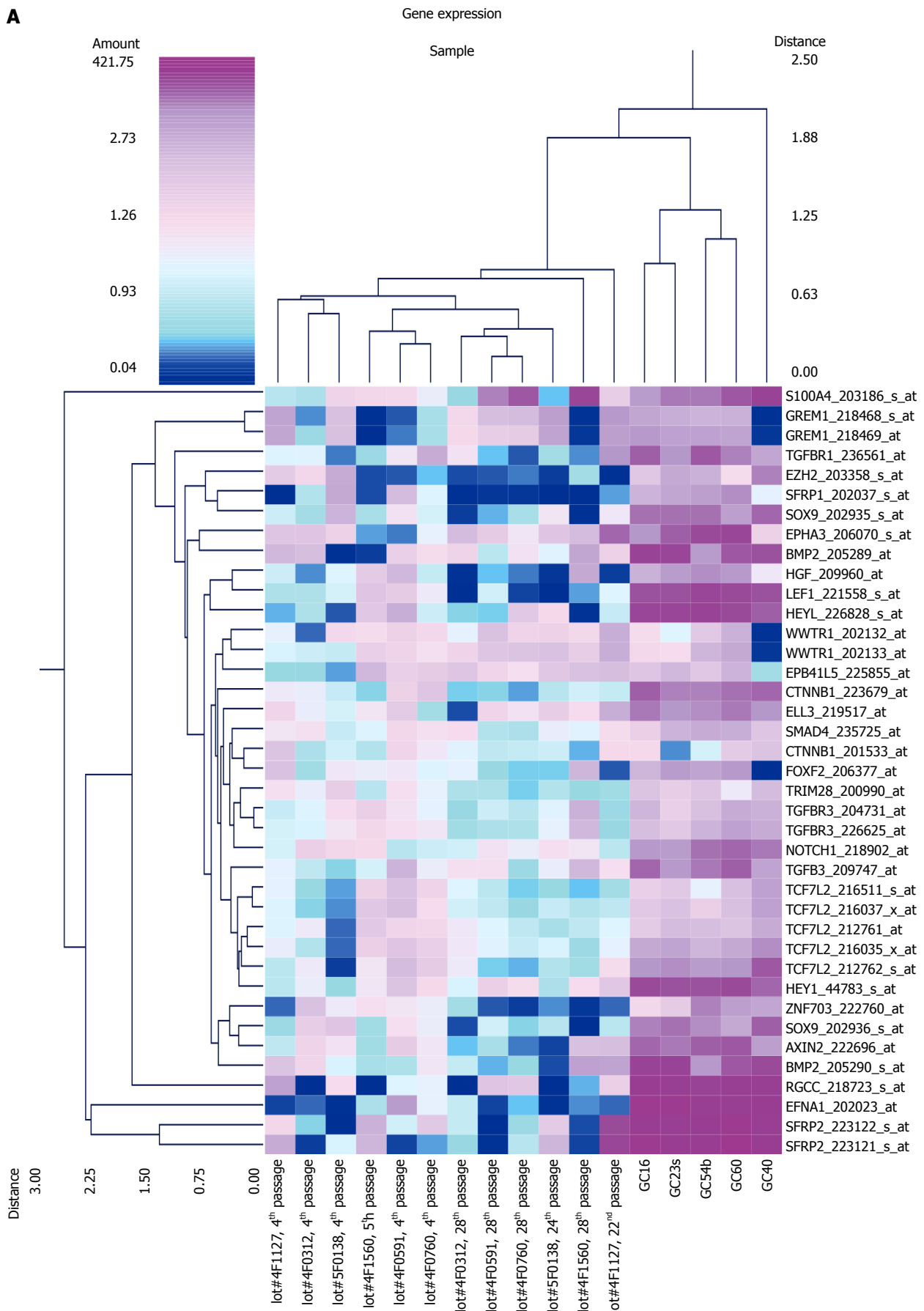
The expression of EMT-related genes in MSCs and

diffuse-type GC cells is shown in Figure 1. The genes for which probe sets included the "EMT" term in the Gene Ontology (GO) Biological Process field were selected as EMT-related genes. The average signal intensity for early-stage MSCs, late-stage MSCs, or GC cells was greater than 500. Panel A shows the results of a cluster analysis of 39 probe sets that were up-regulated in diffuse-type GC cells compared to early-stage MSCs (*n* = 6 in early-stage MSCs, *n* = 6 in late-stage MSCs, *n* = 5 in GC). Panel B shows the results of a cluster analysis of 46 probe sets that were down-regulated in diffuse-type GC cells compared to early-stage MSCs (*n* = 6 in early-stage MSCs, *n* = 6 in late-stage MSCs, *n* = 5 in GC). To evaluate *CTNNB1* expression in cancer and stem cells, the expression of the *CTNNB1* gene was compared in MSCs and diffuse-type GC cells, and the results indicate that *CTNNB1* is up-regulated in GC cells (Figure 2). One of the probe sets was up-regulated more than 8-fold over its expression level in MSCs, whereas the other probe sets showed no increases in expression in GC cells compared to MSCs.

3D complex structures of β -catenin

To verify and explore protein-protein interactions with β -catenin, 3D complex structures of β -catenin were found using the HOMCOS database (<http://homcos.pdbj.org>)^[17] and are summarized in Table 1. Figure 3 shows the superimposed 3D structure of the complex. Most of the proteins bind to the inner concave surface of the armadillo repeat region of β -catenin by using their 40-60 residue length extended peptides [adenomatous polyposis coli protein (APC), E-cadherin 1 (CDH1), catenin beta interacting protein 1 (CTNNBIP1), lymphoid enhancer binding factor 1 (LEF1), transcription factor 7 like 1 (TCF7L1) and transcription factor 7 like 2 (TCF7L2)].

A



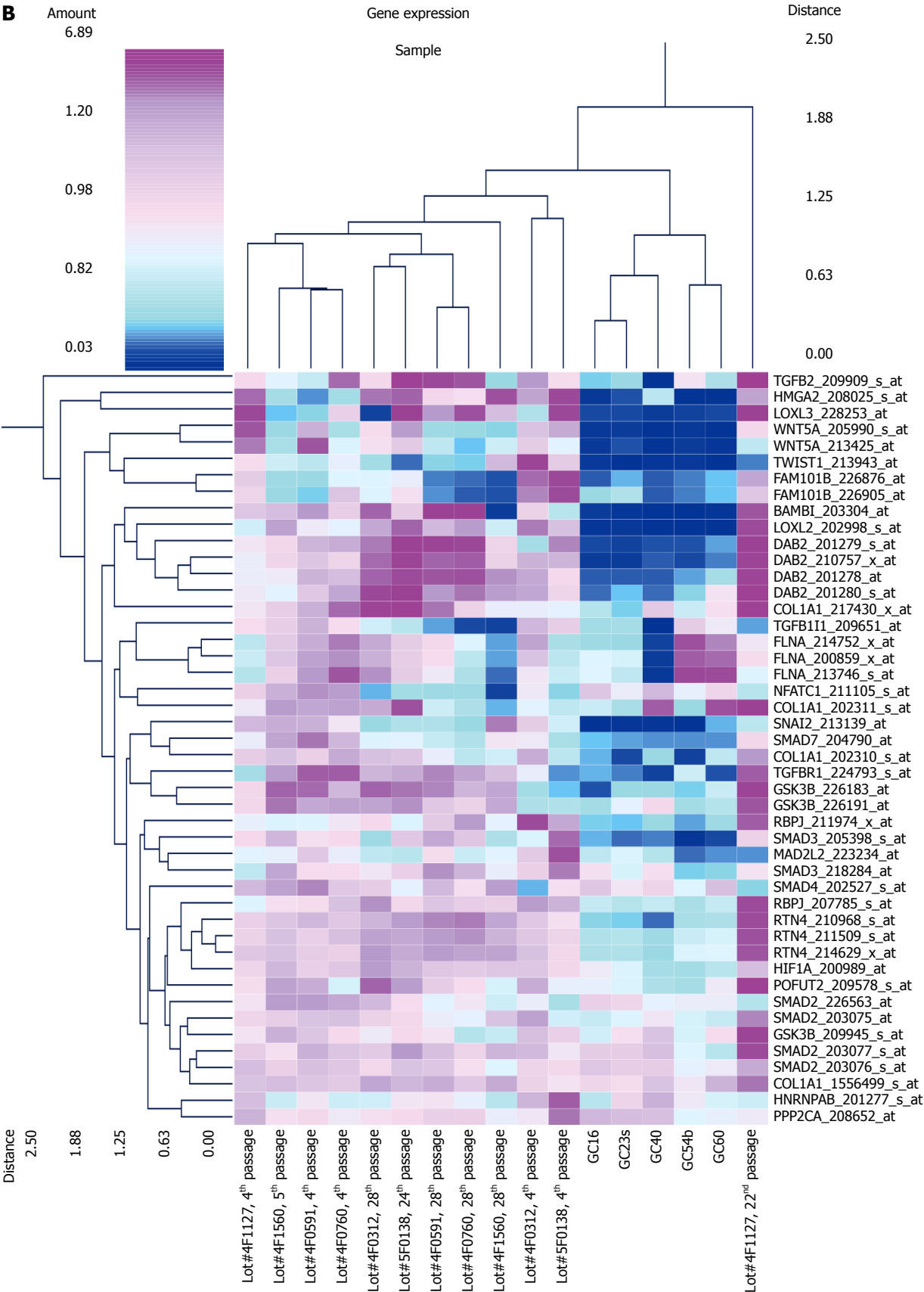


Figure 1 Expression of epithelial-mesenchymal transition-related genes in mesenchymal stem cells and diffuse-type gastric cancer cells. Cluster analysis of gene expression in mesenchymal stem cells (MSCs) and diffuse-type gastric cancer (GC) cells. A: The result of the cluster analysis of 39 probe sets that were up-regulated in diffuse-type GC cells compared to early-stage MSCs ($n = 6$ in early-stage MSCs, $n = 6$ in late-stage MSCs, $n = 5$ in GC); B: The result of the cluster analysis of 46 probe sets that were down-regulated in diffuse-type GC cells compared to early-stage MSCs ($n = 6$ in early-stage MSCs, $n = 6$ in late-stage MSCs, $n = 5$ in GC). The probe sets with epithelial to mesenchymal transition in the Gene Ontology Biological Process were selected (the average signal intensity in early-stage MSCs, late-stage MSCs, or GC cells is greater than 500).

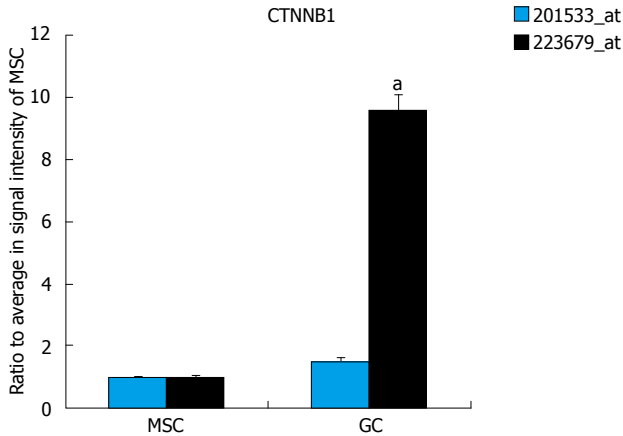


Figure 2 *CTNNB1* expression in mesenchymal stem cells and diffuse-type gastric cancer cells. *CTNNB1* gene expression was up-regulated in GC cells compared to MSCs. The signal intensity of probe set ID 223679_at was up-regulated more than 8-fold in GC cells compared to MSCs, whereas the signal intensity in probe set ID 201533_at was unchanged ($n = 12$ in MSC, $n = 5$ in GC, $^aP < 0.01$ in t test). GC: Gastric cancer; MSCs: Mesenchymal stem cells.

CDH1 and B-cell CLL/lymphoma 9 (BCL9) bind to the N-terminal region of the repeat that has the alpha-helical peptides. The nuclear receptor subfamily 5 group A member 2 (NR5A2) ligand binding domain binds to the middle of the armadillo repeat region. Of these binding factors, the transcription of the *CDH1*, *LEF1* and *TCF7L2* genes was up-regulated in GC cells (Table 1). It has been reported that a small molecule antagonist of the β -catenin/T-cell transcription factor 4 [TCF4; official name is transcription factor 7 like 2 (TCF7L2)] interaction inhibits self-renewal of CSCs and suppresses tumorigenesis^[19]. The 3D complex structures of β -catenin and TCF7L2 are available^[20,21]. The complex structure of NR5A2 has also been reported^[22]. NR5A2 (or liver receptor homolog-1; LRH1) is a member of the nuclear hormone receptor family of transcription factors that play essential roles in development, metabolism, and cancer and are implicated in Wnt/ β -catenin signaling^[22]. NR5A2 is essential for the early development and maintenance of pluripotent mouse embryonic stem (ES) cells^[22,23]. Network models for *CTNNB1*, the Wnt signaling pathway, Hippo signaling pathway and adherens junction signaling in cancer are shown in Figure 4. *CTNNB1* binds to CDH1 near the cellular membrane or to TCF to transcribe anti-apoptotic or pro-proliferation genes, such as SRY-box 2 (*SOX2*) or v-myc avian myelocytomatosis viral oncogene homolog (*MYC*) (Figure 4). Wnt stimulation prevents glycogen synthase kinase 3 beta (GSK3 β) from phosphorylating *CTNNB1* and leads to *CTNNB1* translocation into the nucleus to induce transcription. The 3D complex structure (PDB code: 1m1e) clearly shows how CDH1 binds to *CTNNB1* in the mouse model.

***CTNNB1* pathway (Kyoto Encyclopedia of Genes and Genomes)**

CTNNB1 is listed in 21 pathways in Kyoto Encyclopedia of Genes and Genomes (KEGG), including the Rap1 signaling pathway, Wnt signaling pathway, Hippo signaling

pathway, focal adhesion regulation, adherens junction regulation, tight junction regulation, signaling pathways that regulate the pluripotency of stem cells, leukocyte transendothelial migration, melanogenesis, the thyroid hormone signaling pathway; bacterial invasion of epithelial cells, pathogenic *Escherichia coli* infection, HTLV-I infection, and various cancer pathways. The following conditions use the aforementioned pathways and are also thus implicated: Proteoglycans in cancer, colorectal cancer, endometrial cancer, prostate cancer, thyroid cancer, basal cell carcinoma, and arrhythmogenic right ventricular cardiomyopathy (ARVC) (http://www.genome.jp/dbget-bin/www_bget?hsa:1499). The inhibition of GSK3 β kinase activates β -catenin, which stimulates endoderm induction via the degradation of Tcf7l1 and forkhead box A2 (FoxA2) expression^[24]. Wnt signaling induces intracellular β -catenin signaling via GSK3 β kinase inhibition and dephosphorylation of β -catenin^[25-28]. The inhibition of β -catenin decreases proliferation and induces apoptosis in the mantle cell lymphoma cell line^[29]. Noncanonical Wnt signaling is activated in circulating tumor cells from the prostate that are anti-androgen-resistant^[30].

Mutations in *CTNNB1* and related genes (cBioPortal: Stomach adenocarcinoma)

The Cancer Genome Atlas Research Network project has indicated that there is a characteristic molecular signature for ras homolog family member A (*RHOA*) mutations in diffuse type stomach adenocarcinoma^[16]. Two-hundred and ninety-five primary gastric adenocarcinomas have been investigated, and mutations in *RHOA* have been enriched in genomically stable subtype, diffuse-type GC cells^[16]. The analysis with cBioPortal showed that *CTNNB1* was altered in 24 (8%) of 287 cases/patients in stomach adenocarcinoma: 4 amplifications, 2 deep deletions, 12 missense mutations, 5 truncating mutations and 1 inframe mutation. Several gene mutations occurred concurrently with *CTNNB1* alterations in stomach adenocarcinoma (Table 2). The development of mutations in EPH receptor A8 (*EPHA8*), synovial sarcoma translocation chromosome 18 (*SS18*), interactor of little elongator complex ELL subunit 1 (*ICE1*), patched 1 (*PTCH1*), mutS homolog 3 (*MSH3*) and caspase recruitment domain family member 11 (*CARD11*) occurred alongside the *CTNNB1* alterations (Table 2). Of the mutated genes, *PTCH1* expression was up-regulated in GC cells compared to MSCs (Table 2). The GO of the mutated genes is shown in Table 3. *EPHA8* possesses kinase activity, *SS18* is involved in cell morphogenesis, *ICE1* may play a role in positive regulation of intracellular protein transport, *PTCH1* is involved in morphogenesis and cell growth, *MSH3* is involved in mismatch repair, and *CARD11* regulates B cell proliferation, apoptosis and NF- κ B signaling, according to GO biological process (Table 3). GO biological process terms in Table 3 are based on Affymetrix annotation (<http://www.affymetrix.com/estore/>) and gene information in NCBI (<http://www.ncbi.nlm.nih.gov/>).

β -catenin signaling model

Several β -catenin-binding proteins, such as LEF1 or

Table 2 Genes mutated along with the *CTNNB1* alteration

Gene symbol	Gene title	Cytoband	Mutation percentage		Log ratio	P-value	Ratio of GC cells to MSCs
			In altered group	In unaltered group			
EPHA8	EPH receptor A8	1p36.12	29.17%	2.28%	3.68	1.45E-05	Signal intensity is low
SS18	Synovial sarcoma translocation Chromosome 18	18q11.2	16.67%	0.00%	> 10	3.84E-05	0.6 1.4
ICE1	Interactor of little elongator complex ELL subunit 1	5p15.32	33.33%	4.56%	2.87	4.74E-05	1.5
PTCH1	Patched 1	9q22.3	29.17%	3.42%	3.09	8.16E-05	16.6
MSH3	MutS homolog 3	5q14.1	20.83%	1.14%	4.19	1.28E-04	Signal intensity is low
CARD11	Caspase recruitment domain family, member 11	7p22	29.17%	4.18%	2.8	2.03E-04	Signal intensity is low

Table 3 Gene ontology of mutated genes along with *CTNNB1* alteration

Gene symbol	Gene ontology biological process
EPHA8	Protein phosphorylation // substrate-dependent cell migration // cell adhesion // transmembrane receptor protein tyrosine kinase signaling pathway // multicellular organismal development // nervous system development // axon guidance // phosphorylation // neuron remodeling // peptidyl-tyrosine phosphorylation // regulation of cell adhesion // neuron projection development // regulation of cell adhesion mediated by integrin // positive regulation of MAPK cascade // positive regulation of phosphatidylinositol 3-kinase activity // protein autophosphorylation // ephrin receptor signaling pathway
SS18	Microtubule cytoskeleton organization // cell morphogenesis // transcription, DNA-templated // regulation of transcription, DNA-templated // cytoskeleton organization // response to drug // positive regulation of transcription from RNA polymerase II promoter // ephrin receptor signaling pathway
ICE1	Positive regulation of intracellular protein transport // positive regulation of protein complex assembly // positive regulation of transcription from RNA polymerase III promoter // snRNA transcription from RNA polymerase II promoter // snRNA transcription from RNA polymerase III promoter
PTCH1	Negative regulation of transcription from RNA polymerase II promoter // branching involved in ureteric bud morphogenesis // neural tube formation // neural tube closure // heart morphogenesis // signal transduction // smoothened signaling pathway // smoothened signaling pathway // regulation of mitotic cell cycle // pattern specification process // brain development // negative regulation of cell proliferation // epidermis development // regulation of smoothened signaling pathway // response to mechanical stimulus // organ morphogenesis // dorsal/ventral pattern formation // response to chlorate // positive regulation of cholesterol efflux // response to organic cyclic compound // protein processing // spinal cord motor neuron differentiation // neural tube patterning // dorsal/ventral neural tube patterning // neural plate axis specification // embryonic limb morphogenesis // mammary gland development // response to estradiol // response to retinoic acid // regulation of protein localization // limb morphogenesis // hindlimb morphogenesis // regulation of growth // negative regulation of multicellular organism growth // regulation of cell proliferation // response to drug // glucose homeostasis // negative regulation of sequence-specific DNA binding transcription factor activity // keratinocyte proliferation // negative regulation of osteoblast differentiation // negative regulation of smoothened signaling pathway // negative regulation of smoothened signaling pathway // negative regulation of epithelial cell proliferation // negative regulation of cell division // pharyngeal system development // mammary gland duct morphogenesis // mammary gland epithelial cell differentiation // smoothened signaling pathway involved in dorsal/ventral neural tube patterning // cell differentiation involved in kidney development // somite development // cellular response to cholesterol // cellular response to cholesterol // renal system development // cell proliferation involved in metanephros development // protein targeting to plasma membrane
MSH3	Meiotic mismatch repair // ATP catabolic process // DNA repair // mismatch repair // cellular response to DNA damage stimulus // reciprocal meiotic recombination // somatic recombination of immunoglobulin gene segments // maintenance of DNA repeat elements // negative regulation of DNA recombination // positive regulation of helicase activity
CARD11	Positive regulation of cytokine production // signal transduction // positive regulation of B cell proliferation // T cell costimulation // Fc-epsilon receptor signaling pathway // positive regulation of T cell proliferation // regulation of apoptotic process // positive regulation of I-kappaB kinase/NF-kappaB signaling // thymic T cell selection // positive regulation of interleukin-2 biosynthetic process // innate immune response // regulation of B cell differentiation // regulation of T cell differentiation // nucleotide phosphorylation // regulation of immune response // T cell receptor signaling pathway // positive regulation of T cell activation // positive regulation of NF-kappaB transcription factor activity

TCF7L2, share high mobility group (HMG)-box domains, which suggests that β -catenin signaling switches mechanisms with the binding of different transcription factors. 3D complex structures show that CDH1, LEF1 and TCF7L2 bind to β -catenin. The role of β -catenin signaling in the pluripotency pathway should be investigated to reveal its mechanism in cancer and stem cells. The Wnt pathway is located upstream, and TCF, downstream of CTNNB1 in

the cascade^[31]. The merged network model of the β -catenin signaling network and CDH1, together with molecules in the 3D complex structures and genes mutated along with the *CTNNB1* alteration is shown in Figure 5A. The merged network model of the *CTNNB1*, *Wnt*, and *TCF* signaling networks and *CDH1*, together with molecules in the 3D complex structures and genes mutated along with the *CTNNB1* alteration is shown in Figure 5B. Of

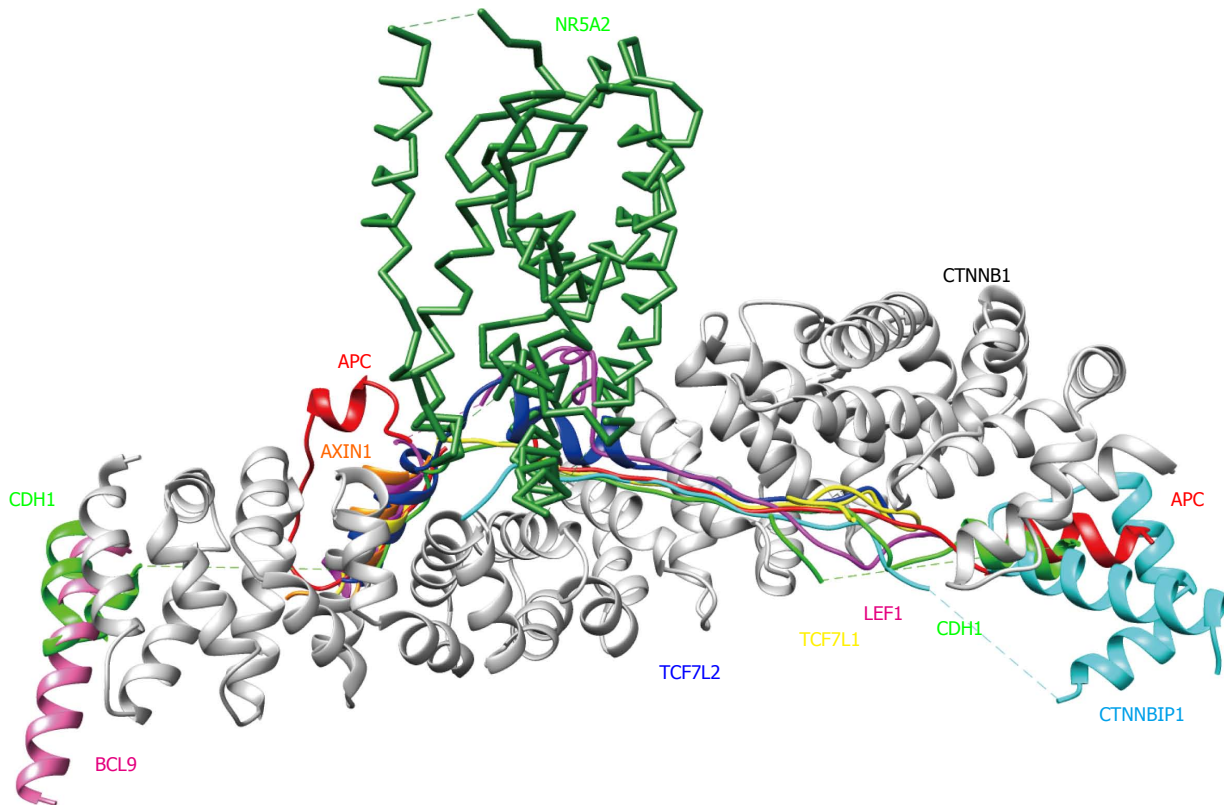


Figure 3 3D structures of β -catenin (CTNNB1) binding proteins. Each CTNNB1 complex structure was superimposed onto the CTNNB1 structure in a complex with APC (PDB code: 1th1), using the program MATRAS (From Ref. [18]). Colors and PDB codes are summarized as follows: White: CTNNB1 (CTNNB1_HUMAN, 1th1); red: APC (APC_HUMAN, 1th1); orange: AXIN1 (AXN_XELNA, 1qz7); hot pink: BCL9 (BCL9_HUMAN, 3sl9); green: CDH1 (CADH1_MOUSE, 1i7w); cyan: CTNNBIP1 (CNBP1_HUMAN, 1m1e); magenta: LEF1 (LEF1_MOUSE, 3oux); forest green: NR5A2 (NR5A2_HUMAN, 3tx7); yellow: TCF7L1 (T7L1A_XENLA, 1g3j); blue: TCF7L2 (TF7L2_HUMAN, 1jdh).

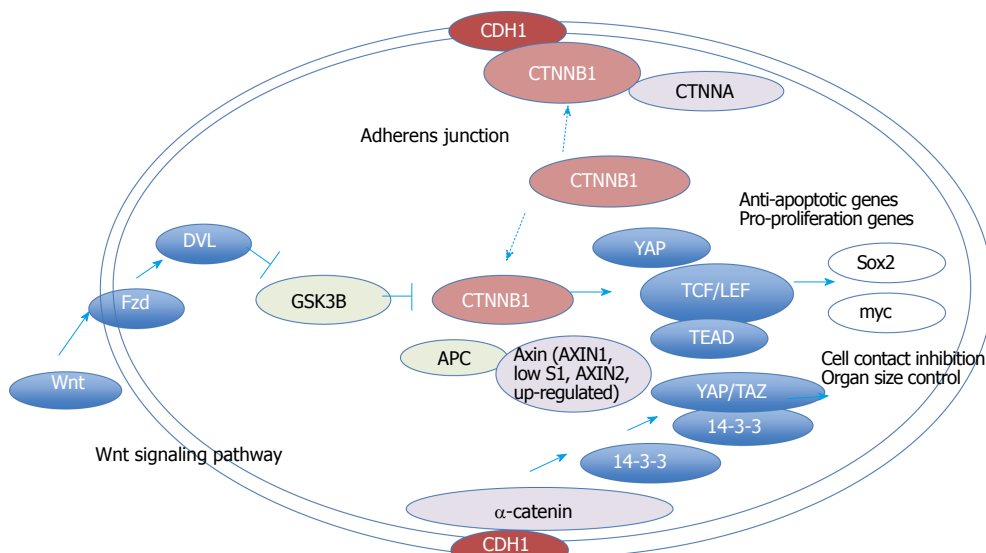


Figure 4 Network model for CTNNB1. The molecular network model for CTNNB1 signaling is shown. The extracted networks for pathways in cancer, Hippo signaling pathway and the Wnt signaling pathway (KEGG) were merged and are shown in a molecular network model. Wnt signaling and adherens junction molecules cross-talk via CTNNB1. Activated CTNNB1 induces the transcription of anti-apoptosis genes and pro-proliferation genes.

the common genes, EPHA8, SS18 and PTCH1 interact with phosphatidylinositol-4,5-bisphosphate-3-kinase catalytic subunit gamma (PIK3CG), SWI/SNF related, matrix associated, actin dependent regulator of chromatin,

subfamily a, member 4 (SMARCA4), and GLI family zinc finger 1 (GLI1), respectively, whereas CARD11, ICE1, MSH3 have no known interactions with molecules in the CTNNB1 network. The networks for stomach

to MSCs are shown in red, whereas the down-regulated genes are shown in light blue (Fold change > 2, $P < 0.05$, $n = 12$ in MSCs, $n = 5$ in GC; the average signal intensity of MSCs or GC cells is greater than 500). The expression of the *CTNNB1*, *CDH1*, notch1 (*NOTCH1*), hepatocyte growth factor (*HGF*), *PTCH1*, discs large homolog 1, scribble cell polarity complex component (*DLG1*), *LEF1*, *CTNND1*, *SMARCA4*, nuclear receptor coactivator 2 (*NCOA2*), SMAD family member 4 (*SMAD4*), *MYC*, junction plakoglobin (*JUP*), *TCF7L2* and *ERBB2* genes

was up-regulated in GC cells compared to MSCs, whereas the expression of twist family bHLH transcription factor 1 (*Twist1*) was down-regulated in GC cells compared to MSCs. The expression of *EPHA2* was up-regulated in some GC samples. The expression of the IQ motif-containing GTPase activating protein 1 (*IQGAP1*), *SS18*, *ICE1*, cortactin (*CTTN*), *RHOA*, CREB binding protein (*CREBBP*) and protein tyrosine phosphatase, non-receptor (*PTPN1*) genes was not altered in MSCs and GC cells. The expression of the *EPHA8*, *PIK3CG*, *CARD11*, *MSH3*, *GLI1*, epidermal growth factor receptor (*EGFR*), snail family zinc finger 1 (*SNAI1*) and *CTNND2* genes was not examined due to a low signal intensity. The alteration frequencies of *CTNND2* and *ERBB2* are relatively high in the *CTNNB1* network (> 15%), according to the cBioPortal for Cancer Genomics. Interestingly, *IQGAP2* was up-regulated in GC cells compared to MSCs.

DISCUSSION

In summary, the *CTNNB1* gene expression was up-regulated in diffuse-type GC compared to MSC. The various molecules are regulated with *CTNNB1*, which suggests the *CTNNB1* signaling network in cancer and stem cells. EMT-related genes have been reported to be induced by transforming growth factor (TGF)- β or epidermal growth factor (EGF), and genes in the Wnt signaling pathway are mutated in non-small cell lung cancer^[32-34]. The expression of β -catenin was up-regulated in the TGF- β 1-induced EMT model and was inhibited by cucurbitacin B treatment^[32]. Solid tumors induce hypoxia, leading to HIF-1 α protein regulation of molecules that are involved in angiogenesis, erythropoiesis, metabolism, cell survival and cell proliferation^[35]. *SNAI2* and *Twist1* were down-regulated in GC cells compared to MSCs, whereas *SNAI1* expression was not detected because of low signal intensity^[9,36,37]. Because *SNAI* and *Twist* are associated with EMT, the regulation of their expression is important for understanding EMT mechanisms. Although 3D complex structures of *SNAI2* and *Twist1* with β -catenin are not available, some indirect β -catenin signaling cascade may be involved in the *SNAI2* and *Twist1* pathway^[38,39]. TGF β is also an important factor in EMT^[40]. TGF β regulates osteoblast differentiation, whereas calycosin-7-O- β -D-glucopyranoside-induced osteoblast differentiation is regulated *via* the bone morphogenetic protein (BMP) and Wnt/ β -catenin-signaling pathway^[41]. The TGF β -induced nuclear translocation of β -catenin has been reported to be one of the key factors that activates the EMT program^[42-45]. Wnt/ β -catenin is regulated in stem cells, and Wnt target genes are controlled by the TCF/ β -catenin complex^[46].

In gastrointestinal cancer, somatic mutations that provoke an immune response have been found in tumor-infiltrating lymphocytes, which may be very specific to the individual and are targets for cancer immunotherapy^[47]. KRAS-mutation-specific T cells, as well as personalized mutation-specific T cells, have been identified, and these

may be useful in the future for individual cancer immunotherapeutics^[47]. It has been reported that *Helicobacter pylori* up-regulates Nanog and Oct4 expression *via* Wnt/ β -catenin signaling^[48]. Wnt/ β -catenin signaling and the phosphorylation of β -catenin may be involved in stemness in gastric cancer^[48].

In conclusion, *CTNNB1* plays an important role in the regulation of stem cell pluripotency and cancer signaling. For future direction, precise analyses of Wnt signaling, Notch signaling, and Ephrin signaling are needed to reveal the entire picture of β -catenin signaling in cancer and stem cells. RHO mutations, and regulator of G-protein signaling, with network analysis tools, such as Cytoscape, must be investigated for a greater understanding of this process.

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COMMENTS

Background

β -catenin signaling is essential in pluripotent stem cells and cancer. It is also involved in the epithelial-mesenchymal transitions (EMT). *CTNNB1* is activated by Wnt, and the binding of *CTNNB1* to transcription factors leads to pluripotent gene regulation.

Research frontiers

The regulation of pluripotency and proliferation is important for elucidating the mechanism of cell phenotype transitions. The EMT mechanism should be investigated to better understand cancer resistance to therapeutics.

Innovations and breakthroughs

The 3D complex structures of β -catenin and related molecules were studied using molecular networks, which is an innovation in the field. The mutated genes that were altered along with *CTNNB1* in stomach adenocarcinoma samples were also investigated.

Applications

These results may affect the study of the pluripotency mechanism and potential therapeutic predictions of gastric cancer. The genes in the molecular network that are related to *CTNNB1* may be the targets of predictive medicine for cancer and disease using pluripotent cells.

Terminology

EMT is a cellular phenotype of a transition from an epithelial to a mesenchymal cell type. EMT is regulated in cancer metastasis and malignancy, and it is related to the acquisition of resistance in cancer cells to therapeutics. It is important to understand the EMT mechanism to understand the mechanisms of cancer resistance.

Peer-review

In general, the manuscript is interesting not only for scientific reasons, but also due to its potential clinical relevance, since it provides some light about the

relationships between stem and cancer cells.

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Role of autophagy in bone and muscle biology

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Abstract

Autophagy in eukaryotic cells is a constitutive process and functions as a homeostatic mechanism; it is up-

regulated in response to specific stress stimuli such as starvation, hypoxia and as oxidative stress. In addition to playing a crucial role in adaptive responses to different stimuli, autophagy is also required for intracellular quality control. This second aspect is important to prevent the activation of pathological processes. Autophagy also plays a central role in cellular development and differentiation because it is involved in the regulation of energetic balance. This final aspect is critical for maintaining proper bone and muscle function as well as to prevent any pathological changes. Therefore, identifying new molecular targets involved in autophagy is critical to assure a good quality of life.

Key words: Autophagy; Bone; Muscle

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Core tip: Autophagy is a major catabolic process in eukaryotic cells in which damaged macromolecules and organelles are degraded and recycled. Several studies have demonstrated its crucial role in bone and muscle cell homeostasis. Deficiency or dysfunction in autophagy can result in pathological conditions such as osteoporosis and sarcopenia, which are associated with ageing. It is important to understand the role of the macromolecules involved in autophagy to devise how to counteract its decline and to hinder irreversible cell damage.

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AUTOPHAGIC MACHINERY

Autophagy is a key process in cellular homeostasis and is involved in removing and recycling misfolded proteins, damaged organelles or dysfunctional cell components. Autophagy may be distinguished into

the following three categories: (1) macroautophagy; (2) microautophagy; and (3) chaperone-mediated autophagy^[1,2]. Macroautophagy is the most prevalent form of autophagy among different cell types^[3,4]. It starts with the formation of a phagophore, which is a membrane protrusion that expands to engulf the cellular cargo, generating an autophagosome. This latter structure matures *via* fusion with lysosomes. TOR kinase is a major player in autophagy and acts as a signalling node downstream of growth factor receptor signalling, hypoxia, changes in ATP levels and insulin signalling. It is activated downstream of Akt kinase, PI3-kinase and growth factor receptors and acts to inhibit autophagy by modulating the Ulk1 (Atg1) complex. In response to autophagy cascade activation, the class III PI3K complex produces PI(3)P and induces other Atg proteins, including Atg12-Atg5-Atg16, as well as the LC3 (Atg8)-phosphatidylethanolamine complex. LC3 is the commonly used term for microtubule-associated protein 1 light chain 3. After translation, proLC3 is proteolytically cleaved by Atg4 protease, which generates LC3- I. Upon induction of autophagy, LC3- I is conjugated to the highly lipophilic phosphatidylethanolamine (PE) moiety by the Atg7, Atg3 and Atg12-Atg5-Atg16L complex to generate LC3- II. Finally, PE promotes the integration of LC3- II into lipid membranes to promote autophagosome formation.

Mice lacking Atg7, which is a specific liver gene for autophagosome formation, developed severe hepatomegaly as a consequence of intracellular accumulation of aggregates and non-functional organelles^[5]. Furthermore, knockout mice bearing a neural-specific deletion of either Atg7 or Atg5, both of which are required for autophagosome formation, developed ataxic gait, abnormal motor coordination and systemic tremor^[3,4]. In addition to recycling amino acids after protein degradation, autophagy may also contribute to energy production through the generation of free fatty acids^[6].

Recent studies have shown that autophagy might play different roles depending on the cellular context, leading to either apoptosis or survival by modulating key genes^[7].

In addition, perturbations of the autophagy machinery have been linked to different disorders such as ischaemic cardiomyopathy and neurodegenerative diseases^[8,9].

AUTOPHAGY IN BONE BIOLOGY

Osteoblasts, the bone-forming cells, secrete the skeletal matrix (osteoid) and have a principal role in bone mineralization. During differentiation, the osteoblast assumes the characteristics of an osteocyte, which orchestrates bone remodelling. At present, 2 families of transcription factors involved in the autophagy process, the forkhead box O (FOXO) and cAMP responsive element binding protein (CREB) families, are known to regulate osteoblasts. FOXO activation promotes autophagy by binding to the promoter regions of target genes, and studies have shown that deletion of *FOXO* genes causes oxidative stress and apoptosis. Conversely, overexpression of FOXO3

reduces bone impairment associated with ageing^[10]. The involvement of *FOXO* genes in the regulation of bone homeostasis may conceivably be due, at least in part, to the induction of autophagy. ATF4 (a member of the CREB family) is required to support osteoblast function and maturation as well as to reduce cellular stress due to amino acid starvation because it increases amino acid trafficking into cells. Reduced ATF4 activity has been associated with skeletal impairments such as Coffin-Lowry syndrome and neurofibromatosis type I^[11]. Another autophagy protein involved in osteoblast biology is the autophagy receptor NBR1 (a neighbour of *BRCA1* gene 1), which interacts with the MAP1LC3 protein family through its LC3-interacting region^[12]. Autophagy is also involved in osteoclastogenesis. The autophagy proteins Atg5, Atg7, Atg4B and MAP1LC3 are required for osteoclast differentiation; these cells are responsible for bone resorption. Among others, Atg5 and Atg7 promote bone remodelling by inducing bone resorption *via* directing lysosomes through the plasma membrane into the extracellular space^[13].

AUTOPHAGY IN MUSCLE

Autophagy plays a crucial role in muscle cells under both healthy and adverse conditions. The highly structured organization of skeletal muscle cells and their function may cause damage to proteins and organelles; mitochondria are particularly susceptible because of the increased generation of reactive oxygen species compared to other tissues. Muscle cells therefore require efficient housekeeping mechanisms that can quickly eliminate unfolded and toxic proteins as well as dysfunctional organelles. Under physiological conditions, basal levels of autophagy guarantee muscle cell homeostasis, whereas upregulation of the autophagic machinery contributes to cell survival under various stress conditions, including calorie restriction (CR), atrophy, and sarcopenia, by maintaining the required amino acid supply and metabolite levels^[14].

AUTOPHAGY AND PHYSICAL EXERCISE

A chaperone-assisted selective autophagy process (CASA) has been characterized as a tension-induced autophagy pathway essential for the adaptation of mechanically strained tissues such as skeletal muscle, heart, lung and kidney. In striated skeletal muscle, the CASA machinery is located at the Z-disk; this is of critical importance because the complex mediates the degradation of large cytoskeleton components (e.g., FLNC, filamin C) damaged during contraction. Recent studies demonstrated that strenuous resistance exercise caused a significant increase in CASA activity in muscles of healthy men who trained moderately^[15]. Impairment of CASA in animal models, however, leads to muscle weakness^[16].

AUTOPHAGY AND MUSCLE DISORDERS

The hallmark of autophagy, autophagosomes, are found

in most myopathies and dystrophies. In many acquired and genetic muscle diseases, however, misregulation rather than upregulation of autophagic and protein degradation machineries appear to be the cause of muscle degeneration^[17].

AUTOPHAGY AND AGEING

Sarcopenia, which is ageing-related skeletal muscle loss, is a common debilitating condition affecting 5%-13% of the elderly population over 60. Imbalance between protein synthesis and protein degradation; the accumulation of denatured, misfolded or aggregated molecules; mitochondrial dysfunction; and excessive apoptosis caused by complex intrinsic and extrinsic factors can result in sarcopenia. Growing molecular evidence supports the idea that dysregulated autophagy may contribute to sarcopenia. Strategies (e.g., CR and life-long exercise) aiming to induce an appropriate autophagic process in muscle cells may reduce age-associated muscle wasting^[18]. Autophagy is therefore a potential target for sarcopenia; bioactive molecules known to modulate autophagy can play a beneficial role at the cellular level in the ageing process^[19].

CONCLUSION

It is not surprising that bone and muscle structures and functions are tightly regulated by endogenous and environmental factors. Autophagy plays a relevant role in physiological and pathological conditions: A well-balanced performance of the autophagic machinery is essential for both bone and muscle homeostasis. Thus, in-depth knowledge of autophagy may provide new molecular targets for maintaining bone and muscle formation and metabolism to assure a good quality of life.

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Involvement of blood mononuclear cells in the infertility, age-associated diseases and cancer treatment

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Abstract

Blood mononuclear cells consist of T cells and monocyte derived cells. Beside immunity, the blood mononuclear cells belong to the complex tissue control system (TCS), where they exhibit morphostatic function by stimulating

proliferation of tissue stem cells followed by cellular differentiation, that is stopped after attaining the proper functional stage, which differs among various tissue types. Therefore, the term immune and morphostatic system (IMS) should be implied. The TCS-mediated morphostasis also consists of vascular pericytes controlled by autonomic innervation, which is regulating the quantity of distinct tissues *in vivo*. Lack of proper differentiation of tissue cells by TCS causes either tissue underdevelopment, *e.g.*, muscular dystrophy, or degenerative functional failures, *e.g.*, type 1 diabetes and age-associated diseases. With the gradual IMS regression after 35 years of age the gonadal infertility develops, followed by a growing incidence of age-associated diseases and cancers. Without restoring an altered TCS function in a degenerative disease, the implantation of tissue-specific stem cells alone by regenerative medicine can not be successful. Transfused young blood could temporarily restore fertility to enable parenthood. The young blood could also temporarily alleviate aging diseases, and this can be extended by substances inducing IMS regeneration, like the honey bee propolis. The local and/or systemic use of honey bee propolis stopped hair and teeth loss, regressed varicose veins, improved altered hearing, and lowered high blood pressure and sugar levels. Complete regression of stage IV ovarian cancer with liver metastases after a simple elaborated immunotherapy is also reported.

Key words: Blood mononuclear cells; Age-associated diseases; Infertility treatment; Regenerative medicine; Transfusion morphostatic treatment; Stem cells; Tissue control system; Immune system; Tissue morphostasis; Cancer immunotherapy

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Core tip: Currently, there are little possibilities to treat age-associated disorders, since the morphostasis of normal tissues and its alteration with age advancement remain poorly understood. The components of the

immune system, beside immunity, exhibit an important morphostatic function in the regulation of tissue physiology and regeneration as participants in the tissue morphostasis management. Age-induced immune system decline is accompanied by gonadal infertility and growing incidence of age-associated diseases and cancers. Utilization of young blood can alleviate aging, and a novel simple elaborated immunotherapy can cause regression of advanced cancers without a need of the debulking surgery and/or exhaustive chemotherapy.

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INTRODUCTION

The morphostasis, or tissue homeostasis, ensures that under appropriate conditions various tissues can regenerate, if needed, and functional cells are prevented to leave the appropriate stage, *i.e.*, stem cells are stimulated to divide and mature into the functional stage, and prevented to exhibit aging and regression. The functional stage of brain neuronal cells is lower compared to moderate functional differentiation of the gut. Squamous epithelial tissues exhibit most advanced differentiation resulting in the functionally needed apoptotic surface epithelial cells^[1]. The immune system is generally considered to exhibit organism protection against nonself substances and ignore self tissues due to the elimination of autoreactive lymphoid cells^[2,3], *i.e.*, it is supposed to exhibit no reactivity toward self cells. Formerly, however, Alexis Carrel demonstrated that extracts of leukocytes, like extracts from embryonic tissues, increase fibroblast divisions *in vitro*^[4]. Next, Burwell^[5] indicated that the immune components play an essential function by managing self-tissue morphostasis and Fidler^[6] proposed that the immunity is one of the many functions of lymphocytes. We have been investigating an involvement of immune components in the rat and human female reproduction since 1977^[7]. Observation of immune components in the ovary resulted in the definition of the morphostatic "Tissue Control System" (TCS)^[8,9]. Of particular interest was a possibility to treat ovarian infertility in older women^[10]. With respect to the essential involvement of the immune components in the regulation of self tissue morphostasis, the term "Immune and Morphostatic System" (IMS) appears to be more appropriate. Beside infertility, continuous aging is associated with the emergence of age-associated diseases, the pathophysiology of which is not yet well understood^[11,12], and with a growing incidence of cancers^[13]. In this article we will review cellular processes accompanying physiology of tissue morphostasis and its alteration accompanying aging and cancer growth. Some

novel approaches for the treatment of gonadal infertility, aging diseases, and cancer will be reviewed.

THE IMS AND TISSUE MORPHOSTASIS

What is the tissue morphostasis?

The IMS involvement in morphostasis of body tissues was introduced by Burwell RG in 1963^[5]. In his Lancet article, Burwell wrote: "The immunology still awaits incorporating into the general pattern of biology". He proposed that immune components exhibit essential contribution to the regulation of morphostasis of body tissues, and defined the morphostasis as a "steady state condition that maintains a particular (tissue) pattern".

Tissue morphostasis and the tissue control system

Appropriate tissue morphostasis should follow the three patterns: (1) preserve tissues in an appropriate functional stage, which is different between distinct tissue types; (2) enable regeneration from tissue stem cells, when required; and (3) maintain the proper tissue quantity. While the first two rules appear to be IMS-dependent^[14], the tissue quantity is regulated by the autonomic nervous system, because separation of small proportions of the cephalic neural crest in 9th or 10th stage of the chick embryos development significantly diminished the thymus size^[15,16].

The TCS includes IMS components regulating proliferation and differentiation of tissue cells, vascular pericytes regulating activity of the TCS units, and autonomic innervation regulating tissue quantity and their vascularization *via* stimulation or inhibition of activity of pericytes. In all functional tissues, the cellular IMS components, monocyte-derived cells (MDCs) and T cells (TC), stimulate proliferation of tissue stem cells, and their further differentiation into functional cells. This can later be accompanied by the IgM, and eventually by IgG binding. The pericytes release the Thy-1 differentiation glycoprotein (Thy-1), which represents the smallest component of the immunoglobulin gene superfamily^[17]. The "morphostimulatory" effect of Thy-1 is *in vivo* controlled by the autonomic innervation accompanying vascular pericytes. For instance in the ovary of young fertile women, the emergence of new germ cells is accompanied by Thy-1 release from vascular pericytes^[18], and ovarian follicular growth and selection depends on the local activity of Thy-1⁺ pericytes.

Figure 1 shows basic TCS unit and its involvement in the early stages of differentiation of tissue cells. The basic TCS units accompany postcapillary venules. Many final axons of autonomic innervation accompany postcapillary pericytes, and they consist of cholinergic and adrenergic axons^[19]. The activity of vascular pericytes and entire TCS units for the particular tissue is inhibited by autonomic innervation when the tissue quantity is reached. The cancers lack autonomic innervation^[20], and the pericytes exhibit extreme activity in supporting cancer neovascularization and tumor growth^[21], regardless of its quantity. From the IMS morphostatic components the

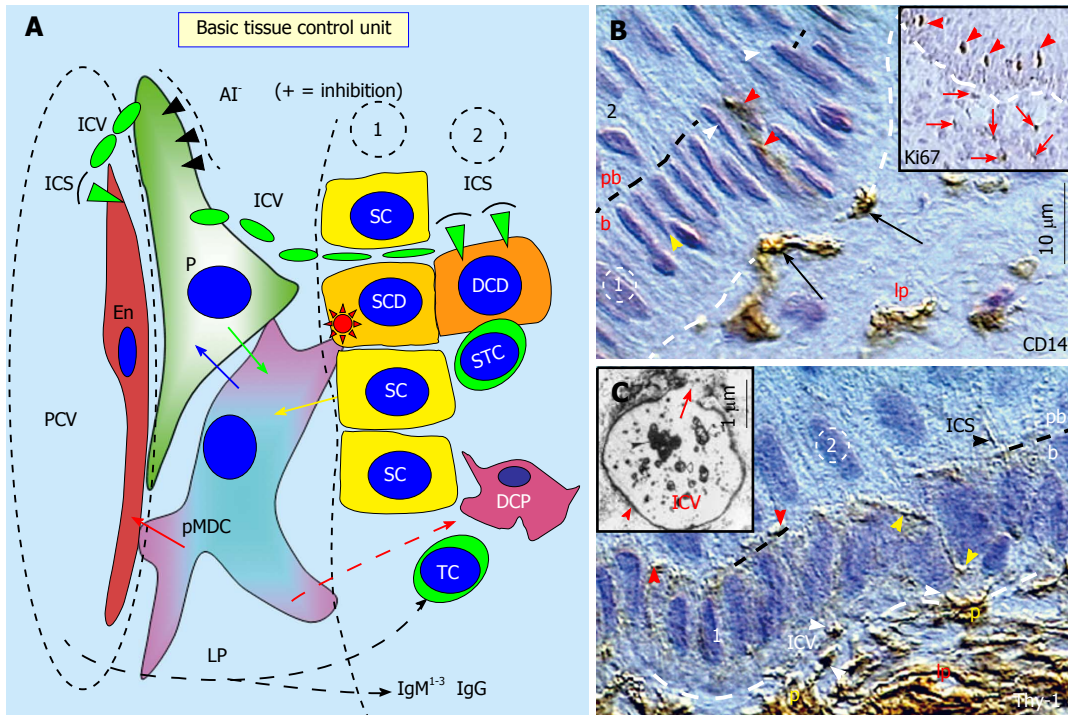


Figure 1 The basic tissue control unit and early cellular differentiation. A: The tissue control unit (TCU) is associated with postcapillary venules (PCV). It consists of CD14⁺ primitive MDCs (pMDCs), pericytes (P) accompanying PCV, and autonomic innervation (AI). The TCU influences properties of Endothelial cells (En) and an involvement of other components of the TCS regulating the differentiation of tissue stem cells into the tissue-specific functional stage by the influence of dendritic cell precursors (DCP), and eventually by T cells (TC), dendritic cells, and immunoglobulins (IgM1-3 and IgG). The pMDCs physically interact with adjacent En (red arrow) and receive requests (yellow arrow) to regenerate from tissue stem cells (SC) when required. The pMDCs communicate with pericytes (blue arrow), and if the pericytes are not blocked by AI, the positive signal (green arrow) is provided to pMDC to stimulate stem cell division. The asymmetric division is initiated by pMDC (red asterisk) and accompanied by a suicidal T cell (STC). It gives a rise to the stem cell daughter (SCD) and differentiating cell daughter (DCD). The pericytes provide by Thy-1⁺ intercellular vesicles (ICV) growth factors and cytokines to the endothelial and tissue cells. After release of ICV content (green arrowheads), the vesicles collapse into intercellular spikes (ICS); B: CD14 MDCs (arrows) in lamina propria (lp) migrate to basal layer (b) of the stratified epithelium, interact with basal stem cells (yellow arrowhead), and migrate to the parabasal layer (red arrowheads). White arrowheads indicate basal epithelial cells moving to the parabasal (pb) layer. Inset shows Ki67⁺ postmitotic parabasal epithelial cells (arrowheads) represented by differentiating stem cell daughters, and postmitotic stromal cells in the lamina propria (arrows); C: Thy-1 P in the lamina propria produce ICV (white arrowheads) migrating (yellow arrowheads) toward postmitotic parabasal cells (red arrowheads) where they release their content and collapse into ICS (black arrowhead). Inset shows transmission electron microscopy of Thy-1 immunolabeling of a pericyte-derived ICV releasing its content (arrow)^[1].

essential role belongs to the MDCs. These cells differentiate from progenitors already present in the embryonic yolk sac^[22], and can follow and remember the stages of development of various embryonic and fetal tissues during the developmental immune adaptation. The earlier the tissue differentiates into the functional stage, the longer its proper function is supported by MDCs during the lifetime. After the termination of developmental immune adaptation, the CD14⁺ primitive MDCs^[23] (pMDCs) regulate homing of circulating TC committed for the particular tissue type. The pMDCs receive signals from tissue stem cells when a regeneration is required and interact with pericytes to realize whether tissue regeneration is feasible. If the tissue quantity does not exceed quantitative limit controlled by autonomic innervation, the pMDCs receive positive signal from pericytes and stimulate asymmetric division of tissue stem cells along with T lymphocytes. The pMDC actions are accompanied by the release of pericyte-derived Thy-1⁺ intercellular vesicles reaching postmitotic tissue cells, where they collapse into intercellular spikes after the release of differentiation promoting substances. The

TC and MDCs may enter among tissue cells to support continuing development of the tissues. This is associated with the IgM binding to tissue cells. Cellular apoptosis is accompanied by the binding of IgG.

The MDCs regulate an involvement of morphostatic IMS components by the so called "stop effect (StE)", which is established during the embryonic and fetal immune adaptation. The appropriately established StE allows differentiation of tissue cells into their proper functional stage and prevents their aging. In hormonally-dependent tissues, *e.g.*, reproductive tract stratified epitheliums, the hormone can bypass the StE^[8], and the cells differentiate toward apoptotic surface epithelial cells. The CD14 pMDCs are present in lamina propria of the epithelium and distributed in the basal and parabasal epithelial cell layers. The postmitotic tissue cells exhibit Ki67 expression. The tissue stem cells are *in vivo* prevented to divide and differentiate by themselves, since these processes are dependent on the involvement of the TCS components. Such inhibition is, however, absent *in vitro*. For instance the stem cells of the ovary proceed *in vitro* toward differentiation into oocyte-

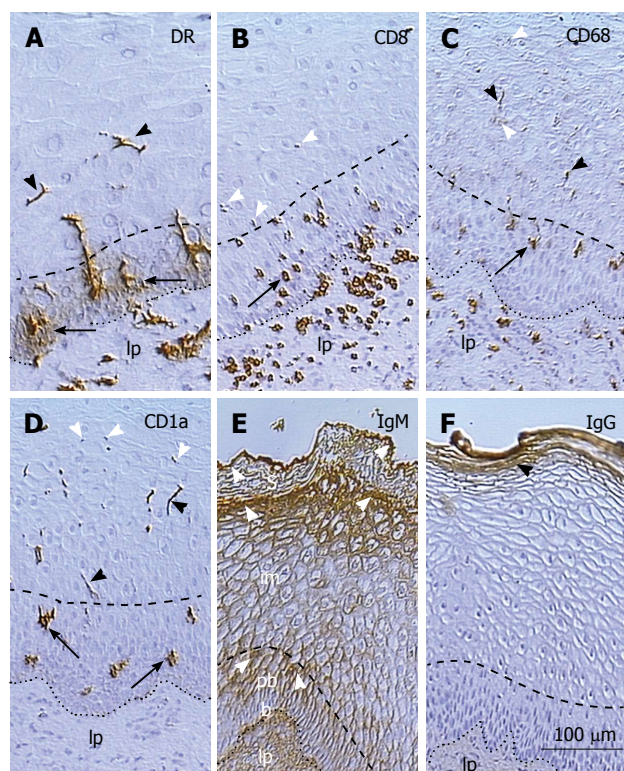


Figure 2 Distribution of monocyte derived cells, T cells, and immunoglobulins in the squamous epithelium of uterine ectocervix. A: Dendritic cell (DC) precursors accompany vessels in the lamina propria (lp), release HLA-DR in the parabasal layer (arrows), and form DC (arrowheads) in the intermediate layer; B: T cells associate with basal epithelial cells, reach parabasal/intermediate interface, and degenerate thereafter (arrowheads); C: The CD68 expression (arrow) accompanies mature DC (black arrowheads), which secrete CD68 (white arrowheads); D: CD1a⁺ DC precursors (arrows) and mature DC (black arrowheads) undergoing fragmentation (white arrowheads); E: The IgM binds to upper parabasal, upper intermediate, and upper superficial layers (arrowheads); F: IgG binds to the entire superficial layer only. The lp indicates epithelium lp, dotted line is basement membrane, and dashed line is parabasal/intermediate interface^[14].

like cells (OLCs), which are able to divide and produce daughter cells in order to collect their organelles required for further growth of the oocyte-like cells. The Thy-1⁺ pericytes produce intercellular vesicles, which release their differentiation promoting content among postmitotic differentiating stem cell daughters to stimulate their early stage of differentiation.

Figure 2 shows hormonally stimulated uterine ectocervix squamous epithelium, which exhibits StE bypass. The MDCs release HLA-DR in the parabasal epithelial layer and exhibit differentiation toward dendritic cells in the intermediate epithelial layer. CD8⁺ TC migrate toward parabasal/intermediate interface, where they regress. Intraepithelial lymphocytes are permanently present in the skin, intestine, biliary tract, oral cavity, lungs, upper respiratory tract, and in the reproductive tract tissues^[24]. Mature dendritic cells secrete CD68⁺ molecules among intermediate epithelial cells and regress thereafter. The presence of CD68⁺ MDCs accompanies the rheumatoid arthritis^[25]. The CD1a dendritic cells reach mid intermediate

epithelial layer, where they regress. The IgM exhibits binding to epithelial cells at the top of parabasal, intermediate, and superficial layer, and IgG is binding to all superficial cells. The adherence of immunoglobulins is considered to represent an autotoxic event, but it just depends where they bind^[26]. Even atrophic ectocervical epithelium lacking hormonal stimulation will be prevented by IgM binding to its surface from microbial infection.

Figure 3 shows that the epithelium infiltrating MDCs and TC do not act alone, but in cooperation among themselves. At the parabasal/intermediate interface, the TC become activated by DR expression and exhibit regression after entering the intermediate layer. This suggests that epithelial cells differentiate upon activation and regression of TC. Along with that, the MDCs differentiate into the dendritic cells accompanying more advanced differentiation of the epithelium, and regress in the mid intermediate epithelial layer. It appears that regression of intraepithelial MDCs promote further differentiation of epithelial cells. The TC accumulate among basal epithelial cells, and some of them may contribute to the asymmetric division of the epithelial stem cells.

Figure 4 describes nine stages, from basal tissue stem cells to the apoptotic surface epithelial cells, and an involvement of the IMS components and their markers for the transitions to the particular stages of differentiating tissue cells. The tissue-committed suicidal T cell required for the *in vivo* induction of asymmetric stem cell splitting enters daughter differentiating cell and regresses to be prevented from causing more than a single asymmetric tissue cell division. To be recognized by particular IMS component, the tissue cell should express a proper receptor.

The temporary presence of TC accompanies tissue regeneration but inappropriate permanent infiltration will cause degenerative disease

In the course of tissue regeneration after a partial (one third) hepatectomy or unilateral nephrectomy, the infiltrating TC are involved, but they disappear when the regenerating organs reach the original tissue extent^[27].

The persistent presence of tissue TC^[28], including DR + TC and microglia^[29,30] (i.e., brain MDCs), and also binding of immunoglobulins^[31,32], however, accompany regressing neuronal cells in the Alzheimer's disease, which is considered to represent an autoimmune disease^[33]. It has been proposed that the Alzheimer's is a consequence of the immune ageing, where the population of autoreactive TC initiates overactivation of the brain microglia, which are supposed of being involved in causing inflammatory neurotoxicity^[34].

Beside Alzheimer's, the available studies in humans and animals indicated an involvement of neuroinflammatory processes in the development of Parkinson disease, depression, schizophrenia, and autism, although the underlying mechanisms remain poorly understood. The progressing neural inflammation can activate microglia,

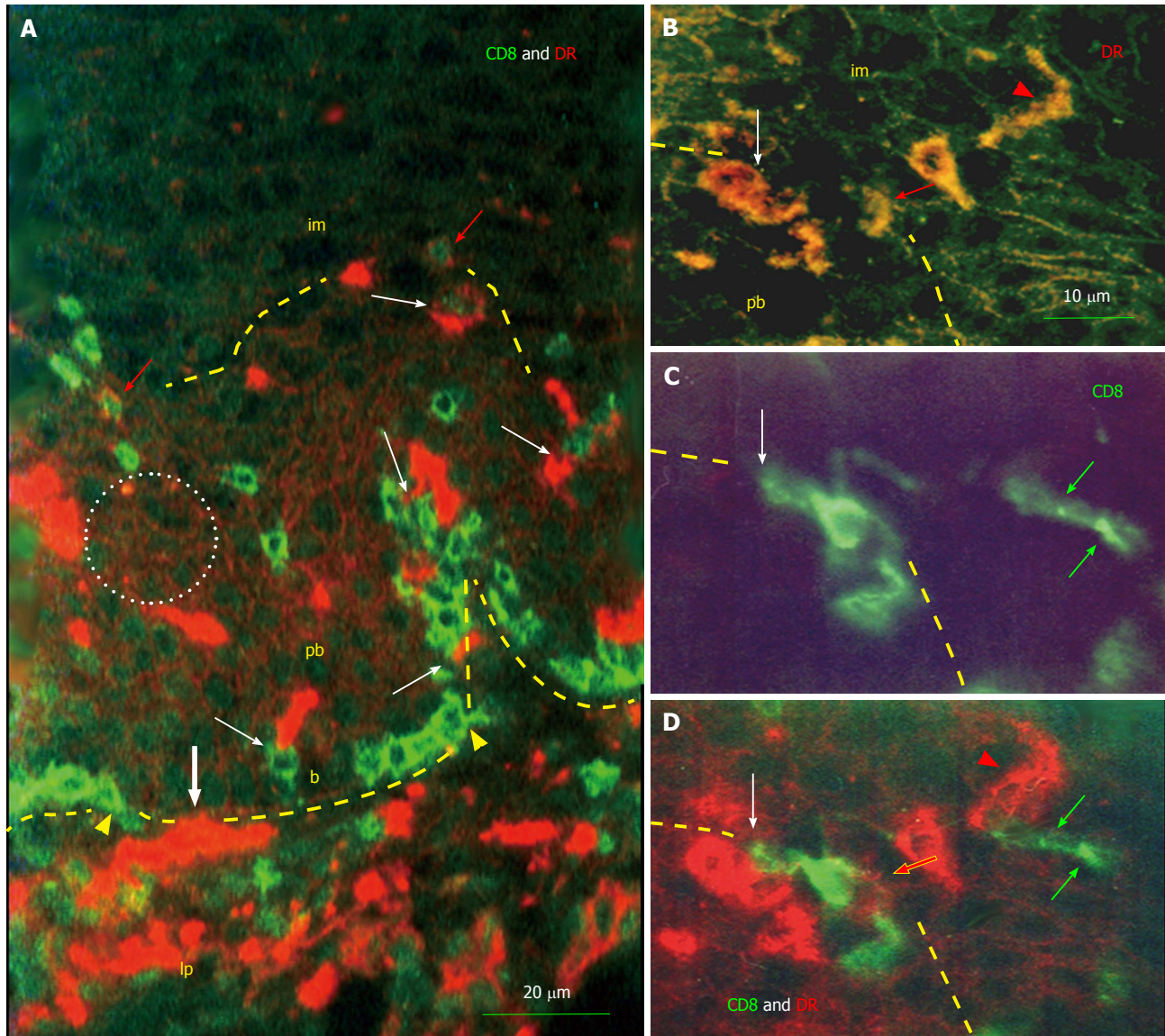


Figure 3 Interaction of monocyte derived cells and T cells in the squamous epithelium. A: HLA DR⁺ monocyte-derived cells (MDCs) (red color) and CD8 T cells (TC) (green color) enter (bold white arrow and arrowheads) basal epithelial layer (b) from the lamina propria (lp). Note accumulation of TC among basal epithelial cells. Both cell types interact by themselves (white arrows). After reaching the parabasal (pb)/intermediate (im) interface (dashed line) TC also express DR (red arrows) indicating their activation. Note binding of DR released from MDCs (see Figure 2A) to parabasal epithelial cells (dotted circle); B-D: Detail of pb/im interface shows MDC/T cell interaction (white arrows), the DR expression by TC (red arrows), transition of MDC into elongated dendritic cell (arrowheads), and remnants of regressing T cell in the lower intermediate layer (green arrows)^[14].

which leads into secretion of inflammatory cytokines causing regression of additional neurons and brain impairment. Consequently, the activation of microglia is supposed to contribute to the development of brain disorders caused by neural degeneration^[35].

From our point of view, the permanent presence of TC and immunoglobulins where inappropriate indicates that the tissue committed MDCs exhibit an altered StE, *i.e.*, higher StE than required for the proper function of that tissue^[36]. In other words, such MDCs will cause homing of committed circulating TC, become activated by interaction with them, and consequently, the tissue cells will leave the functional stage and gradually develop into the aging and apoptotic cells with a binding of immunoglobulins.

Figure 5 shows that certain distinct tissues require a distinct stage of their cellular differentiation, *i.e.*, distinct StE exhibited by the committed MDCs, for their proper function. An improper StE can develop during the prenatal IMS adaptation, causing postnatal functional failure of the given tissue. Shift up or lost StE during adulthood can originate from the latter tissue differentiation in the course the prenatal IMS adaptation, which naturally causes ovarian infertility starting after 35 years, *i.e.*, along with the IMS gradual regression starting from that period^[37]. Complete lack of StE is naturally present for the tissues which are prenatally absent, like the ovarian corpus luteum (CL). The CL has no stem cells, since it originates from the luteinized follicular granulosa cells, and it regresses after a short period of function during

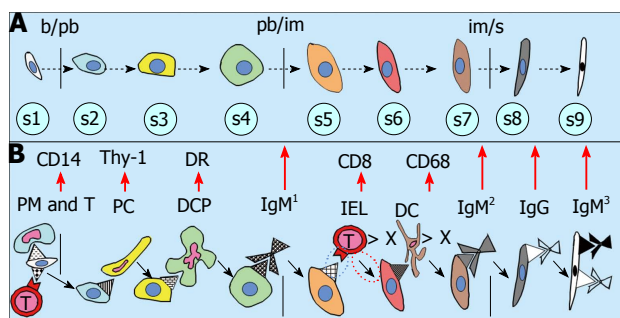


Figure 4 Hierarchy of tissue cell differentiation by an involvement of distinct elements of the tissue control system. A: The differentiation of tissue cells may pass up to nine stages (s1-s9). The epithelial cells pass through basal to parabasal b/pb, from parabasal to intermediate pb/im and from intermediate to superficial im/s layers, with intermediate steps in between; B: Cellular differentiation is regulated by distinct IMS components. The stem cell asymmetric division is mediated by primitive MDC (PM) and T cell (T). The pericytes (PC) cause s2 > s3 transition by substances released from Thy-1 intercellular vesicles. DR⁺ dendritic cell precursors (DCP) stimulate s3 to s4 transfer, s4 > s5 stage is caused by a binding of IgM¹, and regression of intraepithelial T lymphocytes (IEL) induces s5 > s6 transition. Regressing dendritic cells (DC) cause s6 > s7 change, binding of IgM² induces s7 > s8 transfer, and binding of IgG accompanies s8 > s9 stage exhibiting a binding of IgM³. > X indicates the regression IEL and DC^[1].

each ovarian cycle, unless the tolerance of the allogeneic embryo and fetus occurs during the pregnancy. Since the blocking activity of maternal serum is specific for the unknown antigen(s) present^[38] at the beginning of pregnancy, the CL will also survive. Along with the end of gestation the unblocking maternal serum activity develops^[39] and the CL of pregnancy regresses.

Distinct tissues in the healthy individuals apparently differ in their ability to function during the lifespan. The earlier a tissue differentiates during the developmental immune adaptation, the longer its function lasts during the adulthood. There is an apparent relationship between a period of heart and ovary differentiation during prenatal development and lasting of their function thereafter (Figure 6). Delay of fetal ovarian development will cause premature ovarian failure or primary amenorrhea. Acceleration of the rat ovarian follicular development during the developmental immune adaptation by androgens caused permanent anovulation due to the premature aging of rat ovaries and retardation by estrogens caused permanent anovulation due to the persisting ovarian immaturity^[40,41].

The ovarian follicular renewal physiologically ceases between 36-40 years of age, along with the gradual increase of the age-induced ovarian infertility. A retardation of the heart and/or brain development during the embryonic developmental immune adaptation, the age-induced alteration of their function are expected to occur due to the earlier emergence of the StE alteration causing heart or brain so called inflammation. Accordingly, the growing incidence of other age-associated diseases can be associated with the alteration of differentiation of some other tissues during the developmental immune adaptation.

OVARIAN MODEL OF TISSUE MORPHOSTASIS

The cyclical dynamic of ovarian components development and regression are essential for the understanding and managing the tissue morphostasis

The dynamics of ovarian function in the young fertile females represents an essential model for the studies of tissue morphostasis, since the new ovarian germ cells, granulosa cells, and primary follicles are cyclically formed, a proportion of ovarian follicles is selected to differentiate, most of them regress, and new ovarian corpora are formed from the ovulating follicles to function for a short period of time and degenerate, unless the pregnancy occurs. We have been investigating the IMS involvement in the control of ovarian structures development and demise in the rat and human females for 40 years^[7,10,18,42-45]. Recent proposal suggests to use transfer of circulating mononuclear cells from young fertile mammalian females, including humans, to aged females by a partial blood volume replacement to promote the fertility of females with aged or otherwise functionally affected ovaries, and to improve male infertility and some other functional tissue disorders as well^[46].

The former report demonstrated that an essential regulator of mammalian ovarian function is, in addition to the pituitary, the IMS with its homeostatic functions and epigenetically programmed memory for the ovaries. Moreover, the IMS morphoregulatory components appear to regulate, throughout the lifetime, the function of other tissues, and its gradual regression in older individuals is accompanied by an appearance of functional and degenerative disorders^[1]. Similar aging effect may cause testicular infertility^[46].

It is still a prevailing and textbook-presented view that adult mammalian females carry primordial oocytes in primordial follicles, which developed in fetal ovaries. These structures may persist in postnatal ovaries till the menarche, but not thereafter, since they will contain aged oocytes persisting too long, to avoid accumulation of endogenous and/or environmental alterations. In 1995, we had already shown that ovaries of young/adult women during the midfollicular phase exhibit emergence of fresh germ cells developing from the ovarian surface epithelium (OSE) cells, formerly known as germinal epithelium. This germ cell emergence was induced by the IMS-related mononuclear cells (CD14/DR⁺ MDCs and CD8/DR⁺ TC)^[18]. The OSE cells were later called ovarian stem cells (OSCs)^[47], since beside being able to form new germ cells, they have also an ability to be a source of the new granulosa cells in adult^[18] as well in the fetal^[44] human ovaries.

The Senior Editor of the Nature Publishing Group Tracey Baas in 2012 article^[48] indicated: "Up until the 1990s, the central dogma of reproductive biology was that female mammals have a restricted capacity for generating oocytes before birth, and once born the ovaries cannot renew egg cells that die because of

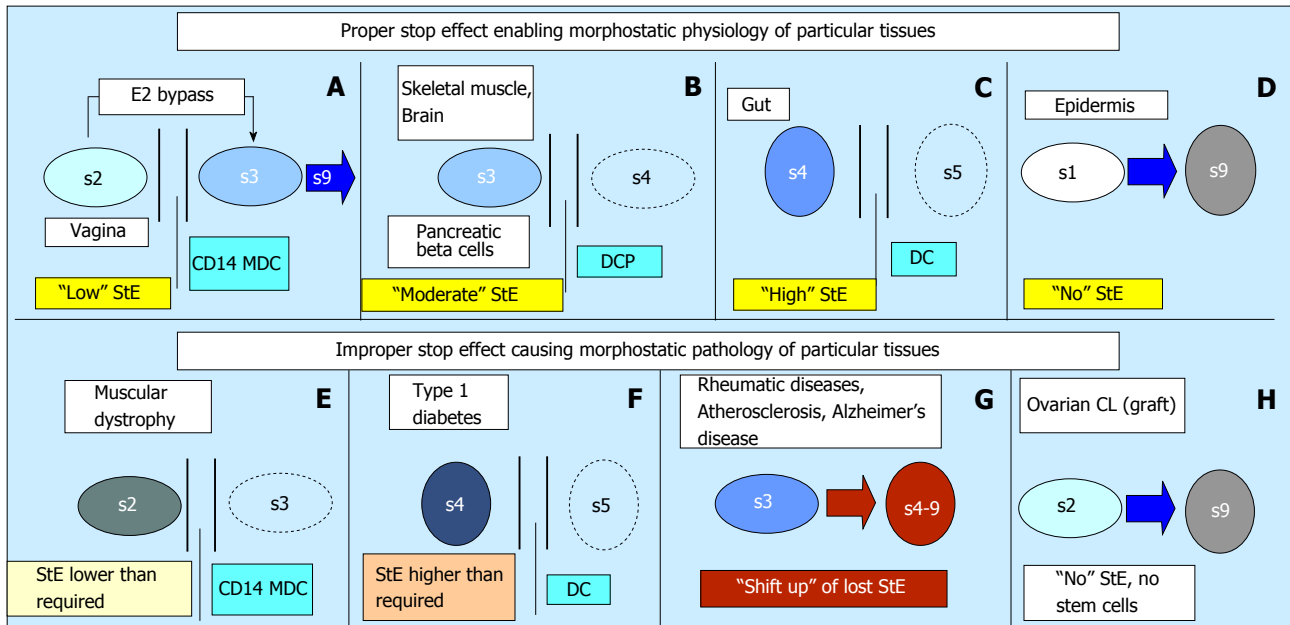


Figure 5 Proper stop effect is distinct for various tissue types and its alteration causes tissue dysfunction. A: Low StE bypass; B: Moderate StE with a lack of T cells (TC); C: High StE with the presence of TC; D: Lack of StE; E: Functional insufficiency > compare with panel B; F: Inappropriate presence of TC > compare with panel B; G: Shift up of lost StE; H: Absence of tissue during the prenatal IMS adaptation (autologous CL graft). Adjusted from^[1]. ©Antonin Bukovsky. DCP: DC precursor; MDC: Monocyte-derived cells; StE: Stop effect.

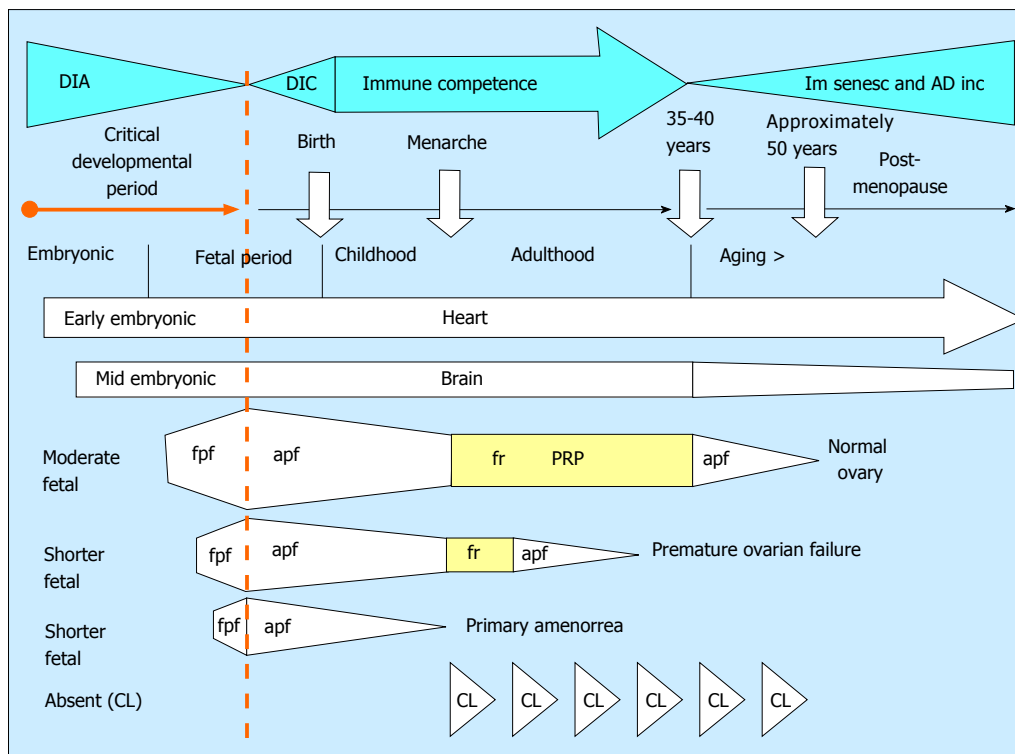


Figure 6 Developmental immune adaptation and the longevity of the tissue control system "stop effect". The functional persistence of various body tissues depends on their proper support by the TCS. The immune components of TCS underwent during an organism development the developmental immune adaptation (DIA), developing immune competence (DIC), mature immune competence (immune competence), and immune senescence and aging diseases incidence (Im senesc and AD inc). The DIA represents a critical developmental period for the preservation of tissue function during the postnatal lifetime. The heart differentiates and functions from early stages of embryonic period (early embryonic) and it can function throughout the life. The brain begins to differentiate a little bit later (mid embryonic), and its function can be deteriorated earlier compared to that of the heart. The fetal ovarian primary follicles (fpf) begin to differentiate during the fetal period (moderate fetal), aging fetal follicles are depleted till menarche, after which they are replaced by the cyclic follicular renewal (fr). Normal function of the ovary lasts from menarche till 35-40 years. Aging primary follicles with altered oocytes are gradually depleted till menopause. The premature ovarian failure (POF) results from shorter development of the ovary during DIA (shorter fetal), and short DIA of ovarian development causes primary amenorrhea. The prenatal lack of corpora lutea causes their cyclic rejection^[1]. TCS: Tissue control system; CL: Corpus luteum; PRP: Prime reproductive period.

aging or disease. Consequently, infertility resulting from oocyte loss had been considered irreversible. However, multiple papers now cast doubt on that belief through the identification of a population of stem cells that give rise to functional oocytes. First, Antonin Bukovsky and colleagues at The University of Tennessee Knoxville published in the American Journal of Reproductive Immunology in 1995 that a subpopulation of human germline stem cells, now known as oogonial stem cells, could be collected from the ovaries of women undergoing surgery and used to generate what was perceived as oocytes in cell culture, based on detection of oocyte markers^[18,43,47].

The role of the TCS in ovarian follicular selection

It appears that initiation of tissue differentiation is primarily dependent on pericytes, the activity of which is prevented by autonomic innervation in the sites, where regeneration is not needed. In other words, the neural system releases inhibition of pericytes when the development of certain amount from the reserve structures present is required to differentiate. This positive behavior of the neural system ceases when the proper tissue quantity has been reached.

Figure 7 shows that the growth of a primary follicle among many resting ones is initiated by vascular pericytes, which exhibit and release Thy-1 differentiation protein from vascular pericytes. This process is apparently straightforward, and indicates that it is not hormonally dependent but regulated by a mechanism which determines how many resting follicles will be allowed to growth among the numerous existing ones. As indicated above, the activity of vascular pericytes is controlled by the autonomic innervation regulating the allowed tissue quantity.

The selection of the single human dominant follicle from multiple large antral follicles available in both ovaries is even more complex or sophisticated. The lack of inhibition of Thy-1 release in follicular theca interna causes follicular regression, since the thecal androgens kill the immature granulosa cells. The dominant follicle exhibits inhibition of thecal Thy-1 release, but its follicular lamina propria exhibits high Thy-1 release from vascular pericytes. This stimulates maturation of granulosa cells. The reason of follicular selection is that the species-specific number of ovulating follicles has to be ensured in the mammalian ovaries^[10]. If the maturation of granulosa cells is exogenously accelerated during the preparation for the *in vitro* fertilization (IVF), the multiple mature antral follicles will develop in both ovaries.

Meiosis I events in the fetal and adult human ovaries

It has been recently indicated that neo-oogenesis in adult mammalian ovaries is unreliable, since the intermediary meiosis I events of the new germ cells have not been demonstrated^[49]. We have described the involvement of ovarian stem cells in the emergence of new germ cells in human midpregnancy fetal ovaries (Figure 8) and shown that new germ cells emerging during the midfollicular phase in the adult human ovaries exhibit meiosis I inter-

mediary events, which are accompanied by CD8⁺ TC and CD14⁺ MDCs (Figures 9 and 10). In the adult human ovaries the germ cells enter blood vessels for their transport to form new adult primary follicles after association with the granulosa cell nests. In addition, the activated MDCs are associated with the formation of new granulosa cells from bipotential OSCs (Figure 11). Similar types of blood mononuclear cells were associated with the formation of new adult germ cells and granulosa cells from the OSCs and with follicular renewal in the adult rat ovaries^[45].

We were unable to detect any persisting primordial germ cells either in midpregnancy fetal or in adult human ovaries. The only detected germ cells were those seen during and after their development from OSCs. The basic involvement of the primordial germ cells appears to be a commitment of OSCs for their potential differentiation into germ cells^[50].

Why the menopause occurs?

In older women the germ cells may still be formed, since they are already present in the embryonic gonads and can regenerate during the lifespan. Figure 12 shows that the association of the circulating small oocyte with granulosa cell nests is essential for its survival. The granulosa cells, which are required for the formation of new primary follicles, appear during the second trimester of the fetal life, and, therefore, their formation during adulthood is terminated between 35-40 years of life. It is apparent that without association with granulosa cell nests, the germ cells will regress immediately in the adjacent ovarian medullary vessels or in the vessels at distant places. Therefore, the lack of granulosa cells causes a lack of follicular renewal, and after a depletion of persisting primary follicles with dysfunctional aging oocytes, the menopause will occur.

The role of secondary Balbiani body in the oocyte maturation

The primary Balbiani body disappears in the resting primary follicles. The oocyte maturation is characterized by expression of zona pellucida glycoproteins. One of them, the zona pellucida glycoprotein 3 (ZP3), is required for the oocyte binding of sperm^[51], and it is absent in the resting follicles, but expressed in the growing primary ones. Nevertheless, the growing preantral follicles are still unable to respond the *in vitro* maturation. In the small antral follicles the secondary Balbiani body was detected (Figure 13), and such follicles are competent to mature *in vitro*^[52].

Human gonadal infertility treatment with the young blood mononuclear cells

There is a growing shift from younger to older women wishing to deliver their own genetic child, but their aged oocytes are unable to produce a successful pregnancy. The IVF technology exhibits a 33% success rate in the young infertile women, but the live birth rate

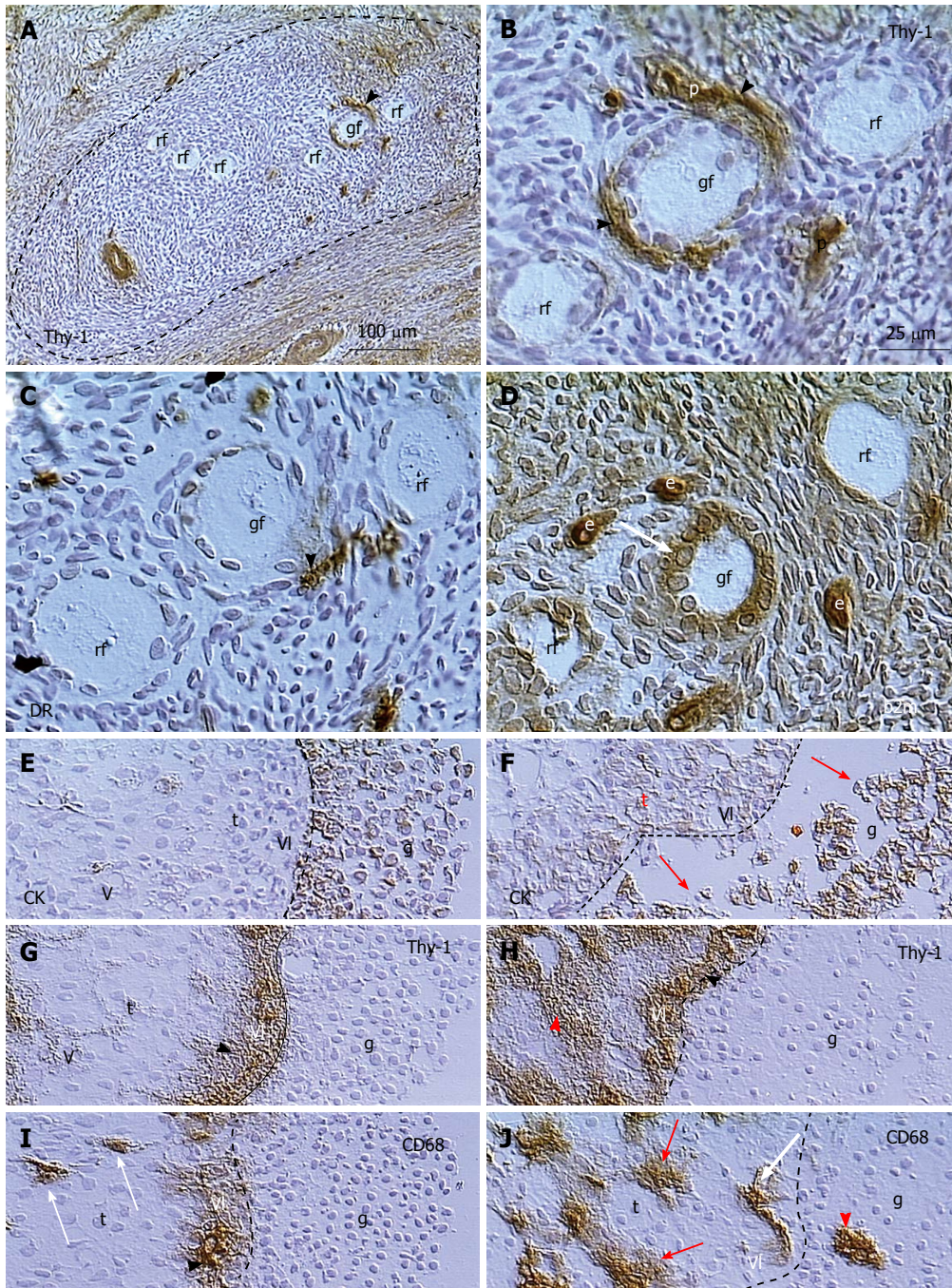


Figure 7 Follicular selection. A: Resting primary follicles (rf) are present in ovarian area without Thy-1 expression by stromal cells, where growing primary follicle (gf) receives Thy-1 supply from vascular pericytes (arrowhead); B: Detail from (A) shows Thy-1 supply (arrowheads) from vascular pericytes (p) for the growing primary follicle; C: Parallel section showing association of DR⁺ activated MDC (arrowhead) with the growing but not resting primary follicles; D: Growing primary follicle exhibits large granulosa cells with strong expression of beta2m; E: Dominant preovulatory follicle granulosa cells (g) with expression of cytokeratin (CK) are attached to the follicular basement membrane (dashed line). Note lack of staining in vascular lamina propria (vl) and theca interna cells (t); F: Regressing large antral follicle in the same ovary exhibits detachment of granulosa cells (arrows) and CK expression in the vascular lamina and thecal cells; G: Thy-1 expression in vascular lamina (arrowhead) but no staining of theca interna in dominant follicle; H: Regressing follicle with Thy-1 staining in both, vascular lamina propria and theca interna (red arrowhead); I: CD68 is released from MDCs in the vascular lamina (arrowhead) of the dominant follicle, but not from thecal MDCs (arrows), and no MDCs are present among the granulosa cells; J: In the regressing follicle the MDCs release CD68 in theca interna (red arrows), and invade among granulosa cells (arrowhead)^[171].

gradually decreases after 35 years of age. Compared to the complex, expensive, and unsolved treatment of infertility of older women by IVF clinics, the transfusion morphostatic treatment (TMT) ameliorating morphostasis of altered tissues, including the ovary, may easily solve such infertility, regardless of the women's age^[46]. Add-

itional possibilities of partial blood volume replacement from appropriate compatible young donors could also be investigated, e.g., treatment of male infertility, which affects approximately half of infertile couples worldwide^[53]. The TMT by a partial (500 mL) blood replacement with the young blood from a fertile healthy

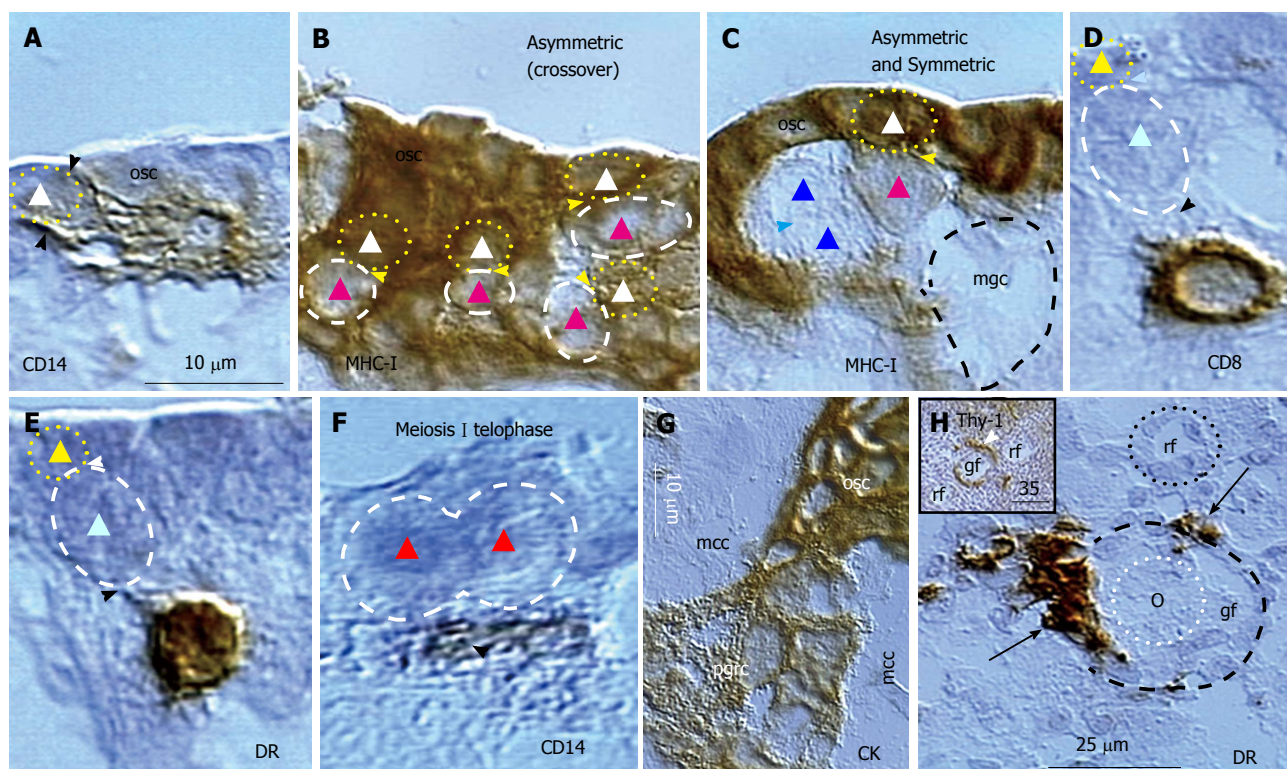


Figure 8 Origin of germ and granulosa cells in the ovary of the human midpregnancy fetus. A: Isolated ovarian stem cell (triangle) is associated with CD14 pMDC (arrowheads); B: Fetal germ cells (red triangles) lacking MHC-I expression originate from MHC-I+ ovarian stem cells (white triangles); C: The symmetric division of germ cells (blue triangles) follows asymmetric division (white and red triangles) of ovarian stem cells, and causes emergence of moving germ cell (mgc); D: The origin of germ cells requires association of CD8⁺ (D) and DR⁺ (E) T cell; F: Meiosis I telophase (red triangles) is associated with a primitive MDC (arrowhead); G: The fetal primitive granulosa cells (pgrc) originate from ovarian stem cells between the mesenchymal cell cords (mcc); H: Small growing follicle (gf) is accompanied by DR⁺ MDCs (arrows), which are absent at the resting follicle (rf). Inset shows Thy-1⁺ pericytes (arrowhead) during follicular selection (Figure 7). Bar in A for A-F. Adjusted from [44] with a permission: ©Springer United States. MDC: Monocyte-derived cell; pMDC: Primitive MDC.

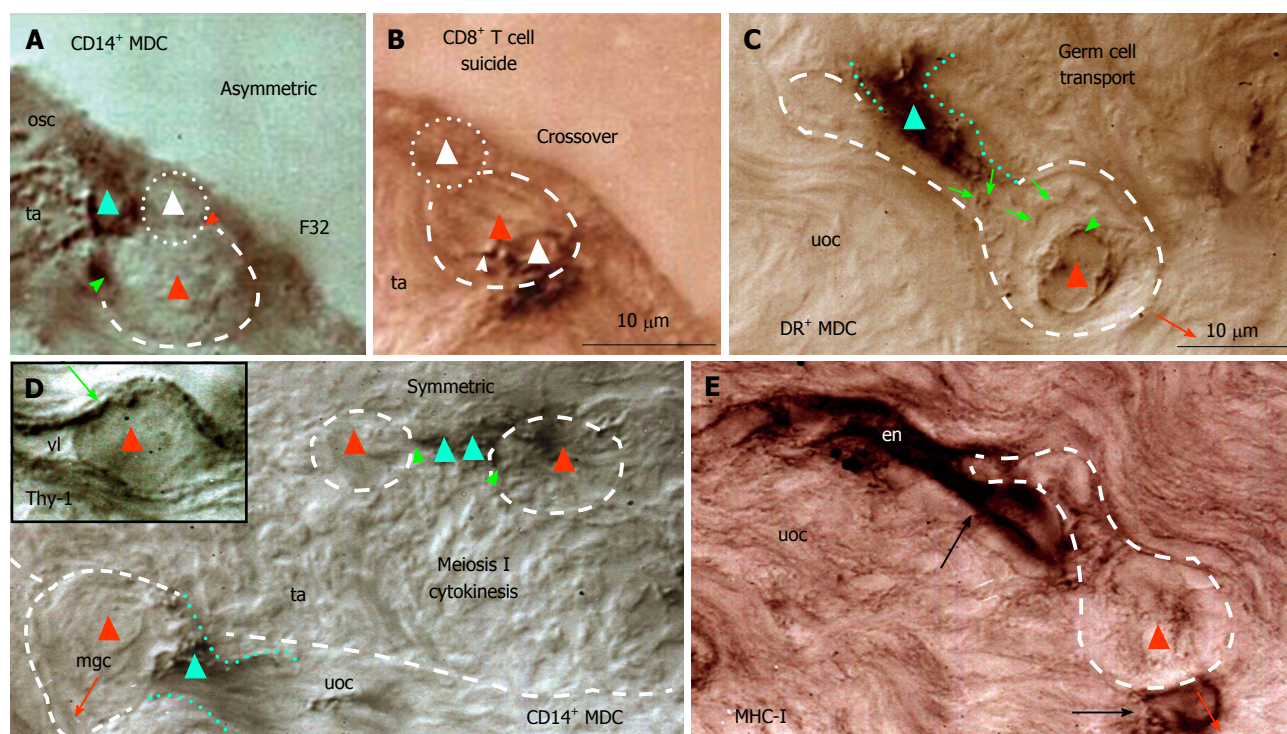


Figure 9 Origin and migration of germ cells during the midfollicular phase in the adult human ovary. A: In the presence of primitive MDC (green triangle) the dividing ovarian stem cell (white triangle) produces a germ cell (red triangle); B: This is accompanied by the presence of CD8⁺ T cell (white triangle) with extensions (arrowhead) within the germ cell; C: Migrating germ cell (dashed line) is accompanied by DR⁺ MDC (dotted lines), which releases DR (arrows) accumulating at the germ cell nucleus (arrowhead); D: In the tunica albuginea (ta) MDCs (green triangles) are associated (green arrowheads) with meiosis I cytokinesis (red triangles) and accompany moving germ cell (mgc) in the upper ovarian cortex (uoc). Inset shows Thy-1⁺ cortical venule containing germ cell (red triangle); E: Migrating germ cell lacking MHC-I expression is associated with stained endothelial cells of a venule in the upper ovarian cortex. F32 indicates a female patient's age [6]. MDC: Monocyte-derived cell.

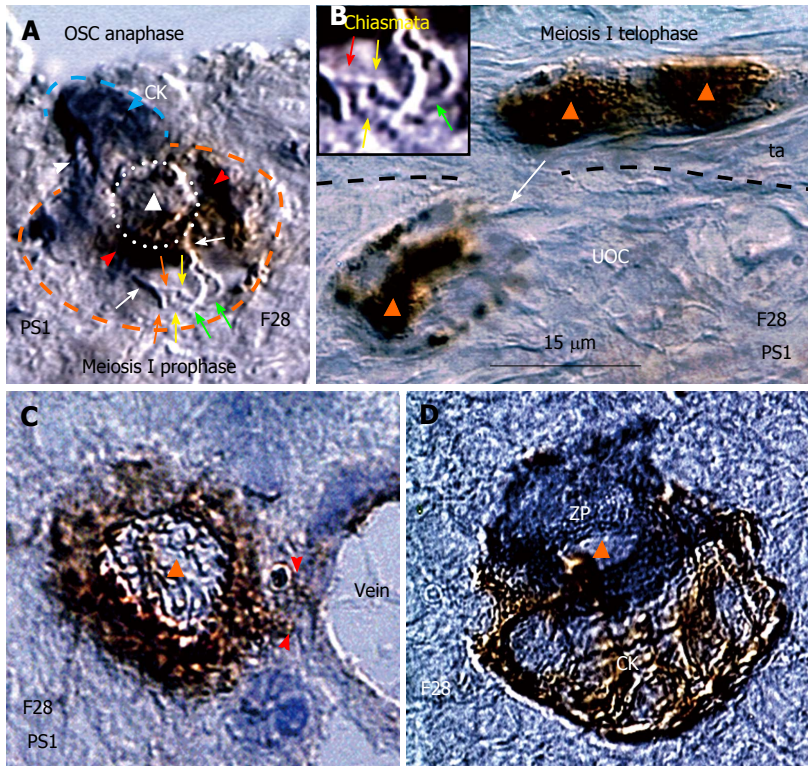


Figure 10 Meiotic events during midfollicular phase are followed by follicular renewal. A: Cytokeratin stained ovarian stem cell (OSC) (blue arrowhead) moves its chromosomes (white arrowhead) during the OSC anaphase to the OSC end. The germ cell expressing PS1 meiotic protein (red arrowhead) exhibits meiosis I prophase with chromosome (white arrows) duplication (red and green arrows) and crossover of sister chromatids (yellow arrows). The triangle indicates putative suicidal T cell (see Figure 9B) inducing expression of PS1 in the emerging germ cell; B: Marked nuclear expression of PS1 accompanies germ cell telophase of meiosis I in the tunica albuginea (ta). Arrow indicates migrating postmeiotic germ cell. Inset shows a detail of interacting chromosomes (red and green arrows) from panel A; C: The germ cell entering (arrowheads) the cortical vein exhibits cytoplasmic but not nuclear (triangle) PS1 staining; D: Association of zona pellucida+ small oocyte with CK⁺ granulosa cell nest during the new primary follicle formation in the lower ovarian cortex^[43]. All panels are from the identical ovary during midfollicular phase of the 28-year-old women.

woman collected about 3 d after menstruation may induce fertility^[46].

Blood parabiosis between young and aged mice was proposed to ameliorate function of tissue cellular progenitors caused by the serum of young blood^[54]. This was supported by additional studies^[46]. The efficiency of the young blood may not be dependent on the serum content alone. The circulating mononuclear cells may contribute to the transfusion-induced benefits for tissue cells^[8].

A single small volume partial blood replacement can induce formation of both, the new germ and granulosa cells. This can result in the development of new primary follicles, which are stimulated by the endocrine system to mature and ovulate. A single partial blood volume replacement from young to the aged animal is not consistent with the heterochronic parabiosis. The animal parabiosis has at least 150 years history^[55]. It represents a situation when the blood of two animals is mixed for a long time period. Although GDF11 serum substance reversed age-related cardiac hypertrophy^[56], more investigations are required to resolve differences between of the young vs aged blood components for possibilities in the long-lasting rejuvenation of various aged tissues.

An advantage of gonads is that a temporary renewal of their function is sufficient for temporary ovarian or

testicular ability to produce functional mature gamete(s). This ability may last for a certain period, since the transfused white blood cells were found to survive in the 7 of 10 trauma patients for 6 mo to 1.5 years^[57]. The partial blood volume replacement-inducing gonadal rejuvenation can be repeated from a distinct suitable individual, if needed. Once the self oocyte is fertilized, the regular pregnancy can be maintained. The eggs from young donors provide 30% live birth rate, regardless of the recipient's age^[58]. This is comparable with a live birth rate in young infertile women treated by the standard IVF technology^[58]. The young healthy egg donors with a regular menstrual cycle can be excellent donors of compatible blood collected during the early midfollicular phase (about 3 d after menstruation^[46]).

It is impossible to accept that the 18 to 35 years old primordial oocytes in primordial follicles will provide healthy babies. The numbers of human ovarian oocytes did not exhibit a significant change between 18-38 \pm 2 years of age, but decreased thereafter^[59,60], when the IVF live birth rate begins to decline. This indicates that 18-38 \pm 2 years of age is the prime reproductive period (PRP). Because even during the PRP more than 60% of the oocytes exhibit degenerative changes^[61], and follicles with altered oocytes are cyclically eliminated by the immune effectors^[43], the presence of follicles with

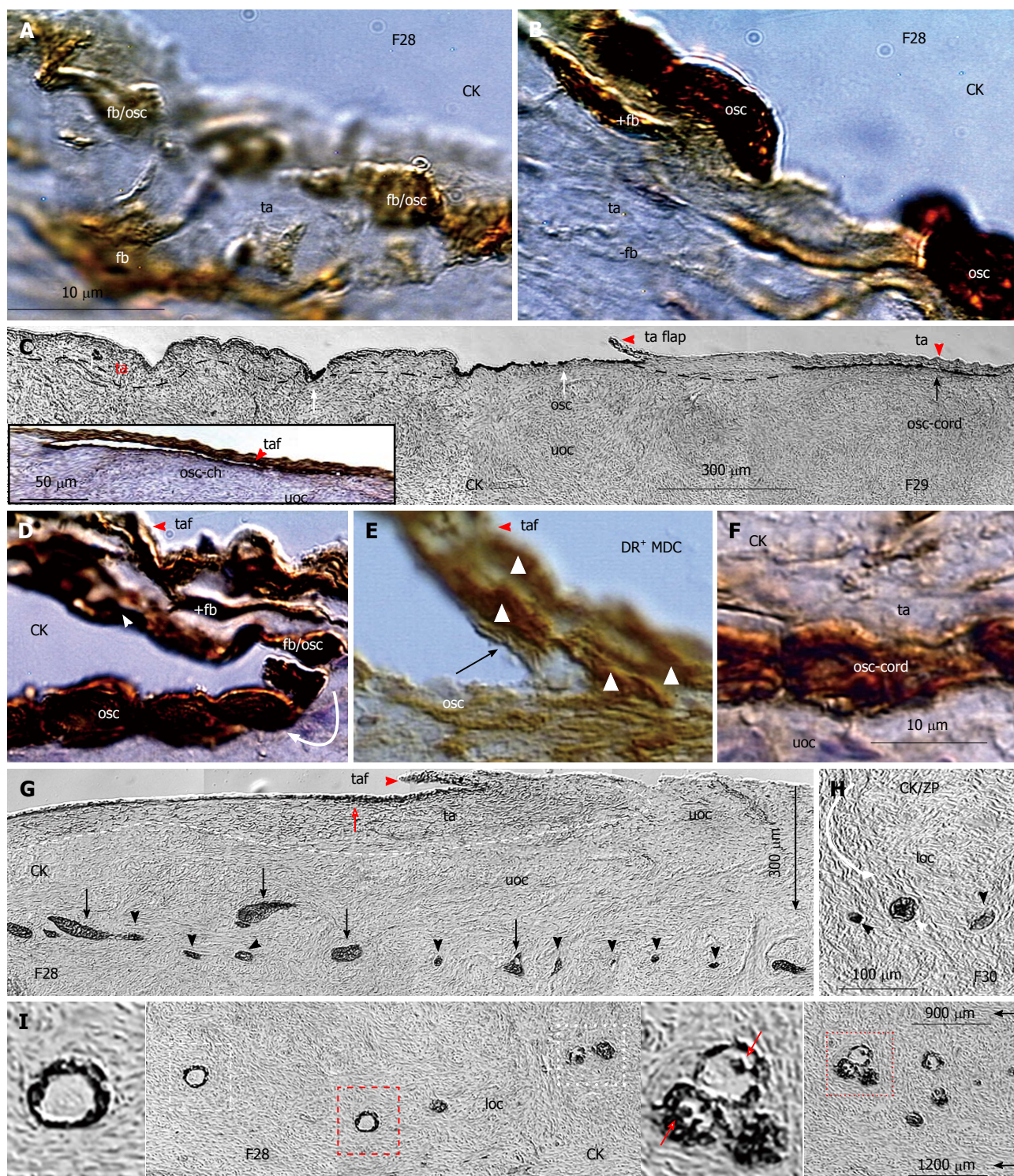


Figure 11 Origin of ovarian stem cells and formation of granulosa cell nests migrating to the lower ovarian cortex in midfollicular ovaries. A: The CK⁺ fibroblast-shape cells (fb) in tunica albuginea develop into ovarian stem cells (OSC) precursors (fb/osc) to form new OSC cells (B); C: Certain segments of ovarian surface are covered by OSCs (white arrows) to form by extensions the tunica albuginea flaps, the OSC channels (inset), and OSC cords (black arrow). The flaps originate from CK⁺ fibroblasts, and are covered by CK⁺ OSC precursors (fb/osc) (D), contain DR⁺ (activated) MDCs (E), and form bilaminar OSC cords (F); G: The CK⁺ OSC-derived clusters (arrows) of granulosa cells undergo fragmentation into granulosa cell nests (arrowheads), which are transferred by stromal rearrangements to the lower ovarian cortex (H) to form new primary follicles (white arrowhead); I: New primary follicles contain CK⁺ primary Balbiani bodies (right inset), which are consumed, and absent in the resting follicles (left inset)^[43]. The panels A, B, G and I are from the identical ovary of 28-year-old women presented in the Figure 10. MDC: Monocyte-derived cell.

unaffected oocytes will not last for more than a few ovarian cycles. Therefore, the follicle numbers cannot remain unchanged during the PRP without a cyclic

follicular renewal. The follicles with altered oocytes, however, persist after 35 years of age, when the IMS begins to exhibit significant functional alterations^[37], thus

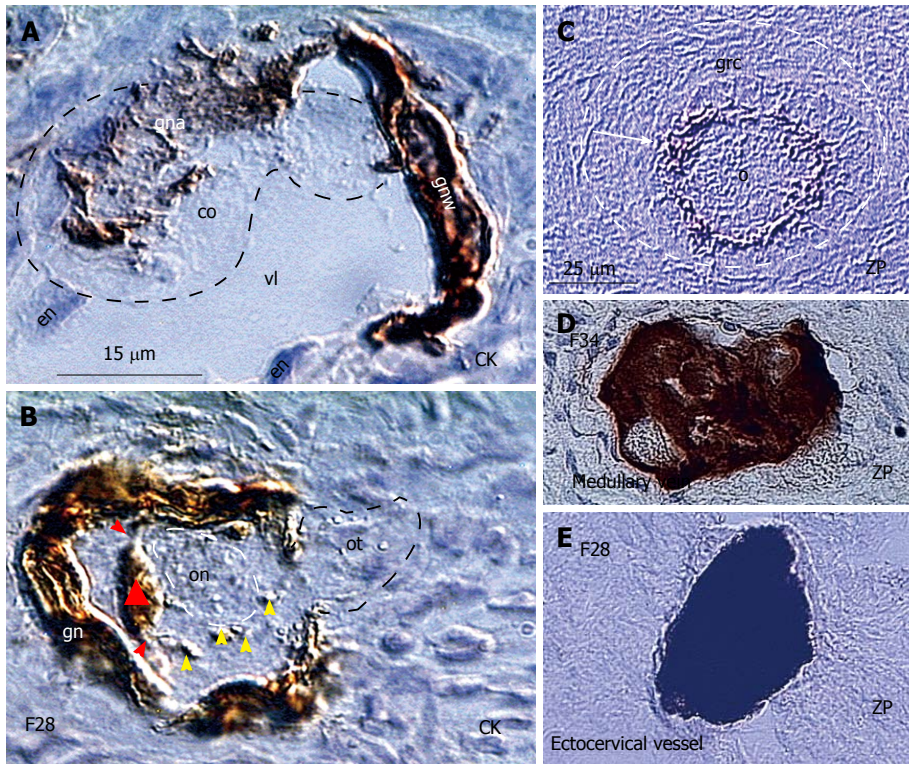


Figure 12 Granulosa cells are essential for the *in vivo* survival of the newly formed oocytes. A: The small circulating oocyte (co) is captured in the lower ovarian cortex by CK* granulosa nest arm (gna) in the vein lumen (vl) lined by endothelial cells (en) and granulosa nest wall (gnw); B: The CK* primary Balbiani body (triangle) adjacent to the oocyte nucleus (on) is formed by granulosa cell extensions (red arrowheads) and disperses its particles (yellow arrowheads) to the freshly captured oocyte, which still exhibits the oocyte tail (ot) outside of the nest; C: Intact oocyte (o) covered by ZP* membrane (arrow) and granulosa cells (grc) in the primary follicle (dashed line); D: The vein in ovarian medulla in the identical ovary contains regressing oocyte with marked cytoplasmic ZP staining; E: Regressing oocyte with strong cytoplasmic ZP expression found in the ectocervical vein in the women showing follicular renewal in A and B panels. Panels A and B adjusted from^[43]. © Antonin Bukovsky; C-E from^[172], with a permission: ©Elsevier/North-Holland Biomedical Press. The panels A, B, and E are from the identical ovary of 28-year-old women

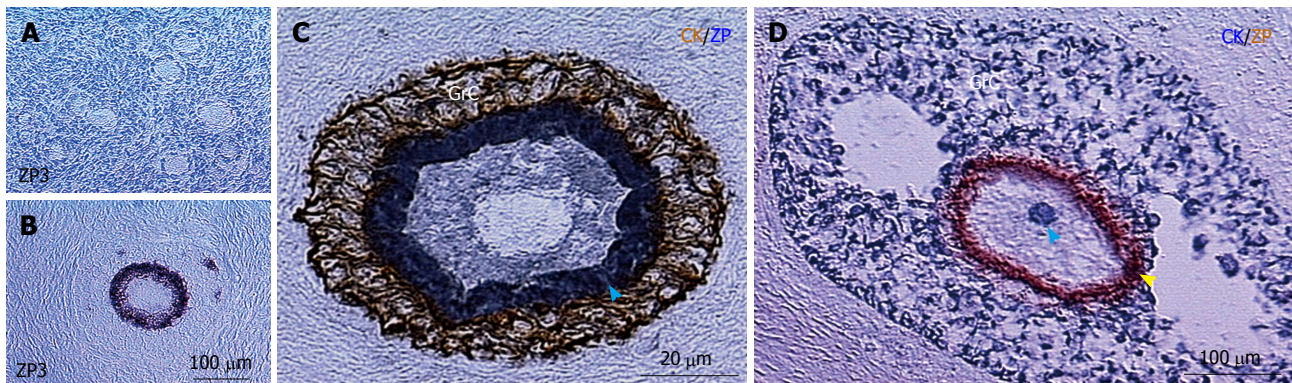


Figure 13 ZP3 expression on growing preantral follicles and development of secondary Balbiani body in small antral follicles. A: Resting primary follicles express ZP1, ZP2 and ZP4^[172], but lack ZP3 expression; Growing preantral follicles show oocyte ZP3 expression (B), but lack the secondary CK* Balbiani body (C); which develops in the small antral follicles (blue arrowhead in D)^[46]. ZP: Zona pellucida glycoprotein.

promoting follicular renewal cessation and persistence of follicles with altered aging oocytes.

Clinically, ovarian infertility begins to affect human females after 35 years of age and increases progressively thereafter. This biology contrasts with the growing shift from young to older women wishing to begin to deliver their own genetic children. The observed live birth rate using patient oocytes in IVF technology markedly declines after the age of 40 years. The live birth rate is 33%

between 18-34 years of age, 27% between 35-37, 20% between 38-39, 13% between 40-42, 5% between 43-44, and 2% between 45-50 years^[58]. Consequently, the infertile women after age of 40 are rarely treated by the IVF clinics.

The TMT represents a simple *in vivo* method without the need of any hormonal stimulation, collection and incubation of oocytes, fertilization and embryo selection, endometrial implantation of embryos, and eventual

purchase of donor oocyte(s). If successful in humans, the partial blood volume replacement will be significantly cheaper compared to IVF costs and will allow women who are unable to cover the IVF expenses to carry their own genetic children. Nevertheless, the IVF clinics still will be needed to manage the preparation for the TMT partial blood volume replacement procedure in infertile women, regardless of their age. The IVF centers may also execute TMT by themselves, or negotiate a cooperation with the blood transfusion centers.

The partial blood volume replacement is recommended instead of a simple blood transfusion, in order to prevent an overdose of erythrocytes. An alternative is the utilization of blood extract without erythrocytes, or use the separated mononuclear cells with serum. This will not require the partial blood volume replacement^[46]. However, a partial fresh blood replacement should be preferred, since it will minimize the alteration of young blood properties, and improve a proportion between the new and old blood functional abilities.

The TMT might also be essential for the treatment of older infertile men, the solution of which remains basically unresolved^[46]. The IVF clinics will also remain essential for the intracytoplasmic sperm injection in the cases where the naturally evolved patient's oocyte fails to be fertilized *in vivo*. In the cases where the first TMT fails to rejuvenate the ovaries within six months, another TMT attempt could be made with the blood from a distinct young fertile female donor. The TMT can also be accompanied by the IVF type hormonal stimulation during the ovarian follicular phase. This will produce more mature preovulatory follicles, a proportion of which may be collected and preserved for the additional fertility rejuvenation of the patient, if needed.

The effect of TMT for the rejuvenation of aged or otherwise infertile human ovaries is not immediate, since even in normal human ovaries, the formation of primary follicles and their differentiation into mature ones will require several months^[46]. This might be faster in perimenopausal or women with premature ovarian failure lacking ovarian cycle and the aged ovarian follicles.

In human ovaries, development of granulosa cell clusters is followed by their transition to the lower ovarian cortex (approximately one thousand microns from the ovarian surface) where the new adult primary follicles are formed by the association with the germ cell-derived small oocytes delivered by the cortical venous blood^[46].

The TMT is also expected to successfully eliminate the persisting ovarian follicles with aged oocytes in the older infertile women with persisting ovarian cycles, which are not suitable for fertilization. The persisting menstrual periods are supposed to disappear until the new mature follicles are formed from the fresh germ and granulosa cells. However, the new adult primary follicles are expected to form new resting primary follicles, which can sequentially differentiate for several additional years^[46]. A possibility also exists that, once started, the follicular renewal may last for a certain period of time, *i.e.*, for the six months to the 1.5 years,

due to the persistence of transfused white blood cells in the recipient's blood.

The in vitro treatment of ovarian infertility

An important option for IVF clinics, which can have a significantly faster effect, is an *in vitro* development of multiple oocytes from the patient's own OSCs. This method was developed ten years ago^[47], but the clinical trial^[10] failed to produce mature oocytes that resumed meiosis II stage after *in vitro* maturation (IVM) which were expected to be suitable for fertilization. A similar failure of the neonatal skin cell-derived porcine and mouse OLCs was reported by others, and their research was abandoned^[62,63].

The human serum, including serum from donor-collected blood, should replace animal serum formerly used in the OSC cultures. In addition, the *in vitro* developing oocytes need additional organelles to enlarge. They either associate with fibroblasts, which causes their regression, or divide to produce sister germ cells, the organelles of which they consume^[50]. It has been recently shown, that consumption of sister germ cell organelles occurs in ovaries of fetal mice^[64]. The oocyte growing *in vitro* uses the same fetal mechanism to grow into the large size with a germinal vesicle (Figure 14), but it is not fertilizable because it lacks granulosa cell organelles essential for the meiosis II resumption.

The main problem of the OLC cultures, that needs to be resolved, is the absence of granulosa cells, which form primary Balbiani bodies during renewal of primary follicles, and secondary Balbiani bodies in the growing small antral follicles^[46]. The granulosa cells-derived Balbiani bodies provide important new organelles to the oocyte, including the Golgi vesicles, nascent forms of smooth endoplasmic reticulum, and endoplasmic reticulum membranes, which are needed by oocytes to functionally mature^[65].

Granulosa cells for the oocyte *in vitro* maturation may be of a second party origin, or they may be developed along with the OLCs *in vitro* from the patient's own bipotential OSCs. The development of the self granulosa cells will require the presence of mononuclear cells, and MDCs in particular, which can be separated for several OSC cultures of the patient from about 20 mL of a young fertile woman compatible blood during her early midfollicular phase (about 3 d after menstruation)^[46].

A review of our former investigations revealed that the fibroblasts in the IVM-treated OSC cultures appropriate the cytoplasmic organelles from the OLCs, including the ZP3 (Figure 15), which is required for the sperm-egg binding^[51]. Consequently, the sperm in such cultures bind to the fibroblasts instead to the OLCs. An improved *in vitro* development of fresh mature oocytes from OSCs, with the presence of granulosa cells and avoidance of fibroblasts^[46], may cause the development of multiple oocytes suitable for IVM, fertilization and implantation, and for the preservation in a frozen stage for the future needs.

Since the testicular infertility has been detected in approximately half of infertile couples^[53], the male partner

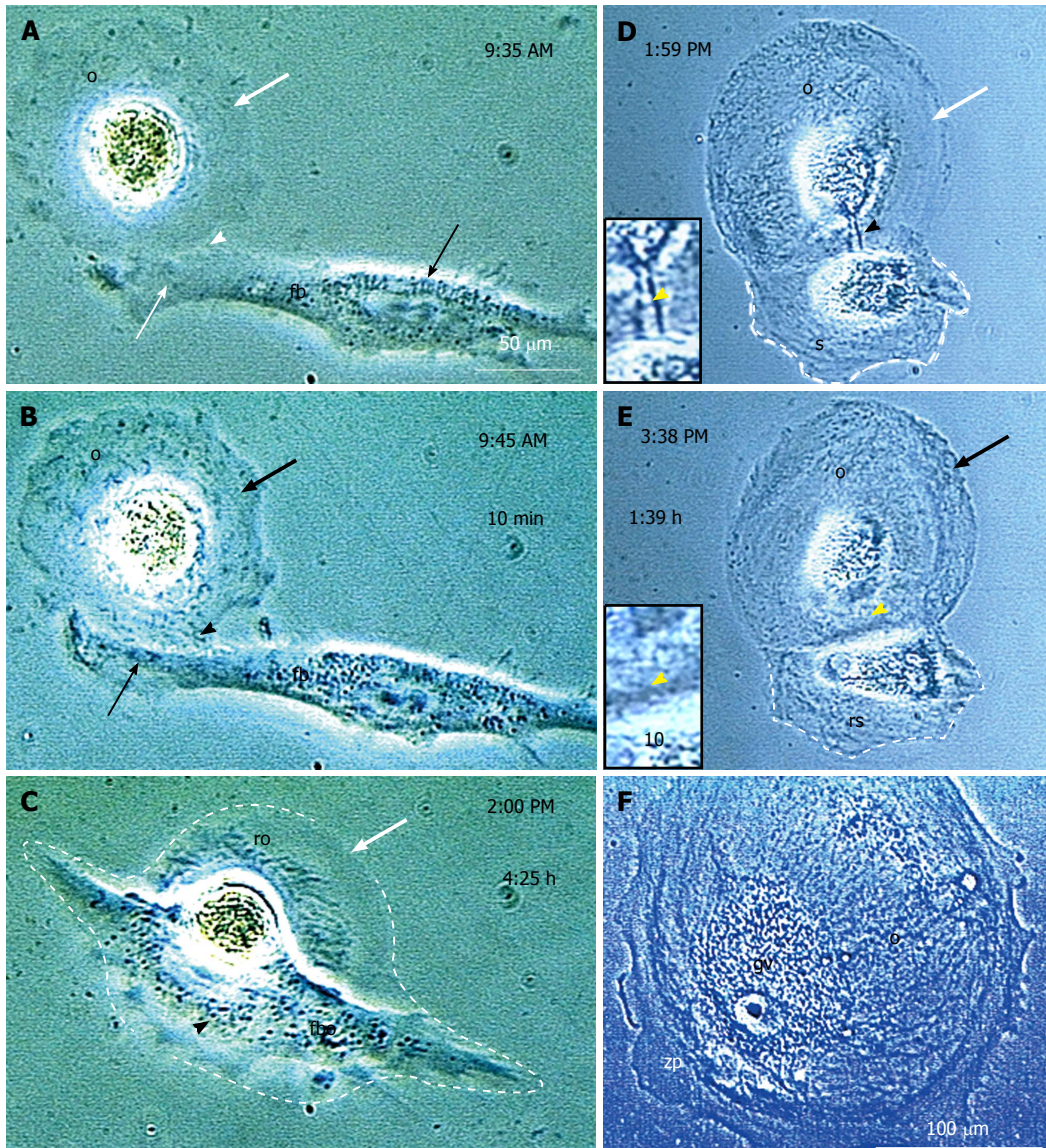


Figure 14 *In vitro* developed oocyte-like cells retrieve meiotically nonfunctional organelles from fibroblasts or satellite daughter cells. A: The *in vitro* developed oocyte-like cell (OLC) (o) with a lack of optically dense organelles (bold white arrow) is joined (arrowhead) by a fibroblast (fb) which provides organelles (B), but forms a fibro-oocyte hybrid causing oocyte regression (C); Alternatively, the OLC forms a satellite daughter (s in D), which is drained (black arrowhead and inset) and regresses (rs in E) when the OLC is saturated (black arrow). This results in the large OLC (F) with germinal vesicle (gv) and thick zona pellucida (zp), but provided organelles are not functional for resuming meiosis II. Panel F reprinted from^[10]. ©Antonin Bukovsky. Other panels adjusted from^[60], with a permission: ©Wiley-Liss, Inc.

should also be investigated for the sperm quality in couples planning TMT in the infertile female partner. If inappropriate, the treatment of the male partner with TMT by the blood from a young sexually active, fertile, and healthy man could be considered. Subsequently, the fresh sperm can develop within three months^[46]. Occasional masturbation in the meantime will be suitable to deplete the aged or altered sperm.

STEM CELL THERAPY IN REGENERATIVE MEDICINE

Are there any perspectives for stem cells in regenerative medicine?

The stem cell therapy attempts to improve function of altered tissues by implantation of *in vitro* developed

tissue-specific stem cells^[66]. It has been recommended that stem cell therapy has to be personalized for the following seven points: The stem cell culture should be free of animal components, exhibit a lack of teratogenic and tumorigenic potential of stem cells, determination of an appropriate dose of cells to be transplanted, ability of stem cells to underwent asymmetric division to differentiate and maintain a pool of stem cells, ability to prevent cell aging resulting in senescence of differentiating cells, determine avoidance of karyotypic abnormalities, and check immunosuppressive activity (immunomodulation) of transplanted stem cells^[67]. According to the available data, such stem cells have not been developed yet.

Embryonic stem cells can differentiate into any cellular type, but their use is virtually abandoned due to their allogeneicity, tumorigenicity, and ethical stem cell con-

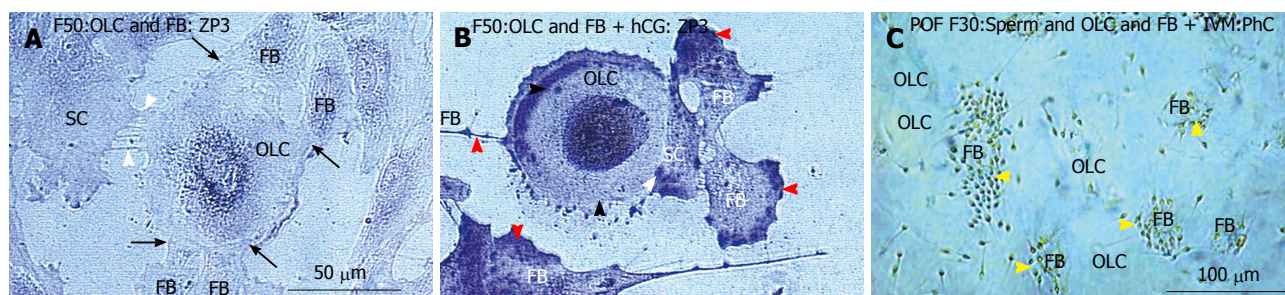


Figure 15 Fibroblasts steal the ZP3 sperm-binding protein from *in vitro* maturation treated oocyte-like cells. A: Untreated culture shows low perinuclear ZP3 in the oocyte-like cell and traces in the satellite cell (SC) and accompanying fibroblasts (FB); B: After hCG, the marked ZP3 staining is apparent, which is drained by FBs (red arrowheads); C: Adding sperm to OSC culture after IVM treatment caused that the sperm were binding to the fibroblasts (arrowheads) instead to the OLCs^[46]. IVM: *In vitro* maturation; ZP3: Zona pellucida glycoprotein 3; OLCs: Oocyte-like cells.

trovery^[67]. The induced pluripotent stem cells (iPSCs) were developed to avoid these issues. They exhibit growth properties, morphology, and embryonic stem cell markers^[68], and can be generated by a few defined pluripotency gene factors from autologous human fibroblast cultures^[69,70]. It has been, however, later reported that human iPSCs are even more efficient and faster in developing teratomas in the immune-compromised mice than human embryonic stem cells^[71]. This may be based on genomic instability of human iPSCs, which may compromise clinical utilization of these cells^[72].

After several decades of the stem cell therapy research^[73], the effective clinical stem cell therapy remains, in most instances, in the stage of possible successful perspectives^[74-78]. The prevailing rationale for the regenerative stem cell therapy is that the newly provided stem cells will develop to replace the dysfunctional cells in the affected tissue(s). This is expected to solve, for instance, a need for the liver or heart replacement, the options for which are very limited due to the lack of the sufficient organ transplant resources. The issue, a knowledge of which is required to be better understood, is why the cells in the affected tissue are dysfunctional. In addition, there are two distinct situations - the tissue dysfunction is already present from the beginning of postnatal life (dysfunction of pancreatic beta cells in type 1 diabetes), or tissue dysfunction develops latter, *e.g.*, hepatic disease caused by viral infections, alcohol, or age-associated degenerative diseases.

The regenerative medicine could be effective in trauma-affected younger human beings, which carry intact stem cell niche, but not in patients with chronic disease accompanied by an altered niche of stem cells. The stem cell niche includes MDCs, which prevent unaffected organs from aging of tissue cells by the proper StE, which stops the cellular differentiation toward aging after the tissue cells attain the functional stage.

The alteration of StE in the brain will cause aging of implanted neuronal stem cells^[79]. The age-altered StE could be repaired by the TMT, which can supply circulating MDCs from young healthy individuals. In this way, the proper differentiation of existing tissue stem cells or their implantation could be effective. The persistence of transfused white blood cells for 6 mo to 1.5 years^[57], or longer^[80], may result from donor stem cell engraftment

resulting in the tolerance of donor leucocytes^[80].

The stem cell therapy without the cells

The neuronal and myocardial regeneration induced by TMT from an appropriate young donor could be enhanced by sex steroid combinations causing the transition of microvascular pericytes (vascular smooth muscle cells) to the stem cells for neuronal or myocardial cells^[79,81,82]. A combination of sex steroids has been detected to circulate in human fetuses^[83]. This observation suggests that circulating sex steroids are not altering the fetal development, but they can be needed, for instance as neurosteroids, to stimulate a regular development of the brain.

The so called "stem cell therapy without the cells"^[84] can eliminate most of the roadblocks accompanying the development and application of the currently considered human stem cell therapies^[84-86].

Consideration of sex steroids for the regeneration of central nervous system and heart

The presence of endogenous pluripotent cells capable to respond physiological substances is of interest. Neuronal type cells have been occasionally found in human ovarian stem cell cultures^[47], and such cultures exhibited the development of numerous neural/neuronal type cells after the treatment with sex steroid combinations^[87]. Additional observations have shown that sex steroid combinations are also capable to transdifferentiate vascular smooth muscle cells (VSMC) into differentiated neuronal cells^[81,88].

Our former *in vitro* studies^[79] have shown that the incubation of vascular smooth muscle cells with 60 mmol/L progesterone alone induced a rare development of cells with early neurite extensions. This concentration of progesterone is relevant to the 16 mg/kg, which reduced consequences of traumatic rat brain alteration^[89]. This indicates that the progesterone alone may have some *in vivo* neuroprotective effect.

The 20 mmol/L dose of progesterone lacked any *in vitro* effect on cultured VSMC. When 60 mmol/L progesterone was combined with 60 mmol/L testosterone, we observed numerous neural stem cells with neurite extensions^[81]. Therefore, a moderate dose of

testosterone combined with moderate dose progesterone significantly accelerated differentiation of neural cells from VSMC. In addition, utilization of 20 mmol/L progesterone along with 60 mmol/L testosterone caused a direct transdifferentiation of VSMC into neuronal type cells with branching neurites. The 20 mmol/L progesterone is relevant to the dose of progesterone suppositories (200 mg progesterone twice a day), which are used to prevent an abortion and a premature labor^[90,91]. The *in vitro* testosterone concentration is relevant to weekly intramuscular injections of 600 mg of androgens which are utilized in human males^[92,93]. Therefore, the use of 400 mg progesterone daily and weekly injections of 600 mg testosterone may be considered for the clinical trials committed to alleviate the brain dysfunctions, preferably along with TMT in degenerative brain disorders.

Figure 16 shows influence of progesterone with testosterone on the cultured human VSMC, causing their transdifferentiation to the neuronal type cells connected by their neurites. Inclusion of a low dose (12 mmol/L) of estradiol with the 20 mmol/L of progesterone and 60 mmol/L testosterone induced a formation of numerous stem-type cells with bubble-like anchors, indicating a readiness for their settlement. Such cells formed within five days fresh new smooth muscle cells^[81]. The vascular pericytes are able to differentiate into cardiomyocytes^[94], which may be plausible for the treatment of heart disorders. The estradiol 12 mmol/L *in vitro* corresponds in humans to 120 mg *in vivo*. The transdermal 50 mg dose of 17 beta-estradiol was utilized twice a week for the hormone-replacement therapy^[95].

Vascular smooth muscle cells influence properties of endothelial cells and maintenance of the blood vessels. They were suggested to exhibit pluripotency for distinct types of cells^[96]. An utilization of all three sex steroid combination (400 mg of progesterone daily + 600 mg of testosterone weekly + 50 mg of estradiol twice a week) for two to four weeks can induce improvement of vascularization after a stroke and regeneration of an altered heart^[79].

It was proposed that in the early aging or at risk patients for the brain or vascular and heart disorders, the progesterone and testosterone combination may induce a preventive or regenerative influence. This could be sufficient for the brain, vascular, and heart regeneration, since it will also cause estradiol formation by the endogenous aromatase. The sex steroid treated vascular smooth muscle cell cultures exhibited significantly higher estradiol concentration in the presence of progesterone and testosterone combination, than when the testosterone or progesterone are used separately^[81]. The sex steroid hormonal combinations may be applicable locally, e.g., in the acute alteration of the spinal cord, stroke, or myocardial infarction in younger individuals or along with TMT in older patients.

The replacement of a partial blood volume from a similar but possibly distinct proper blood donor could be repeated at 6 mo intervals, if needed^[46]. The preservation of regenerated tissues in the functional state

could also be supported by drugs that stimulate the IMS morphoregulatory functions - honeybee propolis treatment influences tested on animals.

CAN THE AGING BE DELAYED?

How to extend the body rejuvenation?

In addition to the gonads, the partial blood volume replacement from appropriate young donor is expected to temporarily rejuvenate the function of other body tissues^[46]. A partial volume compatible blood replacement from a healthy young individual of the same sex and ethnicity could improve functional disorders of tissues, where the IMS components regulate the asymmetric stem cells division, development of differentiating stem cell daughters, and preservation of tissues in the proper functional stage^[46]. This can cause prevention of tissue cell aging and degeneration^[1], e.g., aging of neuronal cells in the Alzheimer's disease. The promising aspect of the chemical approach in the neuronal and heart disorders is based on the lack of a need to implant *in vitro* developed stem cells. The regenerative medicine is supposed to be more effective in younger individuals with an intact morphostatic stem cell niche as compared to the chronic and degenerative disorders caused by an altered stem cell niche^[79].

A longer mitigation of aging will, however, require a continuous rejuvenation of the IMS morphoregulatory functions, particularly the thymic function. Homeostatic functions of the IMS significantly deteriorate with age. The age-induced thymic involution is delayed in women compared to men^[97]. This may be why the women live longer compared to men^[46].

Health advantages of the honey bee propolis and cayenne pepper use

The propolis has been used empirically for centuries to help to create effective health improvements^[98]. The utilization of local propolis treatment for prevention of hair depletion, diminution of varicose veins, and prevention of dental calculus formation have not been, however, reported yet. It depends on how the effect of propolis is tested, in which form it is applied, and on its local and/or systemic use.

Available data indicate that the utilization of propolis stimulates the IMS and causes thymic activation^[99-102]. Propolis has been found to be a potent contact sensitizer in animal experiments^[103], and it causes contact dermatitis in humans^[104].

In a personal case report, the Organic Stakich Propolis (Stakich, Inc., Bloomfield Hills, MI), containing no beeswax remnants, was used. The propolis tincture was obtained by mixing 50 g of solid propolis with 250 mL of 95% pure ethyl alcohol (Golden Grain Alcohol, Luxco, Inc., St. Luis, MO). The maceration for one to two weeks was accompanied by occasional mixing, until the propolis was completely dissolved. The propolis tincture moistened gauze sponge in surgical clamp was used once a week for the local applications (hair and leg skin).

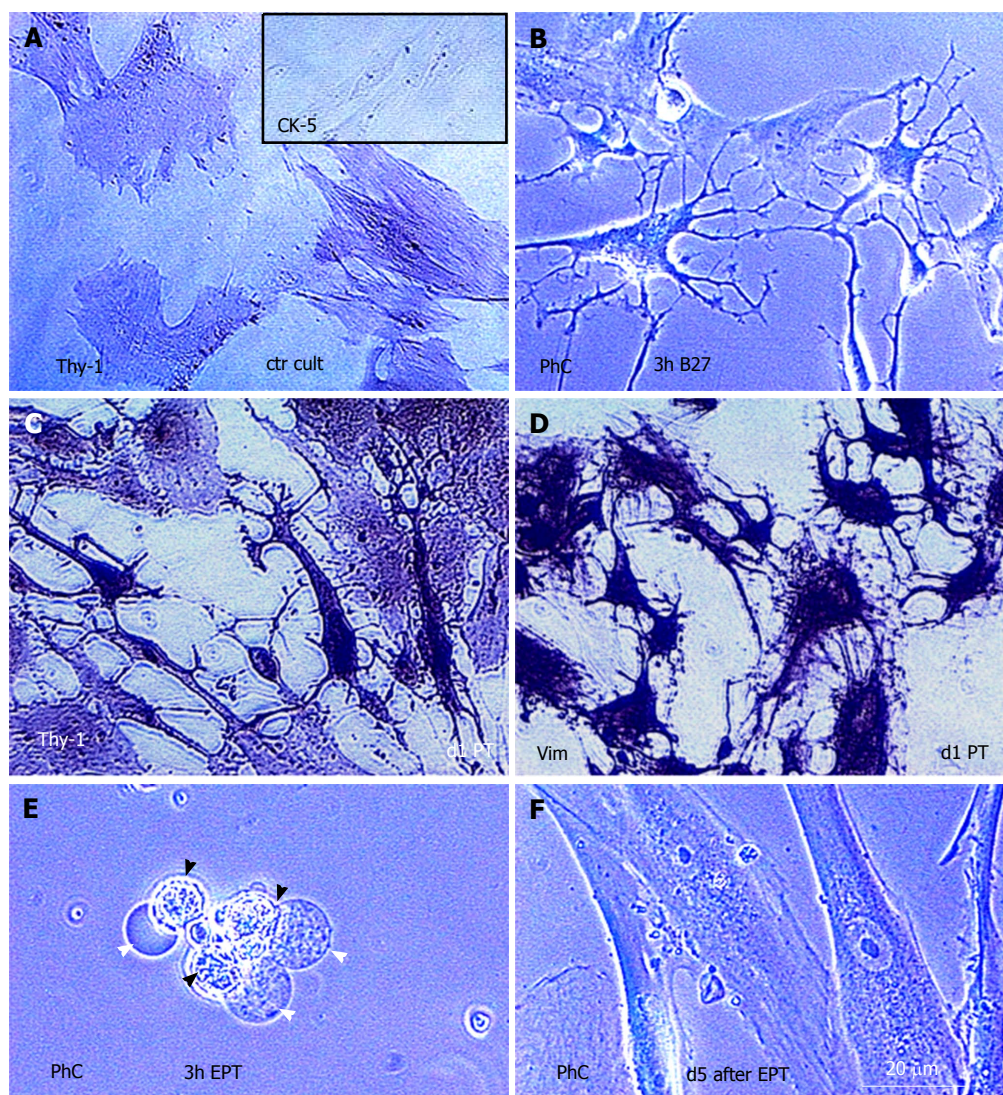


Figure 16 Transdifferentiation of human vascular smooth muscle cells to the neuronal cells and smooth muscle stem cells after the treatment with sex steroid combinations. A: Untreated human smooth muscle cell control culture exhibits a moderate Thy-1 expression characteristic for vascular pericytes. Inset shows a lack of cyokeratin staining; B: Phase contrast (PhC) of smooth muscle cell culture 3 h after the treatment with Neurobasal/B27 medium showed a transdifferentiation of smooth muscle cells into neuronal cells connected multidirectionally by their neurites; C: The neuronal cells exhibited strong expression of Thy-1 glycoprotein characteristic for neuronal cells (compare with panel A) after the treatment with 20 mmol/L progesterone and 60 mmol/L testosterone (d1 PT); D: The so called “brain *in vitro*” shows strong expression of vimentin, which is characteristic for the human neural cells; E: When 12 mmol/L estradiol was added to the progesterone and testosterone, the proportion of human smooth muscle cell culture dedifferentiated in 3 h into human smooth muscle stem cells (black arrowheads), which exhibited anchors of bubble-like type (white arrowheads), indicating their ability for a settlement. These cells redifferentiated in a few days in the fresh new human smooth muscle cells (F - compare with panel A). Adjusted from^[81] with permission: ©Landes Bioscience.

Figure 17 demonstrates utilization of the propolis tincture. For the dental treatment and propolis consumption each day, the 30 mL of a freezer cooled 40% alcoholic distillate was measured and over layered with 1 mL of the propolis tincture. The over layered propolis tincture was sipped to the mouth for a short (2-3 s) wash of the teeth, and poured back to the alcoholic distillate in order to dilute the propolis tincture. The diluted propolis tincture was returned for about 30 min to the freezer, and then gradually consumed for the systemic effect of the propolis. About three years ago, the development of mid-frontal alopecia was observed. This was accompanied by a depletion of hair color on the head top and bottom sides of the hair. Weekly used local propolis

tincture for the developing alopecia caused initially a moderate skin sensitization, which gradually diminished, and the alopecia did not show any further progression. The hair exhibited a persistence of moderate alopecia, without any progress since the beginning of the propolis treatment. This included a restoration of the original hair color. These observations indicate that propolis tincture will not induce a regrowth of lost hairs but will be able to prevent alopecia progression and restores the original color of the propolis treated coat.

Several years ago, before the propolis treatment, attempts to reduce the blood pressure were made by intensive antihypertensive therapy. Subsequently, the varicose veins exhibited a significant swelling accompanied



Figure 17 Propolis tincture preparation for the dental and systemic use, and local effects for the hair, varicose veins, and teeth. A: Glass container with 30 mL 40% alcoholic distillate; B: The distillate is over layered with 1 mL of propolis tincture for a short local teeth treatment (about 2-3 s) and then poured back (C) to be consumed for the systemic propolis treatment; D: Developing frontal alopecia (arrow) before the local propolis treatment; dashed line circle indicates accompanying hair color depletion; E: Persisting unchanged frontal alopecia (arrow) with a restoration of the original hair color (compare with D) but persistence of color depletion in propolis untreated coat sides (yellow arrowhead); F: Hair condition in normal (side to side) hair orientation; G: Hair appearance showing further improvement of hair condition, including partial color regeneration in propolis treated sides (red arrowhead); H: Shrinking (blue arrows) and regressed (yellow arrows) varicose veins on the legs after the several weekly local propolis treatment; I: Upper teeth row after professional dental cleaning; J: Heavy propolis deposits (arrows) thereafter on the dentist densely brushed teeth; K: Residual propolis attachments four days later; L: Teeth's status after five months of daily propolis treatment showing no propolis binding without any dental brushing, which indicates well-regenerated dental enamel. Arrowheads indicate a diminution of dental fissure (compare with panel I). Numbers indicate the image collection dates^[161].

by massive swelling of both legs. This was supposed by family physician to be caused by vena cava obstruction, which was not confirmed by the magnetic resonance imaging. The swellings disappeared after reduction of the antihypertensive therapy (removal of the 2 times daily Verapamil 240 mg ER), but the varicose veins persisted. The local weekly propolis treatment of varicose veins for several months also caused gradually diminishing moderate skin sensitization, which was accompanied by essential shrinking and regression of varicose veins.

The aging is also accompanied by the detrimental loss of teeth. Since mid thirties of age, there was a significant dental calculus formation causing a gum disease in spite of the dental cleaning twice a day. As common, the tartar formation most heavily affected rear side of the front bottom teeth because of the saliva secretion from salivary glands. It required professional dental

cleaning twice a year, but the tartar deposits renewed several weeks after each professional cleaning. The daily propolis tincture teeth wash used during last three years, and accompanied by no dental brushing, completely prevented any dental plaque formation, gum disease, and a need for the professional dental cleaning.

These observations suggest that the age-associated tartar formation (dental plaque) causing gum disease is caused by a diminution of dental enamel regeneration, and by enamel alteration with the toothbrush cleaning. This can be prevented by the local propolis treatment accompanied by the avoidance of the intensive dental cleaning, which enables the dental enamel continuous regeneration.

Improvement of the IMS homeostatic functions can also be induced by honey bee propolis^[105-107]. Available data indicate that the propolis utilization strengthens

the body's IMS and activates the thymus^[99-102].

There are also symptoms of type 2 diabetes, which were treated by antidiabetic therapeutics (Metformin 2 × 1 g and Glimepiride 2 × 4 mg daily). The Glimepiride was reported to induce potentially harmful cell signaling for pancreatic beta cells^[108]. Systemic consumption of propolis during one year allowed gradual reduction of the Glimepiride treatment from two times 4 mg to once 1 mg daily, without any apparent changes in the appropriate blood sugar levels. This suggests that the systemic propolis effect is capable to support the Metformin effect against the insulin resistance. Systemic propolis use also allowed to reduce the use of antihypertensive Losartan/Hctz tablets from 100/25 mg to 50/12.5 mg and Amlodipine from 10 to 5 mg daily. Aging is associated with an alteration and eventual loss of hearing^[109,110]. The alteration of hearing in the left ear was completely improved by systemic propolis use to the regular hearing ability.

Recent Lab Results from annual physical examination of the author indicated: Your lipids were checked recently. Your total cholesterol was 127, HDL (good cholesterol) was 53, LDL (bad cholesterol) was 54, triglycerides were 98. These tests were normal. Your hemoglobin A1c (3 mo average sugar reading) was 6.6, urine microalbumin/creatinine was 30, and the liver panel was checked. These tests were normal. Your kidney blood test and thyroid test were normal. Accompanying examination at The Eye Care Clinic showed a moderate visual improvement as compared to the last year.

The propolis diet in rats induced a distinct lowering of the systolic blood pressure in spontaneously hypertensive animals, but did not affect controls^[105]. The ethanol extract of propolis attenuated blood sugar and plasma cholesterol in ob/ob mice^[106] and caused a decrease of blood sugar levels^[111]. The propolis oral gavage might be beneficial for the treatment of periodontitis^[112]. The propolis flavinoids could activate the IMS in mice^[107]. Regarding the gonads, the propolis prevented toxic effects of methoxychlor on rat ovaries^[113] and had a protective effect for the function of sperm^[114-119]. To our knowledge, the effect of propolis on alleviation of the age-induced infertility was not investigated yet.

The combination of systemic propolis use with a pinch of cayenne pepper (capsaicin) further improved the blood pressure and glucose levels. The systemic use of cayenne pepper also additionally improved vision ability and completely avoided the occurrence of the dry eyes - eye drops are no more needed. The cayenne pepper is a herbal medicine with numerous health benefits^[120,121], and it is used in the most of the Dr. Christopher's herbal medications^[122]. Moderate cayenne pepper capsaicin consumption can have antihypertensive effects^[123]. Blood glucose levels were significantly reduced in rabbits consuming diets containing capsaicin^[124]. Purified capsaicin caused a significant decrease in human blood glucose levels and mediated insulin release^[125].

The pyramids as a natural source of healing power

The natural source of healing power are also the pyra-

mids, when one of their bottom sides is oriented exactly to the earth north pole^[126]. Housing of rats within a small pyramid aligned to the north-south axis reduced the oxydative damage and neuroendocrine stress of rats chronically restrained 14 d (6 h daily) within a wire mesh^[127,128]. Beneficial effect of the pyramid was dependent on its north-south alignment^[129]. It has also been shown that pyramid housing markedly reduced stress effects in the developing rat offspring^[130]. More extended daily pyramid use may, however, have some adverse effects^[131].

One of the often experienced aging problems are the chronic shoulder and back pains. A single night in the bed under a pyramid self constructed from the copper round (Figure 18) eliminated the chronic shoulder and back pains for the periods of 2-3 mo.

WHY THE CANCER CELLS SURVIVE AND HOW TO REJECT THEM?

Malignant cells hybridize with the host cells to survive

The functional decline of the IMS with age is also accompanied by a progressively increasing incidence of malignant diseases.

An unique property of malignant cells is to hybridize with various normal cells of the tumor host^[132]. Such hybridization is believed to convert malignant cells into non-malignant ones^[133]. However, the malignant/normal cell hybrids lie in the proximity of blood vessels, and after entering blood they may cause distant metastases by converting into malignant stem cells after elimination of normal chromosomes^[1]. It has been shown that the cancer stem cell can hybridize with tissue-specific stem cells, or with monocyte/macrophage cells^[134-136]. Figure 19 shows pathways of malignant and normal tissue stem cell or bone marrow-derived cell hybridization. Malignant stem cells exhibit a tropism for association of normal stem cells or MDCs. It causes apposition of their membranes and fusion of both cells. Resulting heterokaryon contains cancer and normal cell nuclei which causes a deregulated cell division resulting in the alteration of normal genome and contributes to the tumorigenesis^[137]. Daughter cells resulting from deregulated cell division are hybrids of the malignant and normal cells. They can develop into malignant cells expressing cell surface markers of normal tissue cells or MDCs, or eliminate chromosomes of normal cells and transform back into malignant stem cells. Breast cancer cells hybridized with breast stem cells resulted in an increased proliferation of the hybrid cells with increased cancer drug resistance and expression of antiapoptotic proteins^[136].

Most of experimental and human cancers express allospecific MHC molecules^[138,139]. It has been proposed that augmentation of mammalian cancer growth is a misinterpretation by the body for the growth support of the semi-allogeneic fetal graft containing the MHC alloantigens of the male partner^[140]. Trophoblast hybri-

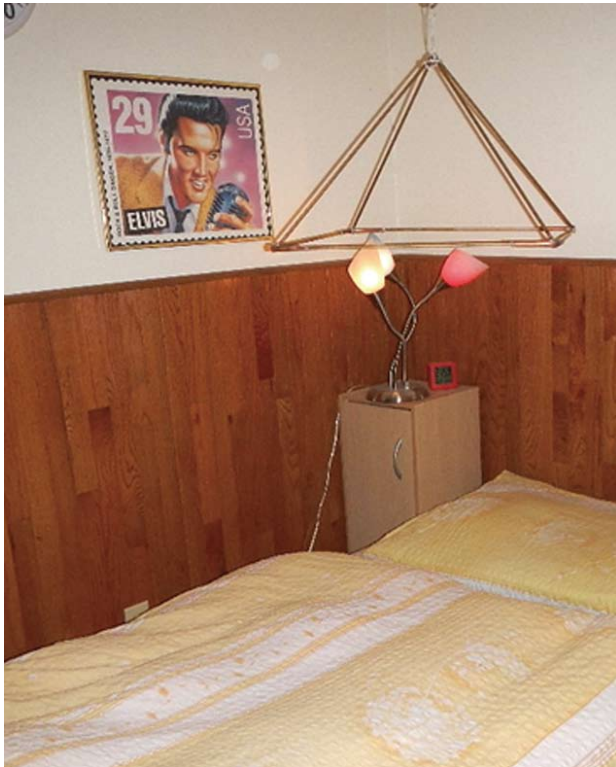


Figure 18 The pyramid above the bed for the natural healing of the shoulder and back pains. The device is hung to the chain, which is fixed to the room ceiling, and oriented by one bottom side exactly to the earth north pole. It was constructed from the copper round (6.4 mm in diameter), measuring 54 cm at the bottoms and 49 cm at the sides.

dization with the uterine cavity surface epithelium was observed in the human, rabbit, and other species^[141-144]. This is essential for the fetal survival^[145]. The trophoblast associates with endometrial vessels^[142], which is similar to the events at the host-tumor interface^[146]. The human and experimental malignancies express allospecific antigens induced by the *MHC* genes^[138,142]. The bone marrow or umbilical cord blood from semiallogeneic donors transplanted to adults with hematologic malignancies was recently reported to improve survival of patients^[147].

The tolerance of semi-allogeneic fetal antigens by the mammalian female is supposed to be caused by blocking antibodies^[148]. PARA-7 tumor-cell inoculation caused significant increase of blocking antibodies in parallel with tumor growth in hamsters. Unblocking was absent in the serum, but when tumor was excised, blocking felt rapidly. Sera obtained one week after surgery neutralized blocking activity of tumor bearing animals^[149].

Malignant tissues exhibit cancer associated-inflammation and it was recommended to reprogram the function of tumor infiltrating immune cell subsets to facilitate cancer rejection^[150]. The issue is, however, more complex, since the "inflammation" like processes accompany regeneration from stem cells in all tissues of normal healthy body, and the maturation and persistence of tissue cells in a proper functional stage in

normal tissues^[1,14]. In the stratified epithelial tissues, the TC and MDCs differentiate and degenerate while the epithelial cells gradually develop into surface cells. This is accompanied by a gradual binding of the IgM and IgG.

Figure 20 demonstrates interaction of ovarian cancer cells with the TCS cellular components. Cancer stem cells interact and hybridize with perivascular MDCs, majority of malignant cells expresses CD14, DR, and CD68 markers of MDCs, and those malignant cells eliminating the MDC markers are dividing cancer stem cells (compare with scheme in Figure 19). Tumor-associated MDCs promote tumor progression, and expression of MDC markers in breast and colorectal cancers correlates with a distant early relapse and shorter survival^[151]. Circulating inflammatory CD8 TC home among cancer cells expressing MDC markers, and regress in order to stimulate tumor growth. Tumor growth is also significantly dependent on the blood supply from the host. Thy-1 pericytes in cancer microvasculature are highly activated. They produce large amounts of Thy-1⁺ intercellular vesicles which collapse into Thy-1⁺ intercellular spikes after releasing their content promoting growth of cancer cells. The cancer cell hybridization with perivascular CD68 MDC and subsequent CD68 expression by cancer cell may cause that the cancer cells become able to stimulate extensive activity of Thy-1 vascular pericytes by obtaining proper abilities from the MDC parent of the cancer/MDC hybrid cell. It has been shown that injection of spleen cells to immunologically incompetent animals stimulates tumor growth^[152]. This indicates that TCS cells, which are required to stimulate proliferation and differentiation of normal cells are capable to enhance growth of cancer cells and participate in the stimulation of extended vascularization enhancing cancer progression. Since suppression of Thy-1 pericyte activity is dependent on autonomic innervation, the persistent pericyte activity in ovarian cancer is also caused by the lack of autonomic innervation in the malignant tissues^[20].

A background for the considerations of an advanced primary ovarian cancer immunotherapy accompanied by a single moderate dose of chemotherapy

Forty years ago, my 61-year-old mother experienced a worsening pain in the left underbelly. Gynecologic examination revealed a large pelvic resistance and she was admitted to our gynecologic clinic at the Institute for the Care of Mother and Child, Prague, Czechoslovakia, for exploring. Available documentation indicates below the following evidences and clinical course approach.

Explorative laparotomy has shown an advanced neoplasia of both ovaries with a presence of ascites, abdominal metastases including the colon, and a severe metastatic alteration of the liver. Histology of ovarian biopsy indicated the proliferating immature adenocarcinoma with a papillary structure.

For stage IV ovarian cancer with an extensive malignant alteration of the abdominal cavity and liver, the debulking surgery was avoided. With respect to the

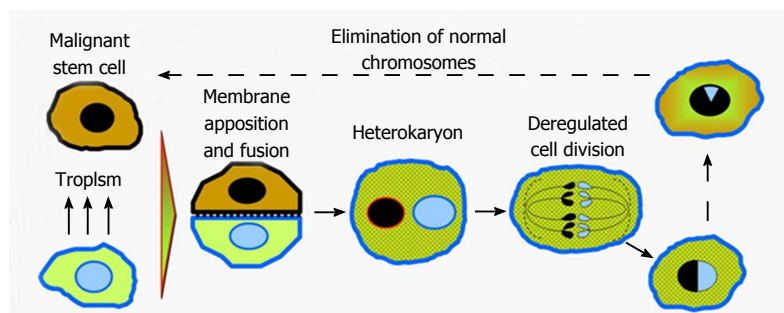


Figure 19 Hybrids of cancer cells with normal cells or monocyte derived cells contribute to the lack of cancer rejection and its progression. The malignant and normal cell exhibit a tropism resulting in apposition and a heterokaryon after the fusion with the surface expression of normal cell markers. After deregulated divisions, the multiple hybrid cells persist and some of them can gradually lose normal cell chromosomes and revert back to the malignant stem cells^[1].

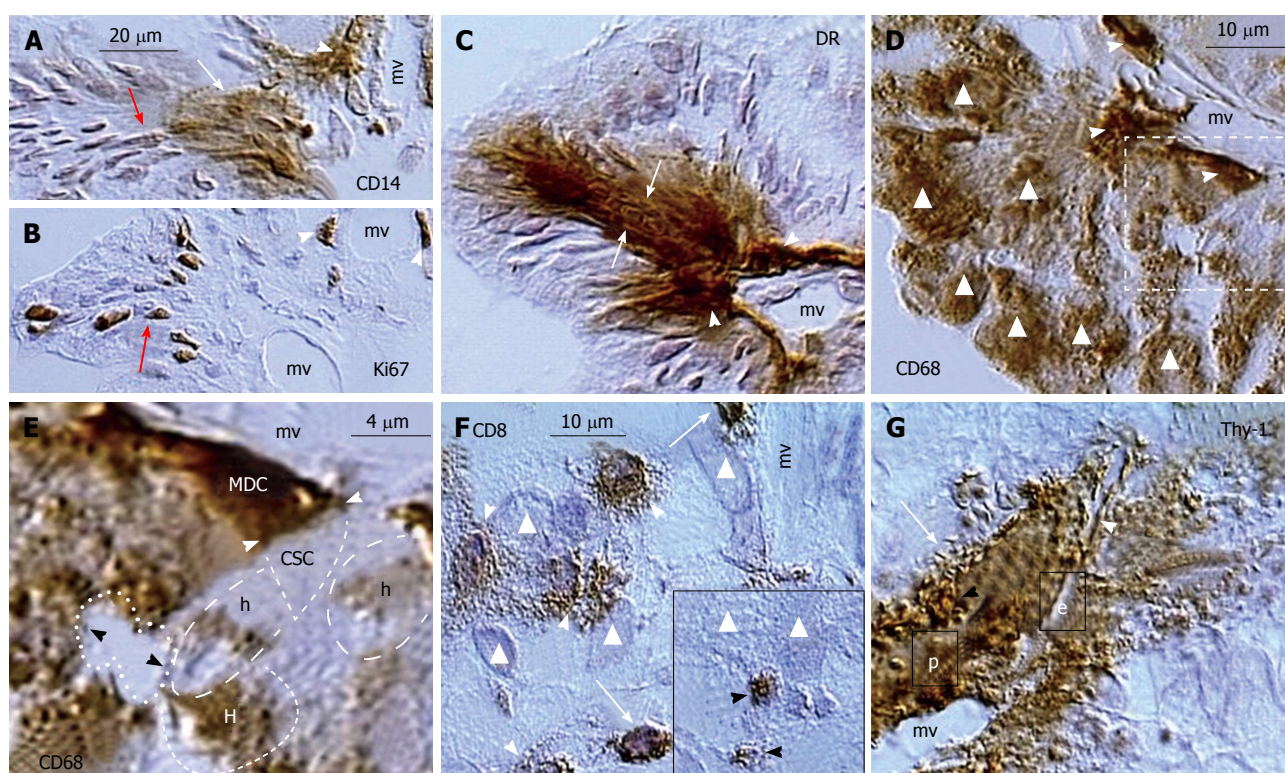


Figure 20 Ovarian cancer augmentation by the immune/morphostatic system. A: Association of CD14⁺ MDC (arrowhead) with cancer microvasculature (mv) results in nuclear and cytoplasmic CD14 expression by cancer/MDC hybrids (white arrow) and nuclear CD14 expression in more distant cancer cells (red arrow); B: Ki67 expression by divided perivascular (arrowheads) and postmitotic malignant cells (arrow); C: HLA DR⁺ expression by perivascular MDCs (arrowheads) and adjacent cancer cells (arrows); D: CD68 MDCs associate with microvasculature (arrowheads) and most malignant cells express CD68 (triangles); E: Detail from panel D shows an apposition (white arrowheads) of perivascular CD68 MDC with unstained cancer stem cell (CSC). The fresh cancer/normal cell hybrids (h) show partial CD68 expression and more advanced hybrids (H) are more distinctly stained. Dividing cancer stem cell (dotted line) is unstained; F: CD8 T cells (TC) evade (arrows) from cancer microvasculature (mv) and exhibit increase in size (arrowheads) among cancer cells (triangles). Inset shows regressing TC (arrowheads) among cancer cells; G: Cancer microvasculature (mv) is accompanied by hyperactive pericytes (p) producing Thy-1⁺ vesicles (black arrowhead) releasing their growth promoting substances among adjacent cancer cells and collapsing into the Thy-1⁺ spikes (arrow). The microvasculature produces new endothelial cells (e) accompanied by new Thy-1⁺ pericytes (white arrowhead). Adjusted from^[21]. ©Antonin Bukovsky.

inoperable metastatic ovarian neoplasia, an unusual novel cancer treatment was approved by the Institute medical leadership. The treatment was based on a review article published a year ago and entitled "Immunological aspects of neoplasia"^[153]. That review article indicated that cancer cells exhibit transplantation specific tumor antigens which are distinct from normal tissues. Lymphocytes from a patient with growing cancer are capable to kill corresponding malignant cells growing *in vitro*, but their

blood serum protects by blocking antibodies *in vitro* reactivity of lymphocytes against malignant cells^[154]. The protection effect of the blocking serum is tumor-specific. From this point of view it appears that the patient's body is involved in the prevention of tumor rejection by cytotoxic lymphocytes^[38]. Alteration of serum blocking activity can be induced by immunosuppression exhibited after splenectomy and presence of unblocking antibodies also eliminates the influence of blocking activity^[155]. The

role of the immune tolerance of the semiallogeneic fetus during pregnancy and utilization of this type of immune tolerance by cancer cells has been discussed^[156].

It has also been shown that intradermal administration of an adequate dose of BCG vaccine preceding the MSVT1 sarcoma transplantation in mice inhibited tumor growth^[157]. Further inspirational evidence for possible immunotherapy was that administration of *Clostridium parvum* following a single injection of cyclophosphamide caused complete regression of fibrosarcomas in 70% of animals^[158]. In addition, cyclophosphamide was reported to eliminate the serum blocking activity^[159]. Final inspiration was that the allosensitization by mouse spleen cells was found to reject mouse tumor allografts^[160].

A simple elaborated immunotherapy of advanced malignancy described below should be considered in the novel cancer cases, without a recent standard cancer treatment, *i.e.*, excessive debulking surgery and/or excessive chemotherapy, as well as radiation treatments affecting body conditions, including the immunity. Advanced malignant stages are not excluded, assuming there is a normal value of the circulating mononuclear cells being capable to be involved. The treatment may involve malignancies regardless of their extent, but lower effectiveness can be anticipated in cancers affecting mononuclear blood cells, and limitations might occur in the brain malignancies^[161]. In the cases with an early malignancy and for prevention of cancer recurrence the weekly consumption of the raw Shiitake mushroom can be effective.

A course of the advanced primary ovarian cancer successful immunotherapy

One week after explorative laparotomy 3.8 mL of human gamma-globulin, which is commonly isolated from the retroplacental blood (IgG; Sevac/Praha), was injected intramuscularly to unblock antibodies against cancer alloantigens. The continuing malignant growth may terminate after this IgG treatment, since the IgG originates from the retroplacental blood usually carrying unblocking antibodies against alloantigens^[39]. Next week 1.9 mL of IgG was injected intramuscularly again. As a part of immunotherapy, the 400 mg single cyclophosphamide dose was injected intravenously at the third week to cause immunosuppression with an additional depletion of blocking antibodies. Fourth week after explorative laparotomy the 500 mL of compatible blood was transfused to stimulate IMS reactivity against alloantigens of cancer cells. Subsequently, bacterial toxins [*Bacterium adnexitidicum* (BA)/SEVAC - also known as Adnexba^[162]] dilutions X-VI were weekly injected exactly intradermally in order to stimulate the reversion of cyclophosphamide-induced immunosuppression. Second 500 mL of compatible blood from distinct donor was transfused at the 8th week to booster IMS alloreactivity. The course of used immunotherapy steps is summarized in Table 1. Beside that, the Metronidazole 500 mg tablets twice a day were also used for the treatment of the

patient's chronic pyelonephritis.

Ten weeks after explorative laparotomy the fist size sensitive resistance was still detected in the left underbelly but a presence of ascites was not detectable. Six months from the beginning of the treatment, a normal liver size in the soft abdomen was found, and irrigoscopy demonstrated normal conditions of the colon.

Subsequent laparotomy found complete regression of abdominal metastases with a liver regeneration to the excellent healthy condition. Ovaries from hysterectomy and bilateral salpingo-oophorectomy histologically exhibited large foci of degenerating malignant adenomatous papilloma. These degenerations exhibited vast necrosis of the malignant tissue. No evidence of a fresh malignant growth was found. Before and during the treatment the blood samples exhibited normal appearance, and the regular counts of red and white blood cells and normal white blood cell differentials.

Possible further development of the advanced primary ovarian cancer treatment

At present, it might be better to perform a simple laparoscopy or computer tomography of abdominal cavity without surgery in about six to twelve months intervals after explorative laparoscopy and cancer biopsy to monitor cancer regression and lack of recurrence. If the results are promising, the administration of bacterial toxins or BCG, which also should be suitable^[163], in two to four weeks intervals should continue to ensure a complete cancer regression and a lack of the relapse. A good regeneration of metastatically affected tissues can be expected to accompany cancer regression. No evidence of the tumor regression, *e.g.*, depletion of ovarian cancer ascites, at three months after the beginning of this simple elaborated immunotherapy, however, should be considered as a failure of the method in a given individual.

The successful cancer regression will not need any additional cytostatics or radiation. A second look surgery to remove regressing tumor remnants is questionable. If performed, it should be followed by the original cancer treatment to prevent recurrence, or other approach listed below.

How to treat and prevent cancer recurrence

Eventual recurrence can be treated with the original method or with a more simple prevention of the malignancy recurrence and immunoprophylaxis of malignant disease, since the weekly consumption of a raw shiitake mushroom could be efficient.

Twenty-five years ago there was a personal experience of early developing colorectal cancer. The signs lasted over six months and consisted of a worsening constipation, resistance at the rectum, and severe inconvenient skin smell resembling protein degradation reflected by emitted volatile organic compounds^[164], that are currently detectable by an electronic nose^[165].

A single larger raw shiitake mushroom was accidentally eaten at a conference meeting. This was followed

Table 1 Course of cancer immunotherapy

Step	Week	Treatment
1	1	3.8 mL of human IgG <i>i.m.</i>
2	2	1.9 mL of human IgG <i>i.m.</i>
3	3	Cyclophosphamide 400 mg <i>i.v.</i>
4	4	First blood transfusion 500 mL
5	5	Bacterial toxins dilution X
6	6	Bacterial toxins dilution IX
7	7	Bacterial toxins dilution VIII
8	8	Second blood transfusion and Bacterial toxins dilution VII
9	9	Bacterial toxins dilution VI
10	10	Bacterial toxins dilutions X to VI continued in weekly intervals till the end of six months since beginning

i.m.: Intramuscular injection; *i.v.*: Intravenous injection.

by a severe skin rash and pruritus lasting over one week. Thereafter, however, a complete regression of the rectal symptoms was observed. The development of dermatitis after the raw shiitake mushroom consumption may indicate the stimulation of the IMS reactivity, eventually accompanied by the good anticancer response. Continuing weekly utilization of the raw shiitake mushroom in salads caused no skin rashes or pruritus and prevented any appearance of the colorectal or other symptoms of malignancy for twenty-five years thereafter^[161].

Raw shiitake mushrooms were recently found to have an antitumor effect by stimulating immunity to kill the cancer cells^[166]. The effective compound of shiitake mushrooms is the thermolabile beta-glucan lentinan^[167]. The raw shiitake mushrooms cause toxic dermatitis by reaction to lentinan thermolabile polysaccharide^[168]. Therefore, the shiitake mushrooms are recommended to be utilized after cooking to prevent emergence of severe skin pruritus and rashes^[169]. Such processing will, however, alter their immunological antitumor effect due to the decomposition of the thermolabile lentinan by heating.

The anticancer properties has also propolis and its active ingredients, caffeic acid phenethyl ester (CAPE) and artemisinin. Propolis, CAPE, and artemisinin C have been shown to activate macrophages, *i.e.*, MDCs, suppress proliferation of cancer cells, decrease population of cancer stem cells, block specific oncogene signaling pathways, and exhibit antiangiogenic effects^[170].

FROM INFECTION AND TRANSPLANTATION IMMUNITY TO THE IMMUNOTHERAPY OF FUNCTIONAL AND AGING DISEASES AND CANCERS

The involvement of the cells and molecules belonging to the classically viewed immune system in morphostasis of normal tissues is reviewed. It is apparent that, beside immunity, the immune system components are also involved in the maintenance of tissue morphostasis. Therefore, the term "immune and morphostatic system"

appears to be more appropriate. In normal tissues the IMS cells induce asymmetric division of stem cells. Various alterations of tissue physiology appear to originate from the altered IMS during its epigenetic formation in early ontogeny (*e.g.*, type 1 diabetes) or with its gradual regression accompanying age advancement. The TC and MDCs may underwent a suicide to enable more advanced differentiation of tissue cells where functionally required, or cause "autoimmune disease" where functionally unsuitable. The individual's overall aging is caused by the age-induced IMS regression starting at 35 years of age. The age-associated diseases (*e.g.*, Alzheimer's) may particularly affect tissues the embryonic and/or fetal development of which was retarded in some individuals. The TMT can induce temporary alleviation of tissue aging, which could be extended by substances stimulating IMS regeneration, like the honey bee propolis. The cancer cells exhibit allogeneic MHC determinants and hybridize with normal host cells in order to express host cell markers and attain IMS support for its expansive growth. Better understanding of the IMS role in the physiology and pathology of various body tissues could bring novel approaches for the efficient treatment of functional, age-associated, and malignant diseases.

CONCLUSION

The optimal morphostasis consists of: (1) tissue regeneration from stem cells; (2) conservation of tissue cells in the functional stage; and (3) preservation of tissue quantity. This is maintained by the TCS consisting of IMS related components, vascular pericytes, and autonomic innervation. The morphostasis is established during the embryonic and fetal IMS adaptation. Postnatal functional disorders, like type 1 diabetes, are caused by an alteration of tissue development during the IMS adaptation. The age-induced IMS regression causes organism's aging, which is accompanied by gonadal infertility and age-associated diseases. The novel approaches in regenerative medicine could be represented by a transfusion of young blood from suitable young donor, which may persist for a period of six months to the one and half years. This can enable temporary treatment of gonadal infertility, which will be sufficient for the production of new mature gametes. Accordingly, the young blood and/or natural substances strengthening the morphostatic function of the IMS, like the honey bee propolis, can extend alleviation of age-associated diseases. The presented successful simple elaborated immunotherapy can cause regression of advanced cancers without a need of a debulking surgery and/or exhaustive chemotherapy.

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