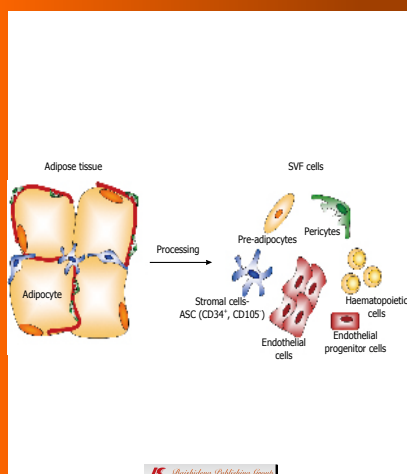
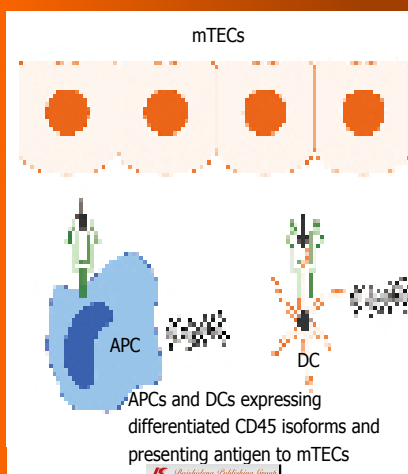


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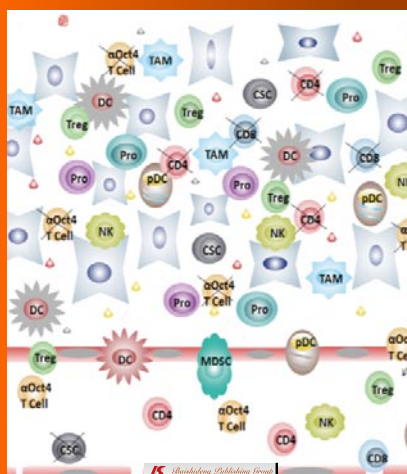
World J Stem Cells 2013 April 26; 5(2): 43-60



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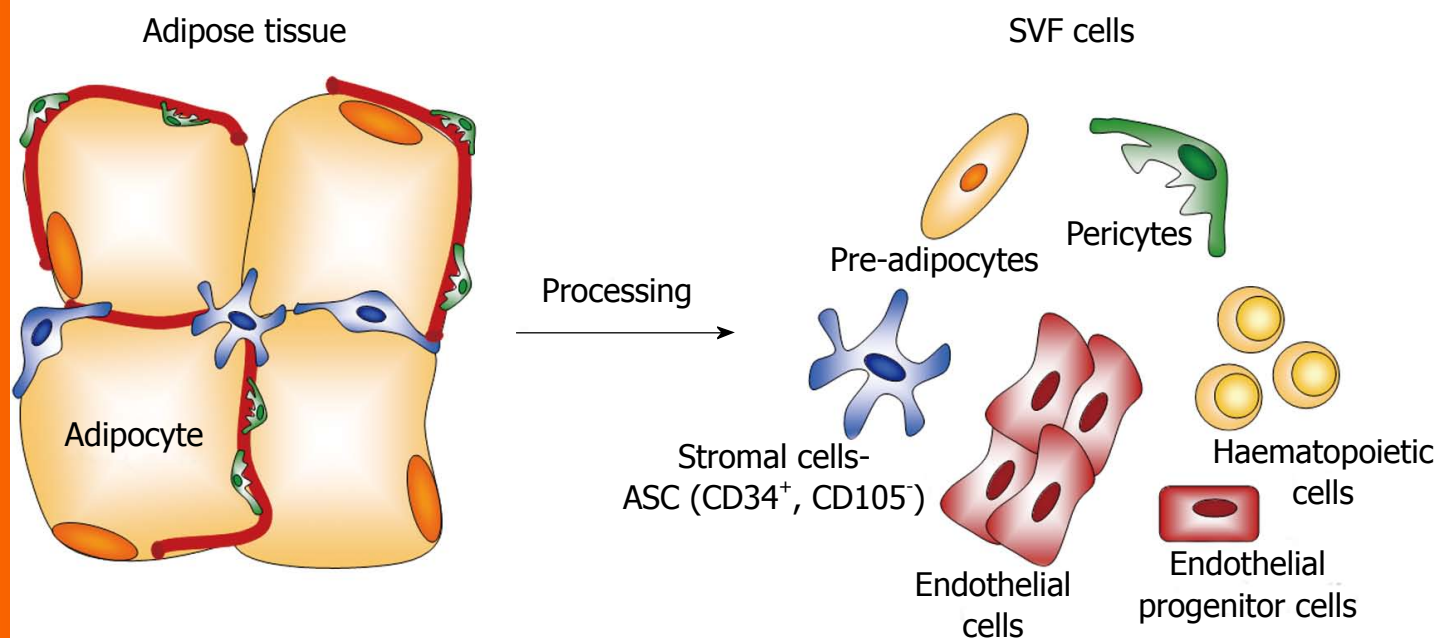


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A familiar stranger: CD34 expression and putative functions in SVF cells of adipose tissue

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a short introduction to the CD34 family of sialomucins and reviews the data from the literature concerning expression and function of these proteins in SVF cells and their *in vitro* expanded progeny.

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Key words: Human adipose tissue; CD34; Sialomucins; Mesenchymal stromal cells; Endothelial progenitors

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Abstract

Human adipose tissue obtained by liposuction is easily accessible and an abundant potential source of autologous cells for regenerative medicine applications. After digestion of the tissue and removal of differentiated adipocytes, the so-called stromal vascular fraction (SVF) of adipose, a mix of various cell types, is obtained. SVF contains mesenchymal fibroblastic cells, able to adhere to culture plastic and to generate large colonies *in vitro*, that closely resemble bone marrow-derived colony forming units-fibroblastic, and whose expanded progeny, adipose mesenchymal stem/stromal cells (ASC), show strong similarities with bone marrow mesenchymal stem cells. The sialomucin CD34, which is well known as a hematopoietic stem cell marker, is also expressed by ASC in native adipose tissue but its expression is gradually lost upon standard ASC expansion *in vitro*. Surprisingly little is known about the functional role of CD34 in the biology and tissue forming capacity of SVF cells and ASC. The present editorial provides

INTRODUCTION

Multipotent stromal cells and various progenitor cells derived from adipose tissue have raised interest for regenerative medicine applications, especially because adipose tissue can be harvested in large quantities (several hundred mL) by a minimally invasive liposuction procedure. This was the topic of several recent reviews^[1-3]. In humans and mice, the stromal vascular fraction (SVF) is a heterogeneous mixture of cells isolated by enzymatic dissociation of adipose tissue followed by gradient centrifugation in order to remove the differentiated adipocytes, which float over the aqueous layer. The pellet of SVF cells contains multipotent mesenchymal cells, which are typically referred to as adipose derived stem/stromal/progenitor cells (ASC). Human ASC (hASC) have biological capacities highly comparable to bone marrow-derived mesenchymal stem/stromal cells (BMSC) and therefore are considered to be a promising alternative source of cells for clinical use in pathological contexts as diverse as cardiovascular disorders, pulmonary diseases, musculoskeletal disorders, soft tissue reconstruction/augmentation, liver dysfunction, gastrointestinal, urogenital or neuronal disor-

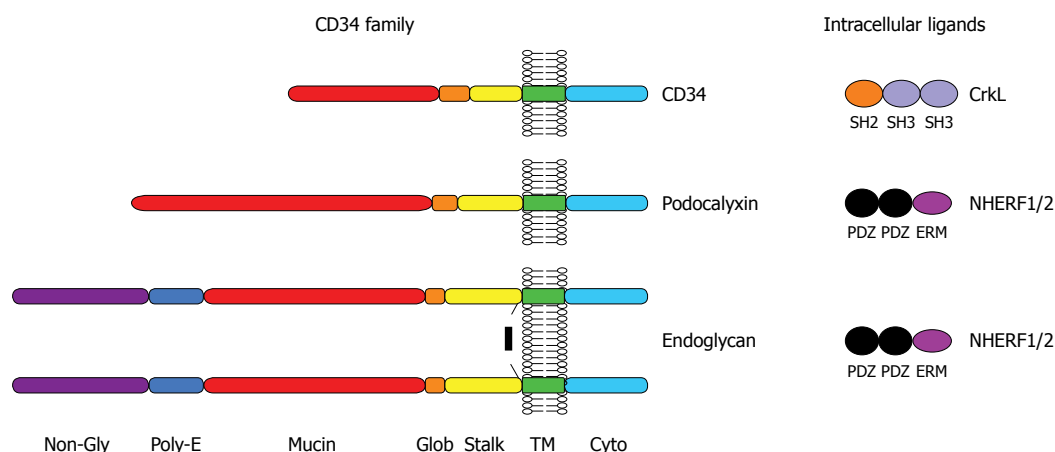


Figure 1 Protein structure of the CD34 family. CD34, podocalyxin and endoglycan are transmembrane proteins which display O-glycosylated and sialylated serine-, threonine- and proline-rich extracellular mucin domain, putative sites of N-glycosylation, a cysteine-containing globular domain and a juxtamembrane stalk region. NHERF1 and 2 bind to the C-terminal tail of podocalyxin and endoglycan but not CD34. Conversely the CrkL selectively binds to a distinct juxtamembrane sequence in CD34.

ders, skin and wound healing, bone regeneration, corneal diseases and immuno-modulation (reviewed in^[1,4]).

In addition to ASC, the SVF also contains blood-derived cells, in particular erythrocytes and leukocytes, characterized by expression of the pan-hematopoietic marker CD45 and/or the monocytic marker CD14. Interestingly, SVF was shown to also include vascular endothelial and mural/pericytic cells, harboring vasculogenic properties *in vitro* and *in vivo* (^[5,6] and recently reviewed in^[7]). The majority of SVF cells (60%-80%^[8,9]) express CD34, which was first used for the identification and isolation of hematopoietic progenitors cells. Very little is known about its possible function in adipose-derived cells, but given the pivotal role played by CD34 in the biology of several stem/progenitor cells, including hematopoietic stem cells^[10], skeletal muscle satellite cells^[11], keratinocyte stem cells^[12], hair follicle stem cells^[13,14] or adipogenic precursors^[15], a function of CD34 also in the biology of adipose-derived stem/progenitor cells can reasonably be hypothesized. The present editorial provides a concise overview of the CD34 family of sialomucins and compiles the data from the literature concerning expression and function of these proteins in SVF cells and their *in vitro* expanded progeny.

The CD34 family of sialomucins: Structure and functions

The CD34 family of cell surface proteins is a discrete subset of the large family of transmembrane sialomucins and comprises 3 members: CD34, podocalyxin (Podxl) and endoglycan (Endgl). A schematic overview of the biochemical features of CD34 family of proteins is shown in Figure 1. Although CD34 family members exhibit relatively limited linear protein sequence identity, they each contain a very similar array of biochemical domains and motifs that distinguishes them as a distinct subfamily from the much larger family of transmembrane mucins. All three contain an N-terminal signal peptide followed by a serine-, threonine- and proline-rich mucin domain that

becomes highly decorated with O-linked and, to a lesser extent, N-linked glycosylation (Endgl is somewhat unique in that it also contains an intervening N-terminal domain that lacks potential glycosylation sites but contains a site for chondroitin sulfate attachment and a poly-glutamic acid motif)^[16-18]. The mucin domain is followed by a disulfide-bonded globular domain, a juxta-membrane “stalk” domain, a transmembrane region and a charged intracellular domain of about 75 amino acids containing consensus phosphorylation sites for PKC or CKII.

At their C-termini, all three proteins contain short motifs (DTHL or DTEL) that resemble binding sites for PDZ-domain scaffolding proteins, which are known to play a role in targeting bound proteins to discrete subcellular localizations and facilitating signal transduction^[19,20]. The DTHL motifs of Podxl and Endgl have been shown to bind the PDZ-domain proteins, NHERF-1 and NHERF-2, which are well known for their ability to bind a large array of G-protein coupled receptors, tyrosine kinases, transcription factors, *etc.*^[17,21]. There is data suggesting that Podxl directs the recruitment of NHERF1, and presumably all of its signaling ligands, to discrete membrane domains within the cell^[22]. Somewhat surprisingly, CD34 does not bind NHERF1 or NHERF2 and it is therefore likely that there is a distinct, yet-to-be-discovered, PDZ domain protein that binds this family member^[21].

Relatively little is known of the intracellular ligands for CD34. In an unbiased phosphoproteomic survey of mast cells, CD34 was identified as one of the most rapidly (10 s) and highly (> 50-fold) tyrosine phosphorylated proteins in response FcεRI crosslinking^[23] providing evidence that CD34 can be modified dynamically in response to extracellular stimuli and could provide a docking site for phosphopeptide binding proteins. In addition, the membrane proximal region of CD34 has been shown to bind the SH2-SH3-SH3 containing adapter protein, CrkL^[24] through a motif that is not present in Podxl or

Endgl. CrkL is known to interact with Abl, Bcr-Abl, C3G, Sos, EPS15, and DOCK180 through its N-terminal SH3 domain whereas CD34 is the first protein known to bind through its C-terminal SH3 domain^[24]. Thus, like its relatives, Podxl and Endgl, there is reason to believe that CD34 can target signal transduction complexes to discrete cell membrane domains, possibly in response to phosphorylation.

Although the biological function of CD34 has not yet been fully clarified, several roles have been attributed to the proteins of the CD34 family. Due to its restricted expression on cycling hematopoietic progenitors, CD34 has been proposed to both promote proliferation and block differentiation (reviewed in^[25]). Indeed, ectopic expression of CD34 in cell lines^[26] and evaluation of progenitors derived from knockout mice^[27] tend to support this view. A similar study showing resistance of CD34 knockout mice to formation of skin tumors posited a similar role for CD34 in the proliferation of precursors^[14]. More recent studies have also linked Podxl and CD34 expression to enhanced trafficking and migration of hematopoietic cells^[10,28,29] (reviewed in^[17]). These studies suggest that the strong negative charge conferred by the highly glycosylated extracellular domain of CD34 family proteins serves an anti-adhesive function and enhance the mobility of cells. This may be further facilitated by a role in aiding chemotactic signaling responses as was recently suggested for CD34⁺ dendritic cell precursors^[30]. The fact that expression of a human transgene was able to rescue the disease phenotype in these mice suggests that the human gene functions in a similar fashion^[30]. While, at face value, it is difficult to reconcile the different roles for CD34 (and Podxl) observed in proliferation and cell migration, it should be noted that these may not be mutually exclusive; it is possible that CD34 plays subtle roles in both pathways. Alternatively, it is possible that by altering cell adhesion, CD34 could alter the downstream sensitivity of cells to entering cell cycle or differentiation. This would be quite compatible with a known role for integrins and other adhesion molecules in regulating cell cycle progression and differentiation (reviewed in^[17]). Finally, through their potent ability to traffic to the apical domains of cells and interact with cytoskeletal signaling proteins, CD34 and its relatives have been speculated to play a role in regulating the recruitment of cell differentiation factors to discrete cellular membranes and thereby to regulate asymmetric cell division of undifferentiated precursors (reviewed in^[17]).

Expression of CD34 in different cell types

CD34 family proteins have both unique and overlapping expression patterns. All three proteins (CD34, Podxl and Endgl) have been described as markers of hematopoietic precursors and vascular tissue (reviewed in^[17]) giving them ample opportunity to serve redundant functions as evidenced in knock-out mice. However, in addition to their overlapping expression on these cell types, these molecules are also uniquely expressed on other cells. In

the case of CD34, it is uniquely expressed by inflammatory cell precursors (mast cells, eosinophils and dendritic cells) and has been shown to be important for facilitating cell trafficking and the development of mucosal inflammatory disease, while in its absence, mice are rendered remarkably resistant to a range of disease including allergic asthma, hypersensitivity pneumonitis, colitis, Salmonella induced inflammation, and colon cancer^[30-34]. As already mentioned in the introduction, CD34 is expressed by various stem/progenitor cells such as muscle satellite cells, adipogenic precursors and by hair follicle and keratinocyte stem cells. Podxl in contrast is expressed selectively by anemic erythroid lineage cells^[28,35], kidney podocytes^[36], a subset of developing neurons^[37], and a variety of embryonic tissues including mesothelial precursors and epithelial precursors^[16,38] (and Hughes *et al*, In Tech - Advances in Cancer Management, in press). Accordingly, deletion of the Podxl gene leads to perinatal lethality due to defective morphogenesis in a number of these cell types^[16,38,39]. In BMSC, the expression and the role of CD34-related sialomucins remain unclear. However, Podxl was shown, together with $\alpha 6$ -integrin (CD49f, VLA-6), to identify early progenitor BMSC with increased clonogenicity and differentiation potential *in vitro* and highly efficient migration to infarcted heart in mice^[40]. In addition, Podxl was shown to be strikingly upregulated in the most life-threatening epithelial tumors and appears to play a role in enhancing the mobility and invasiveness of tumors^[22,38,41] (and Hughes *et al*, In Tech - Advances in Cancer Management, in press).

Expression of CD34 by hASC

Human adipose tissue was shown to turn over^[42] and adipocytic progenitor cells, i.e., ASC in mice have been shown to reside in the adipose vasculature^[43]. However, the precise origin of the native hASC still remains a debated question. Indeed, although it was recently proposed that ASC originate from a pericyte population lacking CD34 expression^[44], Traktuev *et al*^[6] suggested a CD34⁺ pericytic origin for ASC, which have been previously characterized as Lin⁻/CD29⁺/CD34⁺/Sca-1⁺/CD24⁺ cells^[45]. To clarify this question, the expression of CD34 by hASC in native adipose tissue was addressed by characterizing expression of CD34 in subpopulations of cells from the SVF^[46,47]. Initially, two CD34⁺ populations with a difference in the intensity of antigen expression were identified and a majority of the cells expressed CD34 at low intensity. ASC freshly isolated from human SVF were characterized as CD31⁻/CD34⁺/CD45⁻/CD90⁺/CD105⁻/CD146⁻ cells, but were shown to become CD105⁺ when plated^[48]. Upon adhesion to tissue culture plastic, cell expansion and passaging, CD34⁺/CD45⁻ cells from SVF, were shown to gradually lose CD34 expression in monolayer culture, although the kinetics of decrease in CD34 expression seemed to vary strongly with culture conditions, such as plating density or culture medium used^[48,49]. As a consequence, hASC, which are typically expanded as a monolayer on tissue culture plastic for several passages

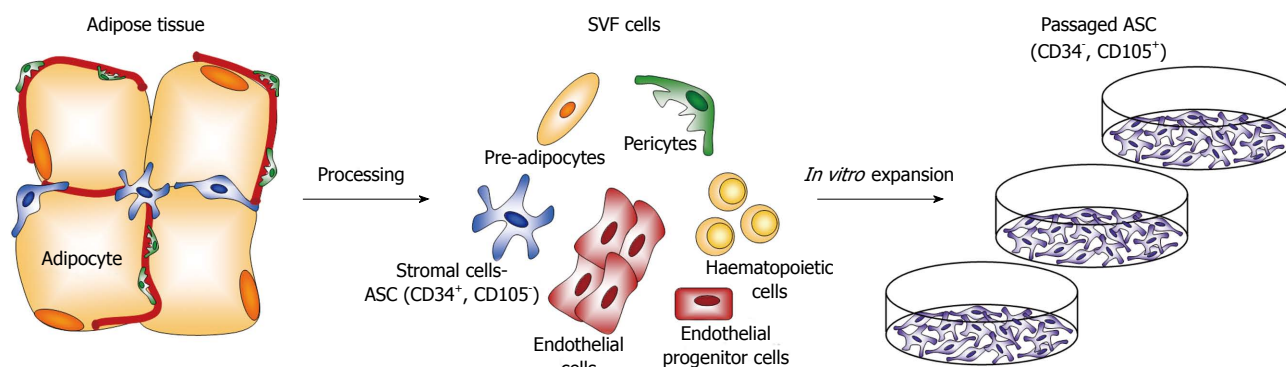


Figure 2 In the adipose tissue, adipose mesenchymal stem/stromal cells are localized perivascularly and are characterized by the expression of CD34. Adipose mesenchymal stem/stromal cells (ASC) are isolated upon collagenase digestion together with other cell types including endothelial and endothelial progenitor cells. Upon adhesion to plastic and active *in vitro* proliferation, ASC change their phenotype, lose CD34 expression and begin to express CD105.

prior to their use, have been reported to be CD34⁺ in most publications so far. This is in accordance with similar reports made on freshly cultured primary human vascular endothelial cells, where endothelial cells initially express CD34, which is later down-regulated with proliferation in continuous culture and is predominantly absent after only nine population doublings^[50]. This was confirmed by the fact that CD34 seems to be especially expressed by endothelial “tip cells”, which are endothelial cells actively participating in the angiogenic process but which do not proliferate^[51]. A follow up study on adipose-derived cells from the group of Yoshimura correlated CD34 expression by freshly isolated hASCs in SVF with a higher clonogenicity and proliferation capacity, reduced differentiation potentials into mesenchymal lineages, namely adipocytic and osteoblastic, increased expression of angiogenesis-related genes^[52]. One limitation of the latter study resides in the fact that CD34⁺ cells were considered as a homogenous population. Our group investigated which cell population in SVF gives rise to hASC in monolayer culture^[5] by sorting SVF cells according to the expression of CD34 and the endothelial marker CD31. We showed that the CD34⁺/CD31⁻ fraction contains progenitors able to differentiate into the osteogenic lineage and colony-forming cells. This indicates that hASC are CD34⁺/CD31⁻ cells in the SVF, which upon adhesion give rise to expanded hASC in culture. A study by another group concomitantly compared freshly isolated hASC and hASC expanded as monolayers on tissue culture plastic with serial passaging and also evaluated hASC morphology in their niche to define their tissue localization within intact human adipose samples^[53]. This study showed that native human ASC (1) are CD34 positive and that its expression is dramatically decreased with *in vitro* proliferation; (2) display both stromal and perivascular positions but (3) do not express *in situ* pericytic markers such as NG2 and CD140b; and (4) exhibit a peculiar cell morphology with long protrusions *in situ*^[53]. In conclusion, hASC are CD34⁺ in the native adipose tissue and in SVF, but rapidly change their phenotype and lose CD34 expression when expanded as monolayer (Figure 2).

Expression of CD34 by vascular cells in human SVF

The presence of vasculogenic cells within the SVF was first proposed in 2004^[54] and thereafter confirmed by different groups^[8,55,56]. The CD34⁺/CD31⁻ cell population from adipose tissue differentiates into endothelial cells and exhibits the capacity to rescue hindlimb ischemia in animal models^[8,57]. Also, both CD34⁺ and CD34⁻ primitive mesodermal progenitors within the SVF of human adipose tissue were shown to exhibit hematopoietic and hemangioblastic activities *in vitro*^[58]. In a recent study, our group investigated the vasculogenic potential of different subpopulations of SVF cells. We showed that neither CD34⁺CD31⁻ nor CD34⁺CD31⁺ from SVF were able to form vascular structures alone, but that a combination of the two resulted in robust vascular structure formation *in vitro*^[5], extending a preliminary report showing tubule formation on Matrigel® by CD90⁺/CD34⁺ cells^[59]. We thereby showed that CD34⁺CD31⁻ cells not only include hASC but also cells with a pericytic phenotype necessary for the stabilization of endothelial capillaries formed by CD34⁺CD31⁺ adipose endothelial cells (hAEC, Figure 1), as previously suggested^[6]. However, despite a clear potential of these vasculogenic cells derived from adipose tissue to initiate vascularization in regenerative medicine applications (reviewed in^[2] or to treat ischemic disorders^[8], their safety for clinical applications should still be considered with caution. For example, the adipose tissue used for autologous lipotransfer procedures, e.g., for breast reconstruction, in some cancer patients (which is rich in CD34⁺ progenitors) was shown to promote tumor growth, angiogenesis and metastases in several orthotopic models of human breast cancer^[60]. This possibly reflects a side-effect due to mast cells, which are present in the adipose transplants, release VEGF and thereby induce tumor angiogenesis^[61].

In summary, it is important to make a distinction between the CD34⁺CD31⁺ hAEC constitutively present in the native adipose tissue and in the SVF and CD34⁺CD31⁺ endothelial-like cells obtained by differentiation of CD34⁺/CD31⁻ expanded hASC^[62,63]. Interestingly, this indicates that, like primary vascular endothelial cells^[50,51], cells

derived from adipose tissue, that lose the expression of CD34 in monolayer culture retain the ability to re-express this marker if stimulated appropriately.

Function of CD34 in SVF cells and hASC

Despite the loss of CD34 expression during *in vitro* culture, expanded hASC retain proliferative capacity and multipotency even after several passages. However, little is known about how CD34 affects the biological features and the functionality of SVF cells, i.e., hASC or endothelial/vasculogenic cells.

With regard to the role of CD34 in the biology of endothelial cells from SVF, it is likely to involve mechanisms mostly similar to the ones demonstrated for lumen formation in the developing mouse aorta^[64], as suggested by our preliminary data showing similar tubes as well as lumen formation by SVF cells *in vitro*^[5]. Moreover, the endothelial CD34⁺/CD31⁺ fraction within SVF cells exhibit the ability to form functional blood vessels *in vivo* and connect to the vasculature of the recipient mice, both after perfusion culture^[9] or if implanted directly after isolation from adipose tissue^[65]. We also find that vasculogenic cells from the SVF can generate highly organized vascular structures *in vitro*, which by anastomosis with the vasculature of the host greatly enhance both the amount and depth of bone tissue formation inside tissue engineered osteogenic constructs *in vivo*^[5]. The functional role of CD34 in vasculogenic cells from SVF, and the mechanisms involved will be further investigated by us in the future.

With regard to CD34 expression in ASC, few studies have addressed the role of CD34 in hASC function, *in vitro*. Suga *et al.*^[52] used SVF cells after a short initial expansion phase on tissue culture plastic and showed that, in sorted SVF-derived cells, both CD34⁺ and CD34⁻ cells are clonogenic and that the impact of CD34 expression on hASC function was limited. Indeed, CD34⁺ cells showed a slightly decreased proliferation and a limited increase in differentiation capacity towards mesodermal lineages. More recently, in 8-d expanded hASC, Maumus *et al.*^[53] demonstrated that CD34⁺ hASC are the only subpopulation of hASC containing clonogenic cells, and the only one able to differentiate into adipogenic and osteogenic lineages. We simultaneously confirmed and extended this finding to unexpanded, SVF cells by showing that it is more specifically the CD34⁺/CD31⁻ cell population which contains the cells with osteoblastic differentiation capacity and also clonogenic, colony-forming cells^[5].

In the context of bone tissue engineering, several reports suggest that, despite the loss of CD34 upon expansion of hASC, they can maintain an actual capacity to form bone tissue *in vivo*, if cultured adequately (reviewed in^[2]). SVF cells were used by our group to generate osteogenic grafts with intrinsic vasculogenic capacity, both if implanted immediately^[65] or when SVF cells were seeded and cultured for 5 d within hydroxyapatite porous scaffolds inside a perfusion-based bioreactor system^[9].

In both cases, it is important to mention that more than 65% of the cells, including hASC, still expressed CD34 in the experimental conditions used. These studies critically established that human SVF cells, similarly to expanded hASC, are capable of generating frank bone tissue *in vivo*. This was possible even in the absence of exogenous osteoinductive signals, when SVF cells were cultured under perfusion^[9], whereas direct implantation of SVF cells required an osteogenic trigger such as bone morphogenetic protein 2 to support ectopic osteogenesis *in vivo* (Mehrkens *et al.*, In press). The potential importance of CD34 in bone formation by hASC is currently being investigated by our group by ectopic implantation of the CD34⁺ and CD34⁻ fractions.

Conclusion and perspectives

The use of CD34 as an adipose-derived cell marker, and the numerous reports about expression of CD34 by hASC and its progressive loss upon *in vitro* cell culture can not compensate for a major lack of references in the literature about the precise role and function of CD34 in hASC and other cells derived from the SVF. This fact should prompt the increasing number of research teams using hASC and SVF cells in regenerative medicine applications worldwide to design new research projects aimed at addressing this question more specifically. In particular, a better understanding of the membrane localization and trafficking of CD34, of the signaling pathways induced by CD34 and of the promoters controlling the expression of CD34 in SVF-derived cells would help in better investigating its role on functions such as adhesion, migration, proliferation or differentiation, and consequently the tissue formation capacities of these cells *in vivo*.

Furthermore, alternative culture methods should be developed to avoid the loss of CD34 expression and to preserve a physiological phenotype. Our group recently investigated the possibility of expanding hASC while maintaining their expression of CD34. This study is based on the hypothesis that cell-extracellular matrix and cell-cell interactions should be favored to mimic the physiological situation. The final aim would be to reconstitute a three-dimensional physiological environment in a controlled setting as it was proposed for the bone marrow niche in one of our recent reports^[66]. The system aimed at recapitulating the complex microenvironment of the niche and establishing the chemico-physical cues required for a physiological stem cell function regulation. By applying this principle to SVF cells, it was possible to maintain CD34⁺/CD31⁻/CD105⁻ hASC, i.e., a phenotype much more similar to native hASC, for up to 6 wk in culture (our preliminary, unpublished data). SVF cells from the same donors, cultured in parallel as standard monolayer with serial, weekly passaging, generated hASC with minimal expression of CD34. A deeper characterization is needed to better understand the biological features and functionality of these cells and is currently ongoing.

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Impact of the antiproliferative agent ciclopirox olamine treatment on stem cells proteome

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Abstract

AIM: To investigate the proteome changes of stem cells due to ciclopirox olamine (CPX) treatment compared to control and retinoic acid treated cells.

METHODS: Stem cells (SCs) are cells, which have the ability to continuously divide and differentiate into various other kinds of cells. Murine embryonic stem cells (ESCs) and multipotent adult germline stem cells (maGSCs) were treated with CPX, which has been shown to have an antiproliferative effect on stem cells, and compared to stem cells treated with retinoic acid (RA),

which is known to have a differentiating effect on stem cells. Classical proteomic techniques like 2-D gel electrophoresis and differential in-gel electrophoresis (DIGE) were used to generate 2D protein maps from stem cells treated with RA or CPX as well as from non-treated stem cells. The resulting 2D gels were scanned and the digitalized images were collated with the help of Delta 2D software. The differentially expressed proteins were analyzed by a MALDI-TOF-TOF mass spectrometer, and the identified proteins were investigated and categorized using bioinformatics.

RESULTS: Treatment of stem cells with CPX, a synthetic antifungal clinically used to treat superficial mycoses, resulted in an antiproliferative effect *in vitro*, without impairment of pluripotency. To understand the mechanisms induced by CPX treatments which results in arrest of cell cycle without any marked effect on pluripotency, a comparative proteomics study was conducted. The obtained data revealed that the CPX impact on cell proliferation was accompanied with a significant alteration in stem cell proteome. By peptide mass fingerprinting and tandem mass spectrometry combined with searches of protein sequence databases, a set of 316 proteins was identified, corresponding to a library of 125 non-redundant proteins. With proteomic analysis of ESCs and maGSCs treated with CPX and RA, we could identify more than 90 single proteins, which were differently expressed in both cell lines. We could highlight, that CPX treatment of stem cells, with subsequent proliferation inhibition, resulted in an alteration of the expression of 56 proteins compared to non-treated cells, and 54 proteins compared to RA treated cells. Bioinformatics analysis of the regulated proteins demonstrated their involvement in various biological processes. To our interest, a number of proteins have potential roles in the regulation of cell proliferation either directly or indirectly. Furthermore the classification of the altered polypeptides according to their main known/postulated functions revealed that the majority of these proteins are involved in molecular functions

like nucleotide binding and metal ion binding, and biological processes like nucleotide biosynthetic processes, gene expression, embryonic development, regulation of transcription, cell cycle processes, RNA and mRNA processing. Proteins, which are involved in nucleotide biosynthetic process and proteolysis, were downregulated in CPX treated cells compared to control, as well as in RA treated cells, which may explain the cell cycle arrest. Moreover, proteins which were involved in cell death, positive regulation of biosynthetic process, response to organic substance, glycolysis, anti-apoptosis, and phosphorylation were downregulated in RA treated cells compared to control and CPX treated cells.

CONCLUSION: The CPX treatment of SCs results in downregulation of nucleotide binding proteins and leads to cell cycle stop without impairment of pluripotency.

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Key words: Stem cells; Differentiation; Hypusination; Ciclopirox olamine; Proteomics; Retinoic acid

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INTRODUCTION

Stem cells (SCs) are cells, which are found in all multicellular organisms, which can continuously divide and differentiate into various specialized cell types and can also self-renew to produce more stem cells^[1]. The therapeutic use of embryonic stem cells (ESCs) has been constrained by problems caused by immune rejection in the patient as well as ethical issues associated with the use of embryos^[2]. Spermatogonial stem cells (SSCs) are self-renewing single cells located in the periphery of the seminiferous tubules whose continuous division maintain spermatogenesis throughout the life of a male individual^[3]. SSCs were isolated from murine testis and cultured for the first time in 2006^[4]. The pluripotency and plasticity of these cultured cells, named multipotent adult germline stem cells (maGSCs), were proven to be similar to ESCs. The ESC-like nature of maGSCs was confirmed on the microRNA level^[5], on the transcriptome level^[6] and on the proteome level^[7]. In a recent study, we investigated the effects of retinoic acid (RA) treatment on the protein expression profiles of maGSCs and ESCs^[8]. The study revealed the important role of Eif5a and its hypusination for stem cell differentiation and proliferation.

Eif5a is a universal translation elongation factor which is highly conserved in all cells. Eif5a has been shown to be associated with translation, viability and proliferation processes^[9-12]. It is the only eukaryotic protein known to have the unusual amino acid hypusine. Hypusine is es-

sential to the function of Eif5a and is involved in protein biosynthesis by promoting the formation of the first peptide bond and translation elongation^[13]. The activation of Eif5a is controlled by a unique post-translational modification called hypusination. It occurs in two steps which are controlled by two different enzymes^[14,15], which inactivation can lead to hypusination inhibition. Ciclopirox olamine (CPX), the ethanolamine salt of 6-cyclohexyl-1-hydroxy-4-methylpyridin-2(1H)-one, is a hypusination inhibitor that controls the second step of the modification, which is catalyzed by deoxyhypusine hydroxylase^[14].

CPX, a synthetic antifungal agent, has been used topically to treat fungal and yeast infection of skin or mucosa for more than 20 years^[16-19]. Apart from its antimycotic activity, CPX is also effective against both gram-positive and gram-negative bacteria^[20]. CPX might also serve as an alternative to recombinant vascular endothelial growth factor (VEGF) treatment or to VEGF gene therapy for therapeutic angiogenesis^[21]. The effect of CPX on several *Saccharomyces cerevisiae* mutants has been screened and tested, and it was suggested that CPX may exert its effect by disrupting DNA repair, DNA replication, cell division signals and a defect in mitotic spindle function. Furthermore CPX can influence the regulation of many processes, including signal transduction, transcription, cell division, and development^[22]. Recent studies demonstrated CPX as a potential anti-cancer agent for the treatment of malignancies, including leukemia and myeloma^[23-25]. However, the mechanism of CPX as a drug in angiogenesis and tumor treatment is poorly understood. CPX works as an inhibitor of the iron-dependent enzymes due to its role as a chelator of intracellular iron^[22,23]. Other studies reported the inhibition of HIV-1 gene expression by CPX^[26], the importance of Eif5a in embryogenesis and cell differentiation^[27], in hepatocellular carcinoma^[28] and in diabetes^[29]. CPX has also been used as an inhibitor of hypusination.

In a recent study, the effect of CPX on the cellular viability and proliferation of ESCs and maGSCs was investigated. CPX treatment of the stem cells resulted in an antiproliferative effect on ESCs and maGSCs *in vitro*, but did not affect the cell pluripotency^[8]. The inhibitory effect of CPX on cell differentiation was reversible and was not associated to apoptosis. The ESCs were found to be more sensitive to CPX than the maGSCs.

The aim of this study was to investigate the proteome changes of ESCs and maGSCs accompanying the treatment with CPX and subsequent inhibition of hypusination using classical proteomic techniques like 2-DE, DIGE and MS. 2D protein maps were generated from control cells and cells treated either with RA or CPX. The resulting protein maps were compared to each other and the differentially expressed proteins were investigated using bioinformatics. We could highlight that a treatment with CPX, involving proliferation inhibition, resulted in an alteration of the expression of 56 proteins compared to non-treated cells, and 54 proteins compared to RA treated cells. The majority of these proteins are involved in nucleotide binding and nucleotide biosynthetic pro-

cesses, metal binding, DNA binding, and other processes which have been linked to CPX.

MATERIALS AND METHODS

Derivation and culture of maGSC and ESC lines

The derivation and culture of maGSCs 129/Sv was described previously^[4]. In brief, testes from adult mice were isolated and digested using collagenase. Single cell suspension was derived after trypsin digestion followed by the culture of the testis suspension cells on a mouse embryonic fibroblasts (MEFs) feeder layer in the presence of GDNF. After appearance of morphological ES-like cells, the colonies were picked and expanded in standard ES cell conditions. In this case, the maGSC line was generated without genetic selection, only by morphological criteria. The ESC R1 line was derived from the 129/Sv mouse^[30]. To maintain maGSCs and ESCs in an undifferentiated state, the cells were cultured under standard ESC culture conditions: DMEM (PAN, Aidenbach, Germany) supplemented with 20% fetal calf serum (PAN, Aidenbach, Germany), 2 mmol/L L-glutamine (PAN, Aidenbach, Germany), 50 mmol/L β -mercaptoethanol (Gibco/Invitrogen, Eggenstein, Germany), $1 \times$ non-essential amino acids (Gibco/Invitrogen), sodium pyruvate (Gibco/Invitrogen), and penicillin/streptomycin (PAN, Aidenbach, Germany). ESCs and maGSCs were cultured on a feeder layer of mitomycin C-inactivated MEFs in the presence of 1000 U/mL recombinant mouse leukemia inhibitory factor (LIF) (Chemicon, Temecula, United States). ESCs were isolated as described previously, and male ESC lines were identified and selected by PCR amplification of Sry gene-specific sequences^[31,32]. In order to differentiate maGSCs and male ESCs, the cells were plated on gelatin-coated dishes and culture medium was supplemented with 1 μ mol/L RA (Sigma-Aldrich, Steinheim, Germany) instead of LIF. Cells were cultured for 48 h before they were lysed and the proteins were extracted. For examining the effect of CPX on the proteome level, ESCs and maGSCs were treated with culture medium supplemented with 2 μ mol/L CPX for 72 h.

Protein extraction

The protein extraction for 2-DE was performed as described previously^[7]. Briefly, 75% confluent cultures were trypsinized and washed three times with PBS. The cells were harvested by centrifugation at $200 \times g$ for 10 min, the pellet was treated with 0.3–0.5 mL lysis buffer [9.5 mol/L urea, 2% CHAPS (w/v), 2% ampholytes (w/v), 1% DTT]. Ampholytes and DTT were added shortly before use. After adding the lysis buffer, the samples were incubated for 30 min at 4 °C. For removing the cell debris, sample centrifugation was carried out at $13\,000 \times g$ and 4 °C for 45 min. The supernatant was recentrifuged at $13\,000 \times g$ and 4 °C for an additional 45 min to get maximal purity. The resulting samples were used immediately or stored at -80 °C until use.

Protein precipitation

To reduce the salt contamination and to enrich the proteins, methanol-chloroform-precipitation according to Wessel *et al.*^[33] was performed. Briefly, 0.4 mL of methanol (100%) was added to 0.1 mL aliquots of protein samples and mixed together. 0.1 mL chloroform was added to the samples and the mixture was vortexed. Subsequently 0.3 mL water was added and the solution was vortexed and centrifuged at $13\,000 \times g$ for 1 min. The aqueous layer was removed, and another 0.4 mL methanol (100%) was added to the rest of the chloroform and the interphase with the precipitated proteins. The sample was mixed and centrifuged for 2 min at $13\,000 \times g$ and the supernatant was removed. The pellet was vacuum dried and dissolved in lysis buffer.

Total protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, United States) according to Bradford^[34]. BSA (Sigma, Steinheim, Germany) was used as a standard.

2D gel electrophoresis (2-DE)

IPG strips (11 cm, pI 5–8) were passively rehydrated in 185 μ L solution containing 150 μ g protein in a rehydration buffer (8 mol/L urea, 1% CHAPS, 1% DTT, 0.2% ampholytes, and a trace of bromophenol blue) for 12 h. The IEF step was performed on the PROTEAN[®] IEF Cell (Bio-Rad, Hercules, CA, United States). Temperature-controlled at 20 °C, the voltage was set to 500 V for 1 h, increased to 1000 V for 1 h, 2000 V for 1 h and left at 8000 V until a total of 50 000 Vhours was reached. Prior to SDS-PAGE, the IPG strips were reduced for 20 min at room temperature in SDS equilibration buffer containing 6 mol/L urea, 30% glycerol, 2% SDS 0.05 mol/L Tris-HCl, and 2% DTT on a rocking table. The strips were subsequently alkylated in the same solution with 2.5% iodoacetamide substituted for DTT, and a trace of bromophenol blue. For the SDS-PAGE, 12% BisTris Criterion precast gels (Bio-Rad, Hercules, CA, United States) were used according to manufacturer's instructions. The gels were run at 150 V for 10 min followed by 200 V until the bromophenol blue dye front had reached the bottom of the gel.

Gel staining

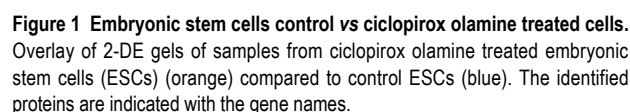
For image analysis, 2-DE gels were fixed in a solution containing 50% methanol and 12% acetic acid overnight and fluorescent stained with Flamingo fluorescent gel stain (Bio-Rad, Hercules, CA, United States) for minimum 5 h. After staining, gels were scanned at 50 μ m resolution on a Fuji FLA-5100 scanner. The digitalized images were analyzed using Delta 2D 3.4 (Decodon, Braunschweig, Germany). For protein visualization, 2-DE gels were additionally stained with colloidal Coomassie blue, Roti-Blue (Roth, Karlsruhe, Germany) overnight.

2D-DIGE

Protein extraction and methanol-chloroform-precipitation

To avoid the dye-specific protein labeling, every pair of protein samples from two independent cell extract preparations were processed in duplicate while swapping the dyes. Thereby four replicate gels were obtained, allowing to monitor regulation factors down to twofold changes^[35]. An internal standard consisting of a mixture of the samples under investigation was labeled with Cy2 and included on all gels to facilitate gel matching, thereby eliminating artifacts from experimental variation. The three differentially labeled fractions were pooled. Rehydration buffer (8 mol/L urea, 1% CHAPS, 13 mmol/L DTT and 1% ampholytes 3-10) was added to make up the volume to 185 μ L prior to IEF. The 2-DE was performed as described above. The CyDye-labeled gels were scanned at 50 μ m resolution on a Fuji FLA5100 scanner (Fuji Photo, Kanagawa, Japan) with laser excitation light at 473 nm and long pass emission filter 510LP (Cy2), 532 nm and long pass emission filter 575LP (Cy3), and 635 nm and long pass emission filter 665LP (Cy5). Fluorescent images were acquired in 16-bit TIFF files format. Spot matching across gels and normalization based on the internal standard was performed with Delta2D software (Decodon, Greifswald, Germany). To analyze the significance of protein regulation, a Student's *t*-test was performed, and statistical significance was assumed for *p* values less than 0.01. For protein visualization, the 2-DE gels were post-stained with colloidal Coomassie blue (Roti-Blue) overnight. Differentially regulated proteins were excised and processed for identification by mass spectrometry.

Manually excised gel plugs were subjected to an automated platform for the identification of gel-separated proteins^[36] as described in the framework of recent DIGE-based^[37] and large-scale proteome studies^[38]. An Ultraflex MALDI-TOF-TOF mass spectrometer (Bruker Daltonik) was used to acquire both PMF and fragment ion spectra, resulting in confident protein identifications based on peptide mass and sequence information. Database searches in the Swiss-Prot primary sequence database restricted to the taxonomy *mus musculus* were performed using the MASCOT Software 2.2 (Matrix Science). Carboxamidomethylation of Cys residues was specified as fixed and oxidation of Met as variable modifications. One trypsin missed cleavage was allowed. Mass tolerances were set to 100 ppm for PMF searches and to 100 ppm (precursor ions) and 0.7 Da (fragment ions) for MS/MS ion searches. The minimal requirement for accepting a protein as identified was at least one peptide sequence match above identity threshold in addition to at



least 20 % sequence coverage in the PMF.

The classification of the identified proteins according to their main known/postulated functions was carried out using DAVID bioinformatics^[39,40]. This classification together with the official gene symbol was used to investigate and categorize the gene ontology (GO)-annotations (biological processes and molecular functions).

Comparative analysis of differentially expressed proteins in RA and CPX treated SCs by 2-DE and ontogenic classification

The identified proteins were classified using DAVID bioinformatics^[39,40] focusing on its information considering the GO (Gene Ontology) annotations. The terms corresponding to the molecular function and biological process were regarded (Figures 5-7).

Examination of all of the proteins, which expression was altered either by CPX or RA treatment, was performed regarding their involvement in biological processes. We found that seven proteins are involved in regulation of

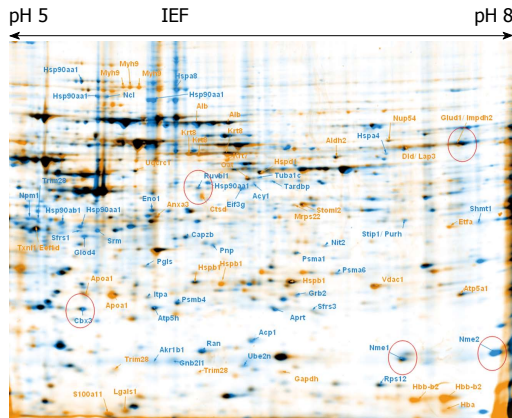


Figure 2 Multipotent adult germline stem cells control vs cyclopirox olamine treated cells. Overlay of 2-DE gels of samples from cyclopirox olamine treated multipotent adult germline stem cells (maGSCs) (orange) compared to control maGSCs (blue). The identified proteins are indicated with the gene names.

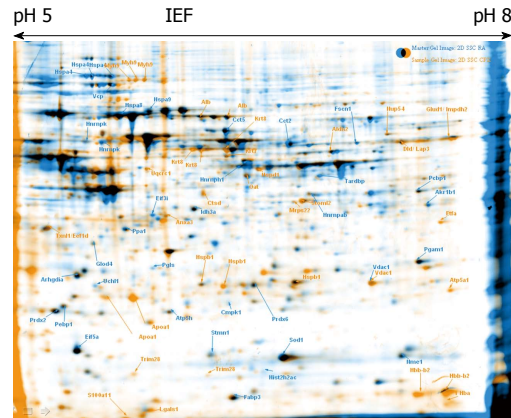


Figure 4 Multipotent adult germline stem cell control vs cyclopirox olamine treated cells. Overlay of 2-DE gels of samples from cyclopirox olamine treated Multipotent adult germline stem cells (maGSCs) (orange) compared to retinoic acid treated maGSCs (blue). The identified proteins are indicated with the gene names.

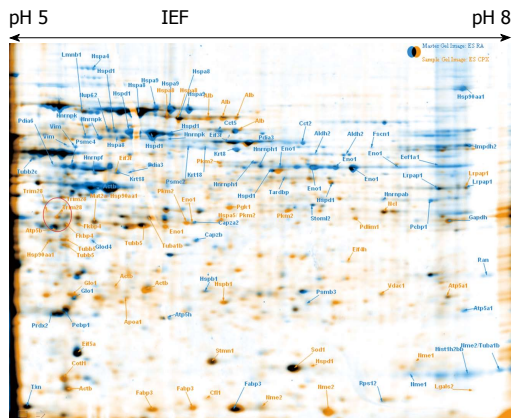


Figure 3 Embryonic stem cell control vs cyclopirox olamine treated cells. Overlay of 2-DE gels of samples from cyclopirox olamine treated embryonic stem cells (ESCs) (orange) compared to retinoic acid treated ESCs (blue). The identified proteins are indicated with the gene names.

transcription. Among these proteins Ube2n, Tardbp, Cbx3 and Hnrnpab were downregulated in CPX treated cells compared to control, whereas Nup62 was upregulated in CPX treated cells compared to control (Table 2). Two other proteins Trim28 and Ruvbl1 were downregulated in RA treated cells compared to control. Detailed information is given in Table 2 and the protein expression regulation folds are given in Tables 3-6.

When we looked at the molecular function of the regulated proteins, we observed that a major part of the proteins are involved in nucleotide binding (Table 7). Approximately half of these proteins were downregulated and the other half was upregulated upon CPX treatment compared to RA treated cells. About 13 proteins are involved in metal ion binding, of these five proteins (Acy1, Uqcrc1, Sfrs1, Trim28, Glo1) are involved in transition metal ion binding, like Fe^{3+} , which is known to be important in case of CPX, as CPX works as an inhibitor of the iron-dependent enzymes due to its role as a chelator of

intracellular iron. Three of the proteins involved in transition metal ion binding (Sfrs1, Trim28 and Glo1), were up-regulated upon CPX treatment compared to RA-treated cells.

Overall, it could be observed that most of the proteins of interest were downregulated in either CPX or RA treated cells compared to control.

Treated cells compared to control

About 56 of the 125 identified proteins showed different expression as a reaction to CPX treatment compared to control. Of these, 14 proteins were upregulated as a reaction to CPX treatment (Table 3), whereas 44 proteins were downregulated (Table 4). The expression of 52 proteins was found to be altered in both cell types, ESCs and maGSCs, under RA treatment compared to control (Tables 5 and 6). Of these proteins, 11 were upregulated and 41 were downregulated as a reaction to RA treatment.

In both experiments the majority of the regulated proteins were downregulated as a reaction to one of the treatments. Although mainly different proteins were regulated, bioinformatics analysis revealed that the downregulated proteins in both experiments are primarily involved in the same molecular functions (Figure 5). The downregulated proteins upon CPX treatment are mainly involved in nucleotide binding, GTP binding, peptidase activity and metal ion binding, particularly magnesium ion binding. The proteins which were downregulated upon RA treatment are involved in transition metal ion binding instead of magnesium ion binding, and furthermore involved in enzyme binding. Proteins, which were upregulated upon CPX treatment, are mainly involved in nucleotide binding, whereas proteins which were upregulated upon RA treatment are involved in nucleotide and metal ion binding.

When we look at the involvement of the regulated proteins in biological processes, more differences were observed (Figure 6). Both treatments showed downregu-

Table 1 Non-redundant proteins

Protein name	Gene name	Swiss-prot	Nominal mass	CPI	PMF-score	PMF sequence coverage	MS/MS-score	MS/MS-sequence coverage
Low molecular weight phosphotyrosine protein phosphatase	Acp1	PPAC_MOUSE	18 636	6.4	96	65	80	24
Actin, cytoplasmic 1	Actb	ACTB_MOUSE	42 052	5.2	170	70	312	15
Aminoacylase-1	Acy1	ACY1_MOUSE	45 980	5.9	167	56	44	5
Aldose reductase	Akr1b1	ALDR_MOUSE	36 052	6.9	128	43	136	10
Aldehyde dehydrogenase, mitochondrial	Aldh2	ALDH2_MOUSE	57 015	8.6	221	54	131	7
Annexin A3	Anxa3	ANXA3_MOUSE	36 520	5.2	84	47	111	14
Adenine phosphoribosyltransferase	Aprt	APT_MOUSE	19 883	6.4	88	67	216	27
Rho GDP-dissociation inhibitor 1	Arhgdia	GDIR1_MOUSE	23 450	5	123	54	66	11
ATP synthase subunit α , mitochondrial	Atp5a1	ATPA_MOUSE	59 830	9.7	100	28	53	4
ATP synthase subunit β , mitochondrial	Atp5b	ATPB_MOUSE	56 265	5.1	90	30	167	10
ATP synthase subunit d, mitochondrial	Atp5h	ATP5H_MOUSE	18 795	5.4	122	70	169	36
F-actin-capping protein subunit α -2	Capza2	CAZA2_MOUSE	33 118	5.5	148	69	19	9
F-actin-capping protein subunit β	Capzb	CAPZB_MOUSE	31 611	5.4	117	61	129	8
Chromobox protein homolog 3	Cbx3	CBX3_MOUSE	21 013	5	38	36	67	6
T-complex protein 1 subunit β	Cct2	TCPB_MOUSE	57 783	6	248	61	75	9
T-complex protein 1 subunit epsilon	Cct5	TCPE_MOUSE	60 042	5.7	186	60	138	6
Cofilin-1	Cfl1	COF1_MOUSE	18 776	9.1	95	45	87	13
UMP-CMP kinase	Cmpk1	KCY_MOUSE	22 379	5.6	74	52	29	10
Coactosin-like protein	Cotl1	COTL1_MOUSE	16 048	5.1	86	60	116	14
Cathepsin D	Ctsd	CATD_MOUSE	45 381	6.9	160	41	95	4
Dihydrolipoyl dehydrogenase, mitochondrial	Dld	DLDH_MOUSE	54 751	9	112	48	81	2
Elongation factor 1- α 1	Eef1a1	EF1A1_MOUSE	50 424	9.7	68	34	115	8
Elongation factor 1- δ	Eef1d	EF1D_MOUSE	31 388	4.8	86	54	79	9
Elongation factor 2	Eef2	EF2_MOUSE	96 222	6.4	52	26	29	1
Eukaryotic translation initiation factor 3 subunit F	Eif3f	EIF3F_MOUSE	38 090	5.2	109	45	106	14
Eukaryotic translation initiation factor 3 subunit G	Eif3g	EIF3G_MOUSE	35 901	5.6	54	35	23	7
Eukaryotic translation initiation factor 3 subunit I	Eif3i	EIF3I_MOUSE	36 837	5.3	228	78	89	16
Eukaryotic translation initiation factor 4H	Eif4h	IF4H_MOUSE	27 381	7.5	83	51	65	8
Eukaryotic translation initiation factor 5A-1	Eif5a	IF5A1_MOUSE	17 049	4.9	115	58	170	22
α -enolase	Eno1	ENOA_MOUSE	47 453	6.4	183	64	170	13
Electron transfer flavoprotein subunit α , mitochondrial	EtfA	ETFA_MOUSE	35 330	9.5	138	59	100	9
Fatty acid-binding protein, heart	Fabp3	FABPH_MOUSE	14 810	6.1	86	77	212	39
Peptidyl-prolyl cis-trans isomerase FKBP4	Fkbp4	FKBP4_MOUSE	51 939	5.4	122	38	168	9
Fascin	Fscn1	FSCN1_MOUSE	55 215	6.5	129	45	26	6
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	G3P_MOUSE	36 072	9.2	62	38	40	8
Lactoylglutathione lyase	Glo1	LGUL_MOUSE	20 967	5.1	134	66	114	20
Glyoxalase domain-containing protein 4	Glod4	GLOD4_MOUSE	33 581	5.2	167	69	115	13
Glutamate dehydrogenase 1, mitochondrial	Glud1	DHEF3_MOUSE	61 640	8.8	70	37	60	5
Guanine nucleotide-binding protein subunit β -2-like 1	Gnb2l1	GBLP_MOUSE	35 511	8.9	116	55	20	5
Growth factor receptor-bound protein 2	Grb2	GRB2_MOUSE	25 336	5.9	73	54	36	17
Histone H2B type 1-B	Hist1h2bb	H2B1B_MOUSE	13 944	10.8	52	41	93	19
Histone H2A type 2-C	Hist2h2ac	H2A2C_MOUSE	13 980	11.4	50	55	67	12
Heterogeneous nuclear ribonucleoprotein A/B	Hnmpab	ROAA_MOUSE	30 926	8.7	83	30	107	5
Heterogeneous nuclear ribonucleoproteins C1/C2	Hnmpc	HNRPC_MOUSE	34 421	4.8	57	32	57	6
Heterogeneous nuclear ribonucleoprotein F	Hnmpf	HNRPF_MOUSE	46 043	5.2	163	56	207	12
Heterogeneous nuclear ribonucleoprotein H	HnmpH1	HNRH1_MOUSE	49 454	5.9	166	61	134	15
Heterogeneous nuclear ribonucleoprotein K	Hnmpk	HNRPK_MOUSE	51 230	5.3	144	46	251	11
Heat shock protein HSP 90- α	Hsp90aa1	HS90A_MOUSE	85 134	4.8	131	31	130	5
Heat shock protein HSP 90- β	Hsp90ab1	HS90B_MOUSE	83 615	4.8	62	25	78	6
Heat shock 70 kDa protein 4	Hspa4	HSP74_MOUSE	94 872	5	242	54	102	3
78 kDa glucose-regulated protein	Hspa5	GRP78_MOUSE	72 492	4.9	78	25	122	5
Heat shock cognate 71 kDa protein	Hspa8	HSP7C_MOUSE	71 055	5.2	234	58	154	4
Stress-70 protein, mitochondrial	Hspa9	GRP75_MOUSE	73 768	5.8	219	50	272	7
Heat shock protein β -1	Hspb1	HSPB1_MOUSE	23 057	6.1	144	55	344	24
60 kDa heat shock protein, mitochondrial	Hspd1	CH60_MOUSE	61 088	5.8	334	69	232	10
Isocitrate dehydrogenase (NAD) subunit α , mitochondrial	Idh3a	IDH3A_MOUSE	40 069	6.3	70	31	158	12
Inosine-5'-monophosphate dehydrogenase 2	Impdh2	IMDH2_MOUSE	56 179	7	173	50	107	7
Inosine triphosphate pyrophosphatase	Itpa	ITPA_MOUSE	22 225	5.5	84	72	128	15
Keratin, type I cytoskeletal 18	Krt18	K1C18_MOUSE	47 509	5.1	199	65	58	9
Keratin, type II cytoskeletal 7	Krt7	K2C7_MOUSE	50 678	5.6	137	52	55	4
Keratin, type II cytoskeletal 8	Krt8	K2C8_MOUSE	54 531	5.6	245	55	237	9
Cytosol aminopeptidase	Lap3	AMPL_MOUSE	56 505	8.7	126	47	58	5
L-lactate dehydrogenase B chain	Ldhb	LDHB_MOUSE	36 834	5.6	84	46	30	6

Galectin-1	Lgals1	LEG1_MOUSE	15 198	5.2	109	70	172	25
Galectin-2	Lgals2	LEG2_MOUSE	14 984	7.9	120	88	60	14
Lamin-B1	Lmnbl	LMNB1_MOUSE	66 973	5	265	60	134	5
α -2-macroglobulin receptor-associated protein	Lrpap1	AMRP_MOUSE	42 189	7.9	139	49	177	14
S-adenosylmethionine synthase isoform type-2	Mat2a	METK2_MOUSE	44 003	6	73	41	59	3
28S ribosomal protein S22, mitochondrial	Mrps22	RT22_MOUSE	41 281	9.2	112	45	97	9
Myosin-9	Myh9	MYH9_MOUSE	227 429	5.4	73	15	103	2
Nucleolin	Ncl	NUCL_MOUSE	76 734	4.5	113	26	179	7
Omega-amidase NIT2	Nit2	NIT2_MOUSE	30 825	6.5	112	59	75	9
Nucleoside diphosphate kinase A	Nme1	NDKA_MOUSE	17 311	7.7	125	72	223	30
Nucleoside diphosphate kinase B	Nme2	NDKB_MOUSE	17 466	7.8	160	84	287	30
Nucleophosmin	Npm1	NPM_MOUSE	32 711	4.5	53	33	136	10
Nuclear pore complex protein Nup54	Nup54	NUP54_MOUSE	55 812	6.6	55	21	23	3
Nuclear pore glycoprotein p62	Nup62	NUP62_MOUSE	53 336	5.1		13	43	5
Ornithine aminotransferase, mitochondrial	Oat	OAT_MOUSE	48 723	6.2	174	64	125	9
Poly(rC)-binding protein 1	Pcbp1	PCBP1_MOUSE	37 987	6.8	175	69	115	12
Protein disulfide-isomerase A3	Pdia3	PDIA3_MOUSE	57 099	5.8	254	55	90	5
Protein disulfide-isomerase A6	Pdia6	PDIA6_MOUSE	48 469	4.9	75	40	47	2
PDZ and LIM domain protein 1	Pdlim1	PDL1_MOUSE	36 208	6.4	200	73	56	7
Phosphatidylethanolamine-binding protein 1	Pebp1	PEBP1_MOUSE	20 988	5.1	130	79	107	11
Phosphoglycerate mutase 1	Pgam1	PGAM1_MOUSE	28 928	6.8	157	66	192	21
Phosphoglycerate kinase 1	Pgk1	PGK1_MOUSE	44 921	9	136	52	128	7
6-phosphogluconolactonase	Pgls	6PGL_MOUSE	27 465	5.5	102	49	148	17
Pyruvate kinase isozymes M1/M2	Pkm2	KPYM_MOUSE	58 378	7.9	178	49	106	9
Purine nucleoside phosphorylase	Pnp	PNPH_MOUSE	32 541	5.8	119	67	138	13
Inorganic pyrophosphatase	Ppa1	IPYR_MOUSE	33 102	5.3	126	66	26	7
Peroxiredoxin-2	Prdx2	PRDX2_MOUSE	21 936	5.1	103	62	285	22
Peroxiredoxin-6	Prdx6	PRDX6_MOUSE	24 969	5.6	156	67	101	17
Proteasome subunit α type-1	Psma1	PSA1_MOUSE	29 813	6	71	52	140	17
Proteasome subunit α type-6	Psma6	PSA6_MOUSE	27 811	6.4	72	38	108	10
Proteasome subunit β type-3	Psmb3	PSB3_MOUSE	23 235	6.2	110	51	187	30
Proteasome subunit β type-4	Psmb4	PSB4_MOUSE	29 211	5.3	60	42	109	10
26S protease regulatory subunit 7	Psmc2	PRS7_MOUSE	49 016	5.6	166	60	72	8
26S protease regulatory subunit 6B	Psmc4	PRS6B_MOUSE	47 366	5	144	55	109	9
GTP-binding nuclear protein Ran	Ran	RAN_MOUSE	24 579	7.8	124	51	139	11
40S ribosomal protein S12	Rps12	RS12_MOUSE	14 858	7.7	77	62	95	11
RuvB-like 1	Ruvbl1	RUVB1_MOUSE	50 524	6	61	35	106	10
Protein S100-A11	S100a11	S10AB_MOUSE	11 247	5.1		36	147	27
Splicing factor, arginine/serine-rich 1	Sfrs1	SFRS1_MOUSE	27 842	10.8	80	43	156	18
Splicing factor, arginine/serine-rich 3	Sfrs3	SFRS3_MOUSE	19 546	12.3			87	14
Serine hydroxymethyltransferase, cytosolic	Shmt1	GLYC_MOUSE	53 065	6.5	98	43	19	2
Superoxide dismutase [Cu-Zn]	Sod1	SODC_MOUSE	16 104	6	83	45	126	31
Spermidine synthase	Srm	SPEE_MOUSE	34 543	5.2	141	73	129	15
Stress-induced-phosphoprotein 1	Stip1	STIP1_MOUSE	63 170	6.4	184	55	89	4
Stathmin	Stmn1	STMN1_MOUSE	17 264	5.7	28	24	69	8
Stomatin-like protein 2	Stoml2	STML2_MOUSE	38 475	9.5	144	61	165	15
TAR DNA-binding protein 43	Tardbp	TADBP_MOUSE	44 918	6.3	68	30	107	7
T-complex protein 1 subunit α	Tcp1	TCPA_MOUSE	60 867	5.8	61	27	28	4
Transcription intermediary factor 1- β	Trim28	TIF1B_MOUSE	90 558	5.4		10	139	4
Tubulin α -1B chain	Tuba1b	TBA1B_MOUSE	50 804	4.8	128	39	152	9
Tubulin α -1C chain	Tuba1c	TBA1C_MOUSE	50 562	4.8	53	24	52	6
Tubulin β -2A chain	Tubb2a	TBB2A_MOUSE	50 274	4.6	126	55	111	11
Tubulin β -2C chain	Tubb2c	TBB2C_MOUSE	50 255	4.6	150	56	49	8
Tubulin β -5 chain	Tubb5	TBB5_MOUSE	50 095	4.6	169	57	237	9
Thioredoxin	Txn	THIO_MOUSE	12 010	4.6	63	67	92	22
Thioredoxin-like protein 1	Txn1l	TXNL1_MOUSE	32 616	4.7	144	78	39	2
Ubiquitin-conjugating enzyme E2 N	Ube2n	UBE2N_MOUSE	17 184	6.2	119	71	20	6
Ubiquitin carboxyl-terminal hydrolase isozyme L1	Uchl1	UCHL1_MOUSE	25 165	5	77	64	16	8
Cytochrome b-c1 complex subunit 1, mitochondrial	Uqcrc1	QCR1_MOUSE	53 420	5.7	95	40	46	6
Transitional endoplasmic reticulum ATPase	Vcp	TERA_MOUSE	89 950	5	310	61	40	5
Voltage-dependent anion-selective channel protein 1	Vdac1	VDAC1_MOUSE	32 502	9.2	159	57	80	24
Vimentin	Vim	VIME_MOUSE	53 712	4.9	218	64	47	8

CPI: Calculated isoelectric point; PMF: Peptide mass fingerprint; MS/MS: Tandem mass spectrometry.

lation of proteins involved in protein complex biogenesis, nucleotide biosynthetic process, cell death and positive regulation of biosynthetic process. Additionally, proteins involved in proteolysis and positive regulation of protein

metabolic process were downregulated in SCs upon CPX treatment. Proteins which were downregulated in SCs upon RA treatment are, among others, involved in cell cycle, RNA processing, glycolysis and negative regulation

Table 2 Gene Ontology functional annotation of proteins which were regulated in this experiment according to their involvement in different biological processes

Biological process	Proteins	CPX > RA	CPX < RA	CPX > c	CPX < c	RA > c	RA < c
Monosaccharide metabolic/ catabolic processes	5	Pgls Gapdh Eno1 Pkm2	Eno1		Eno1 Pkm2		Eno1 Ldhd Pkm2
Nucleobase, nucleoside, nucleotide, and nucleic acid biosynthetic processes	7	Atp5a1	Aprt Nme2 Nme1	Atp5a1	Aprt Atp5a1 Impdh2 Nme2 Nme1 Pnp	Nme2 Nme1	Aprt Atp5a1 Atp5h Impdh2
RNA and mRNA processing	6	Sfrs1			Hnrnpk Tardbp Pcbp1		Hnrnpc Sfrs3
Regulation of transcription	7	Ruvbl1 Trim28		Nup62	Ube2n Tardbp Cbx3 Hnrnpab		Trim28 Ruvbl1
Embryonic development	5	Sfrs1 Eno1	Psmc4 Eno1	Atp5a1 Myh9	Eno1 Atp5a1	Myh9 Psmc4	Eno1
Gene expression	16	Trim28 Sfrs1 Ruvbl1	Rps12	Eif3f Eif3i	Eef1a1 Eif5a Eef1d Cbx3 Hnrnpk Hnrnpab Tardbp Pcbp1		Sfrs3 Eif3i Hnrnpc Ruvbl1 Eef1d Trim28
Cell cycle processes	6	Ruvbl1		Myh9		Krt7 Myh9	Npm1 Tubb5 Stmn1 Ruvbl1
Cell morphogenesis involved in differentiation	4	Trim28		Myh9	Uchl1 Hnrnpab	Myh9	Stmn1 Trim28
Regulation of cell proliferation	4			Nup62	Nme2 Pnp	Nme2	Npm1
Regulation of signal transduction	4			Nup62	Ube2n		Npm1 Hspa5

Ciclopirox olamine (CPX) > retinoic acid (RA): Proteins which were more than 2-fold higher expressed in CPX-treated cells compared to RA-treated cells; CPX < RA: Proteins which were more than 2-fold higher expressed in RA-treated cells compared to CPX-treated cells; CPX > c: Proteins which were more than 2-fold higher expressed in CPX-treated cells compared to control; CPX < c: Proteins which were more than 2-fold higher expressed in control compared to CPX-treated cells; RA > c: Proteins which were more than 2-fold higher expressed in RA-treated cells compared to control; RA < c: Proteins which were more than 2-fold higher expressed in control compared to RA-treated cells.

of protein metabolic process.

Proteins which were upregulated in SCs upon CPX treatment are involved in nucleotide binding, regulation of cell death and protein transport, whereas proteins which were upregulated upon RA treatment are involved in nucleotide binding, metal ion binding and proteolysis.

Proteins in CPX treated cells compared to RA treated cells

When the proteins in RA treated SCs were compared to CPX treated SCs, we observed that 54 proteins are differently regulated (Tables 8 and 9). Of these proteins, 31 were upregulated and 26 downregulated upon CPX treatment. In some cases, different forms of one protein, e.g., Actb, Eno1, and Hsp90aa1 were observed and showed different regulation.

The bioinformatics analysis of these proteins, focus-

ing on biological processes, showed involvement of the proteins in different categories (Figure 7). Proteins which were downregulated in CPX treated cells are involved in processes like protein complex biogenesis, nucleotide biosynthetic process, proteolysis, intracellular transport and regulation of cell death. Proteins which were downregulated as a reaction to RA treatment are involved in protein complex biogenesis, cell death, positive regulation of biosynthetic process, response to organic substance, glycolysis, anti-apoptosis and phosphorylation.

To get a better focus on proteins, which may play a key role in proliferation, we also focussed on proteins, which showed contrary regulation upon CPX treatment and RA treatment compared to control. This resulted in 15 proteins, of which eight were upregulated upon CPX treatment and concurrently downregulated upon RA treatment compared to control, and seven proteins,

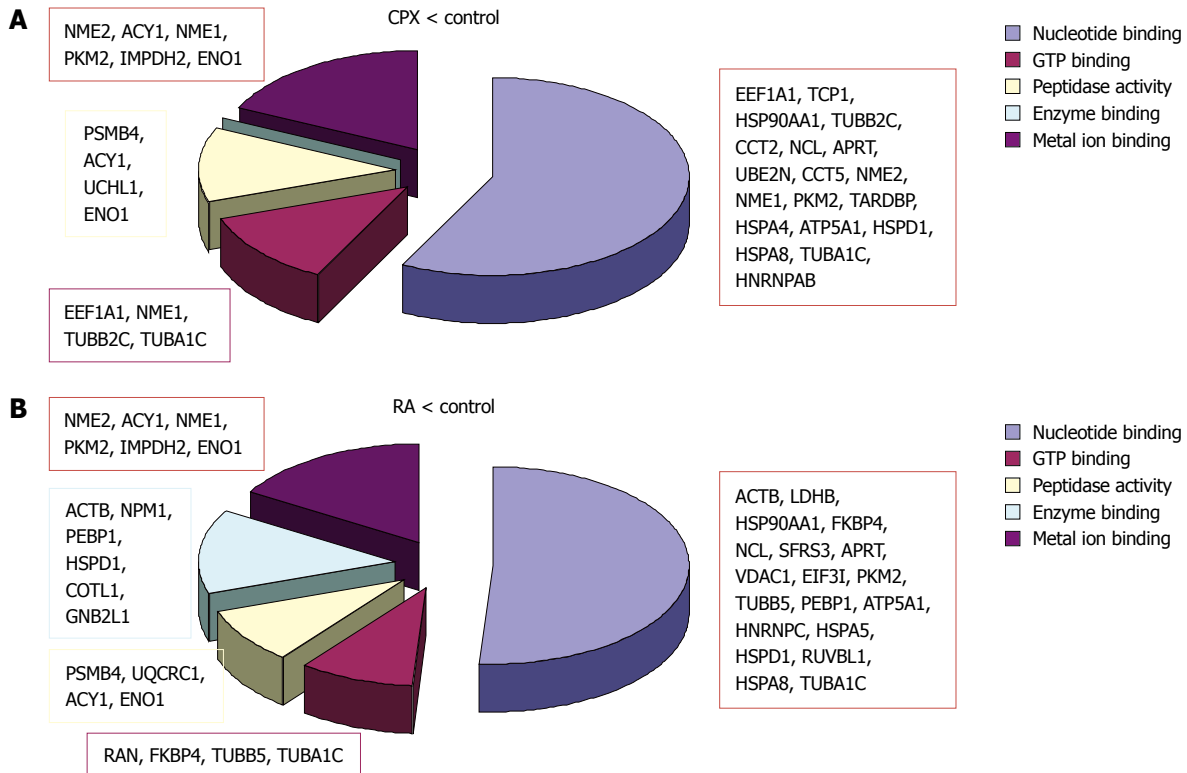


Figure 5 Molecular function. Classification of the downregulated proteins upon treatment with ciclopirox olamine (CPX) (A) or retinoic acid (RA) (B) according to their molecular functions.

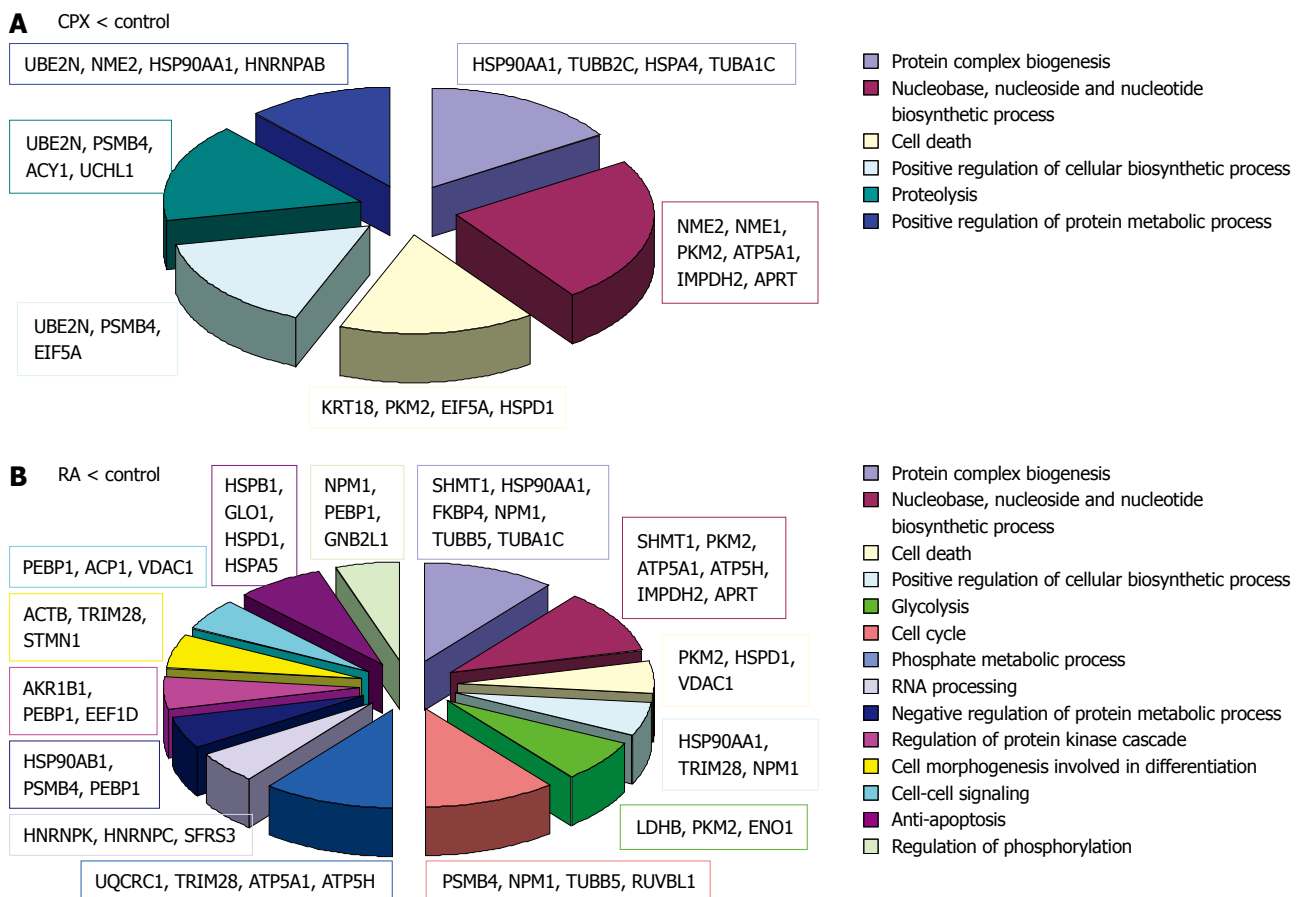


Figure 6 Biological process. Classification of the downregulated proteins upon treatment with ciclopirox olamine (CPX) (A) or retinoic acid (RA) (B) according to their biological processes.

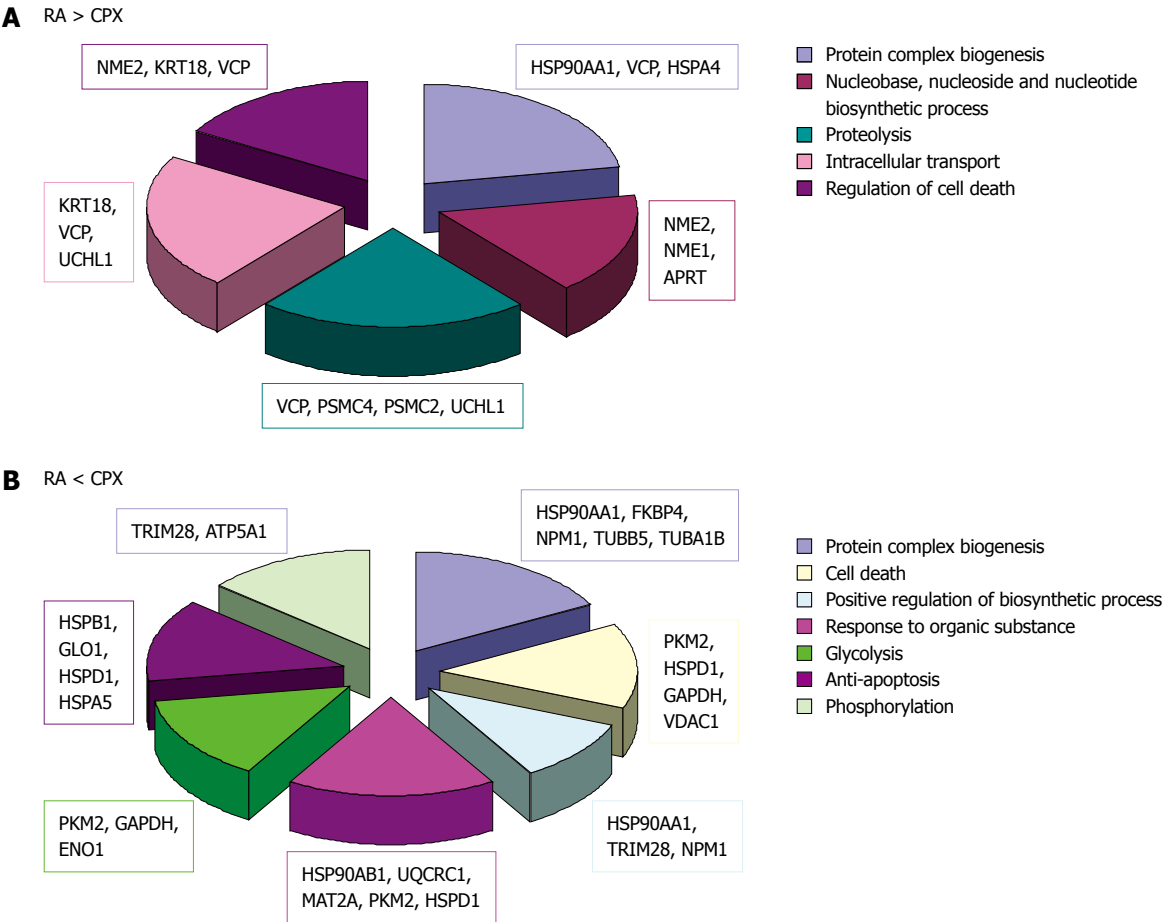


Figure 7 Biological process. Classification of the differently regulated proteins upon treatment with ciclopirox olamine (CPX) (A) or retinoic acid (RA) (B) according to their biological processes.

Table 3 Proteins which are upregulated upon ciclopirox olamine treatment compared to control		
	k/CPX	
	ESC	maGSC
Actb	0.13	0.19
Atp5a1 ¹	0.41	0.54
Ctsd	0.97	0.09
Eif3f ¹	0.94	0.43
Eif3i ¹	0.49	0.95
Etfp	0.63	0.39
Hspa9	0.92	0.31
Hspb1	0.63	0.05
Hspd1	0.19	0.21
Hspd1	0.36	0.69
Myh9 ¹	0.63	0.09
Nup62 ¹	0.30	0.60
S100a11		0.21
Tubb2a	1.00	0.21
Vdac1	0.58	0.18

¹The proteins are referred to in the text and following tables. CPX: Ciclopirox olamine; ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

which were downregulated upon CPX treatment and concurrently upregulated upon RA treatment compared to control (proteins marked by asterisk in Tables 8 and 9).

Bioinformatics analysis of the proteins, which were downregulated upon CPX treatment along with upregulated upon RA treatment were primarily involved in metabolic processes (Nme2, Hsp90aa1, Psmc4, Rps12, Cct2 and Eno1) like protein folding (Hsp90aa1, Cct2), whereas proteins, which were upregulated upon CPX-treatment and concurrently downregulated upon RA-treatment were additionally involved in developmental processes (Psmc4, Eno1) and transport/localization (Vdac1, Hspa9).

Analysis of the molecular function of the differently regulated proteins upon CPX and RA treatment showed their important role in nucleotide binding (Nme2, Hsp90aa1, Psmc4, Hspa4, Cct2, Actb, Pkm2, Hspa5, Vdac1 and Hspa9) and metal ion binding (Pkm2, S100a11, Eno1).

DISCUSSION

CPX is a synthetic antifungal drug, which is currently used for the treatment of superficial mycoses^[41]. Since two decades CPX has also been used as an antitumor agent^[42]. It has been shown that CPX can be used to treat solid tumors due to its strong antiangiogenic activity^[43,23]. CPX might inhibit the cell proliferation and work as an antitumor agent due to its iron chelating function, as iron is essential for cell proliferation and function^[24]. In

Table 4 Proteins which are downregulated upon ciclopirox olamine treatment compared to control

	k/CPX	
	ESC	maGSC
Acp1	1.29	6.01
Acy1 ¹	1.38	2.81
Akr1b1	2.07	13.44
Aprt ¹	4.80	3.60
Atp5a1 ¹	3.50	1.21
Capzb	3.04	2.35
Cbx3 ¹	1.72	2.12
Cct2 ¹	12.00	1.28
Cct5 ¹	1.06	2.02
Eef1a1 ¹	2.47	1.74
Eef1d ¹	1.46	2.03
Eif5a ¹	1.31	2.07
Eno1 ¹	3.56	1.60
Fscn1	3.31	1.49
Glod4	3.35	1.60
Gnb2l1	2.61	12.92
Hist1h2bb	2.31	2.10
Hist2h2ac	17.33	67.90
Hnrnpab ¹	2.41	3.36
Hnrnpk ¹	2.00	1.17
Hsp90aa1 ¹	1.19	6.79
Hsp90aa1 ¹	1.84	3.02
Hspa4 ¹	3.28	1.51
Hspa4	1.13	3.14
Hspa8	1.74	7.17
Hspd1	1.67	3.07
Impdh2 ²	2.59	2.13
Impdh2 ²	> 100	27.94
Krt18 ¹	2.31	1.44
Lgals2	2.82	8.45
Ncl ¹	1.33	2.61
Nit2	1.24	2.13
Nme1 ¹	6.25	1.56
Nme2 ¹	4.77	4.51
Pcbp1 ¹	2.21	1.64
Pkm2 ²	3.75	3.27
Pnp ¹	1.20	2.62
Psmb4 ¹	1.01	2.32
Ruvbl1 ¹	1.02	2.14
Srm	1.64	3.63
Shmt1 ¹	> 100	> 100
Tardbp ¹	1.38	3.52
Tcp1	1.47	3.76
Tuba1c ¹	1.87	3.11
Tubb2c ¹	1.38	3.41
Ube2n ¹	1.31	6.45
Uchl1 ¹	2.66	1.66

¹The proteins are referred to in the text and following tables. CPX: Ciclopirox olamine; ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

a recent study, we investigated the effect of CPX on the cellular viability and proliferation of SCs. The study demonstrated that in contrast to RA, CPX treatment resulted in a reversible antiproliferative effect^[8]. The present study was conducted to understand the anti-proliferative effect of CPX on stem cells in terms of proteins and molecular processes which are involved in its mode of action.

With proteomic analysis of ESCs and maGSCs treated with CPX and RA, we could identify more than 90 single proteins which were differently expressed in both cell

Table 5 Proteins which are downregulated upon retinoic acid treatment compared to control

Label	RA/k	
	ESC	maGSC
Acp1	0.61	0.50
Actb ¹	0.53	0.13
Acy1 ¹	0.13	0.70
Akr1b1	0.43	0.11
Aprt ¹	0.46	0.39
Atp5a1 ¹	0.76	0.38
Atp5h ¹	0.69	0.40
Cbx3	1.01	0.47
Cotl1 ¹	0.50	0.44
Eef1d ¹	0.70	0.15
Eif3i ¹	0.09	0.92
Eno1 ¹	0.24	0.04
Eno1 ¹	0.55	0.22
Fabp3		0.45
Fkbp4 ¹	0.90	0.40
Glo1 ¹	0.74	0.41
Glod4	0.82	0.30
Impdh2 ²	0.76	0.35
Impdh2 ²	0.54	0.20
Gnb2l1 ¹	0.66	0.15
Hnrnpc ¹	0.76	0.43
Hsp90aa1	0.75	0.08
Hsp90aa1	0.49	0.06
Hsp90aa1	0.76	0.12
Hspa5 ¹	0.32	0.22
Hspa8 ¹	0.69	0.50
Hspb1 ¹	0.36	0.47
Hspb1 ¹	0.46	0.88
Hspb1 ¹	0.90	0.41
Hspd1 ¹	0.16	0.67
Hspd1 ¹	0.34	0.95
Itpa	0.57	0.07
Ldhib ¹	0.42	0.43
Lgals2	0.29	0.03
Ncl ¹	0.26	0.71
Npm1 ¹	0.46	0.04
Pebp1 ¹	0.89	0.42
Pkm2 ²	0.38	0.15
Pkm2 ²	0.32	0.65
Pkm2 ²	0.42	0.76
Pkm2 ²	0.21	0.43
Psmb4 ¹	0.62	0.43
Ruvbl1 ¹	0.63	0.22
Sfrs3 ¹	0.41	0.46
Shmt1 ¹	0.01	0.00
Srm	0.68	0.24
Trim28 ¹	0.23	0.11
Trim28 ¹	0.40	0.37
Tuba1c ¹	0.27	0.71
Tubb5 ¹	0.70	0.25
Uqcrc1 ¹	0.24	0.22
Vdac1 ¹	0.30	0.52

¹The proteins are referred to in the text and following tables. RA: Retinoic acid; ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

lines. Bioinformatics analysis of the regulated proteins demonstrated their involvement in various biological processes. To our interest, a number of proteins have potential roles in the regulation of cell proliferation either directly or indirectly.

One of the possible mechanisms of CPX action on

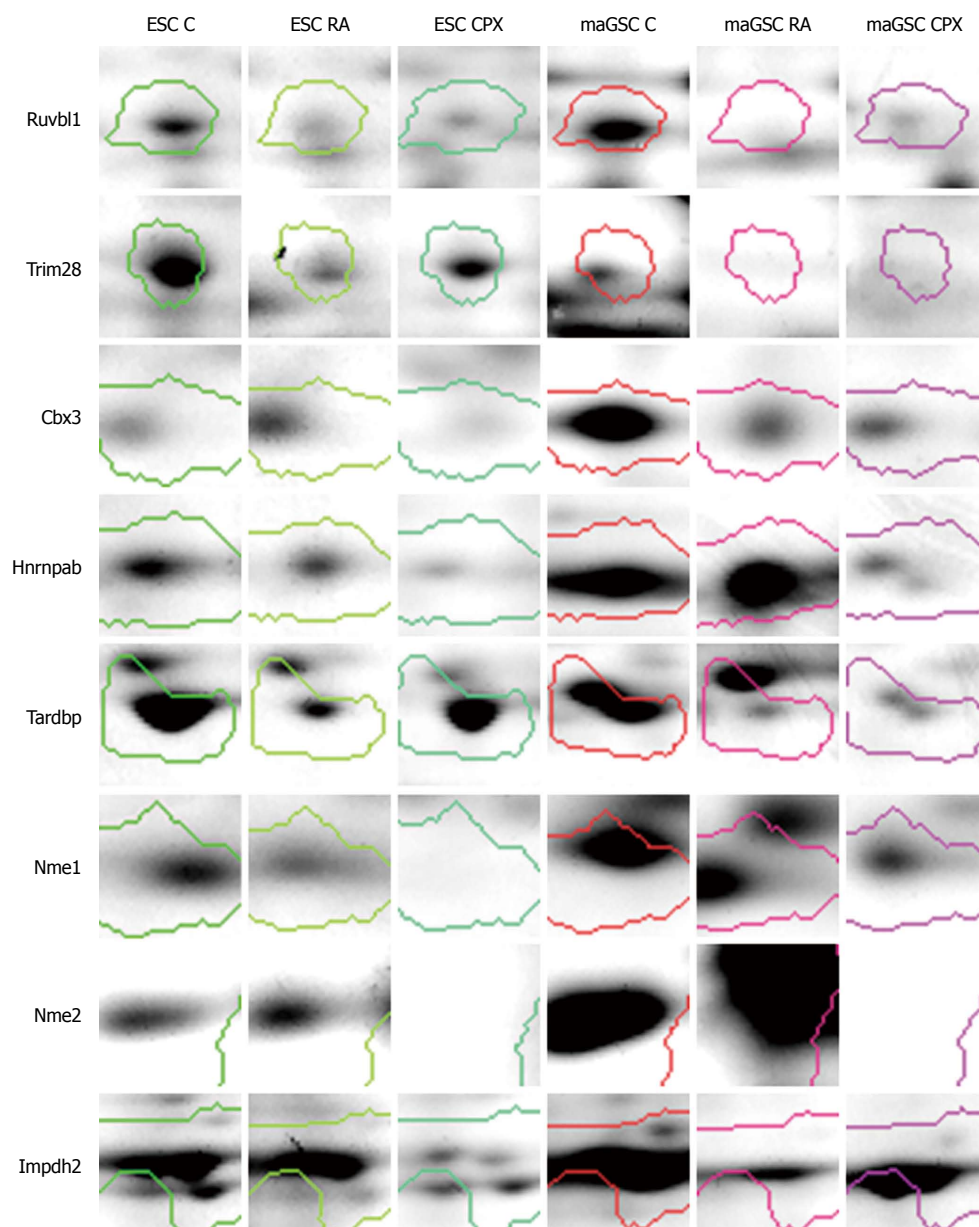


Figure 8 Enlargement of the gel spots of some proteins of interest. ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell; CPX: Ciclopirox olamine; RA: Retinoic acid.

Table 6 Proteins which are upregulated upon retinoic acid treatment compared to control

	RA/k	
	ESC	maGSC
Cct2	1.14	2.16
Hspa4	3.96	2.47
Krt7 ¹	1.01	38.11
Krt8	1.97	1.78
Myh9 ¹	3.06	3.24
Nme1 ¹	2.52	3.81
Nme2 ¹	1.20	2.48
Pdia6	1.72	20.48
Psmc4 ¹	2.17	2.17
Vcp	8.30	4.13
Vim ¹	3.85	1.16

¹The proteins are referred to in the text and following tables. RA: Retinoic acid; ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

cell proliferation is through controlling the progression of the cell cycle^[44]. We identified a number of proteins which are involved in cell cycle processes. Ruvbl1 is one of the differentially regulated proteins which is involved in cell cycle processes, gene expression and transcription regulation. It was found to be downregulated in CPX and RA treated cells compared to control (Figure 8). Ruvbl1 is an evolutionarily highly conserved eukaryotic protein belonging to the AAA+ family of ATPase^[45]. It plays an important role in various cell cycle processes such as chromatin remodeling^[46], gene activation^[47], transcriptional regulation, DNA repair and transcription factor c-Myc^[48]. It also controls Wnt signaling pathway through transcription-associated protein β -catenin^[49,50]. Another protein, which was higher expressed in CPX treated cells compared to RA treated cells, is Trim28. Trim28 is involved in regulation of transcription and silencing gene

Table 7 Gene Ontology functional annotation of proteins which were regulated in this experiment according to their involvement in different molecular function

Molecular function	Proteins	CPX > RA	CPX < RA	CPX > c	CPX < c	RA > c	RA < c
Nucleotide binding	41	Hsp90ab1 Fkbp4 Tubb5 Hspa5 Tubb1b Gapdh Etfa Hspa9 Actb Hsp90aa1 Sfrs1 Vdac1 Pkm2 Atp5a1 Ruvbl1 Hspd1	Atp5b Cct2 Tardbp Hspa4 Actb Hsp90aa1 Ncl Aprt Nme2 Vcp Psmc4 Nme1 Psmc2	Tubb2a Etfa Hspa9 Actb Myh9 Vdac1 Atp5a1 Hspd1	Cct2 Tardbp Hspa4 Tuba1c Hspa8 Hnrnpab Eef1a1 Tcp1 Hsp90aa1 Ncl Aprt Ube2n Nme2 Cct5 Nme1 Pkm2 Atp5a1 Ruvbl1 Hspd1	Cct2 Hspa4 Myh9 Nme2 Vcp Psmc4 Nme1	Ldhb Fkbp4 Tubb5 Hnrnpc Hspa5 Tuba1c Hspa8 Actb Hsp90aa1 Ncl Sfrs3 Aprt Vdac1 Pkm2 Pebp1 Atp5a1 Ruvbl1 Hspd1
GTP binding	8	Fkbp4 Tubb5 Tuba1b	Nme1	Tubb2a	Eef1a1 Nme1 Tuba1c	Nme1	Fkbp4 Tubb5 Tuba1c
ATPase activity	8	Atp5a1	Vcp Psmc4 Atp5b Psmc2	Atp5a1 Myh9	Atp5a1 Hspa8	Vcp Psmc4 Myh9	Atp5a1 Atp5h Hspa8
Enzyme binding	8	Actb Npm1 Hspd1 Cotl1 Hspa9	Actb Gnb2l1	Actb Hspd1 Hspa9	Gnb2l1	Vim	Actb Pebp1 Hspd1 Cotl1
Cofactor binding	5	Gapdh Etfa		Etfa	Shmt1		Ldhb Shmt1
Peptidase activity	6	Ctsd Eno1	Uchl1 Eno1	Ctsd	Psmb4 Acy1 Uchl1 Eno1		Psmb4 Uqcrc1 Acy1 Eno1
Metal ion binding	13	Trim28 Sfrs1 Pkm2 Glo1 Eno1	Atp5b Pdia6 Nme2 Nme1 Eno1		Acy1 Nme2 Nme1 Pkm2 Impdh2 Eno1	Pdia6 Nme2 Nme1	Acy1 Uqcrc1 Trim28 Pkm2 Glo1 Impdh2 Eno1

Ciclopirox olamine (CPX) > Retinoic acid (RA): Proteins which were more than 2-fold higher expressed in CPX-treated cells compared to RA-treated cells; CPX < RA: Proteins which were more than 2-fold higher expressed in RA-treated cells compared to CPX-treated cells; CPX > c: Proteins which were more than 2-fold higher expressed in CPX-treated cells compared to control; CPX < c: Proteins which were more than 2-fold higher expressed in control compared to CPX-treated cells; RA > c: Proteins which were more than 2-fold higher expressed in RA-treated cells compared to control; RA < c: Proteins which were more than 2-fold higher expressed in control compared to RA-treated cells.

expression through its ability to bind to DNA through interaction with a KRAB-ZFP protein. Other proteins, like Cbx3, Tardbp, and Hnrnpab, which are important in gene expression and regulation of transcription, were down-regulated due to treatment with CPX. Tardbp is a DNA and RNA-binding protein, which regulates transcription and splicing. It is also involved in the regulation of CFTR (Cystic fibrosis transmembrane conductance regulator), microRNA biogenesis, apoptosis and cell division. It can repress HIV-1 transcription by binding to the HIV-1 long terminal repeat. Cbx3 seems to be involved in transcriptional silencing in heterochromatin-like complexes. It

recognizes and binds histone H3 tails methylated at K9, which leads to epigenetic repression. It is suggested that these proteins, which are involved in cell cycle processes, transcription regulation and gene expression, might be potential candidates for cell proliferation regulation and their repression through down-regulation might result in cell cycle stop without impact on stem cell pluripotency.

Proteins, which are involved in nucleotide biosynthetic process and proteolysis, were downregulated in CPX treated cells compared to control, as well as in RA treated cells (Figures 6A and 7A). Nucleoside diphosphatase kinases A and B (Nme1 and Nme2) are some of the

Table 8 Proteins which are upregulated in stem cells upon ciclopirox olamine treatment compared to retinoic acid treatment

	RA/CPX	
	ESC	maGSC
Actb ^{*1}	0.12	0.10
Actb ¹	0.14	0.15
Atp5a1 ¹	0.43	0.48
Cotl1	0.19	0.67
Ctsd	0.95	0.16
Eif3i	0.04	0.87
Eno1 ^{*1}	0.13	0.03
Eno1 ¹	0.57	0.40
Etfa	0.68	0.16
Fkbp4 ¹	0.46	0.43
Gapdh ¹	0.35	0.59
Glo1	0.31	0.66
Glod4	0.85	0.36
Hsp90aa1	0.28	0.38
Hsp90aa1	0.43	0.13
Hsp90ab1	0.41	0.26
Hspa5 ^{*1}	0.17	0.13
Hspa9 ^{*1}	0.50	0.28
Hspb1 ^{*1}	0.29	0.04
Hspb1 ^{*1}	0.20	0.42
Hspb1 ¹	0.82	0.50
Hspd1 ¹	0.49	0.74
Hspd1 ¹	0.42	0.33
Itpa	0.21	0.08
Mat2a ¹	0.41	0.14
Npm1 ¹	0.28	0.18
Nup62	0.57	0.26
Pgls ¹	0.40	0.68
Pkm2 ¹	0.22	0.25
Pkm2 ¹	0.29	0.86
Prdx6	0.46	0.94
Ruvbl1 ¹	0.64	0.42
S100a11 ^{*1}	²	0.17
Sfrs1 ¹	0.70	0.30
Trim28 ¹	0.44	0.08
Trim28 ¹	0.42	0.44
Trim28 ¹	0.33	0.28
Tuba1b ^{*1}	0.42	0.65
Tubb5 ¹	0.42	0.54
Tubb5 ¹	0.43	0.58
Vdac1 ^{*1}	0.17	0.09

¹The proteins are referred to in the text and following tables; ²The protein was not identified in embryonic stem cells. Proteins, which are assigned an asterisk, were upregulated upon ciclopirox olamine (CPX) treatment compared to control and concurrently downregulated upon retinoic acid (RA) treatment compared to control. ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

proteins which are involved in nucleotide biosynthetic process. These proteins are known to be involved in the synthesis of nucleoside triphosphatases^[51] as well as in cell proliferation^[52], differentiation^[53] and development^[54], signal transduction, G protein-coupled receptor endocytosis and gene expression. Nme1 was downregulated in CPX treated cells compared to control and RA treated cells (Figure 8). This may explain the slowdown of the proliferation of CPX treated SCs. Impdh2 is a rate limiting enzyme in the *de novo* synthesis of guanine nucleotides and is therefore involved in the regulation of cell growth

Table 9 Proteins which are downregulated upon ciclopirox olamine treatment compared to retinoic acid treated stem cells

	RA/CPX	
	ESC	maGSC
Actb ¹	2.17	1.10
Aldh2	2.61	2.17
Aldh2	2.43	1.21
Aprt ¹	2.20	1.42
Atp5b	1.15	2.17
Capzb	2.79	2.07
Cct2 ^{*1}	13.70	2.77
Eno1 ¹	2.48	2.02
Eno1 ¹	2.71	1.51
Fscn1	2.20	2.14
Gnb2l1	1.81	2.24
Hist1h2bb	7.53	1.62
Hist2h2ac	3.89	211.81
Hnrnpk	2.37	1.58
Hsp90aa1 ^{*1}	2.69	6.36
Hsp90aa1	6.36	4.33
Hspa4 ^{*1}	12.98	3.72
Hspa4	1.35	3.42
Krt7	> 100	1.14
Krt18 ^{*1}	4.04	1.76
Ncl	1.80	3.48
Nme1 ¹	2.63	1.57
Nme2 ^{*1}	5.72	11.15
Pdia6	1.74	6.33
Psmc2 ¹	2.87	1.20
Psmc4 ^{*1}	3.06	2.26
Rps12 ^{*1}	²	2.05
Tardbp	1.11	3.85
Uchl1 ¹	2.02	1.10
Vcp ¹	8.94	2.57

¹The proteins are referred to in the text and following tables; ²The protein was not identified in embryonic stem cells. Proteins, which are assigned an asterisk, were downregulated upon ciclopirox olamine (CPX) treatment compared to control and concurrently upregulated upon retinoic acid (RA) treatment compared to control. ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

and differentiation^[55-58]. It may have a role in the development of malignancy and the growth progression of some tumors. Impdh2 was downregulated in CPX treated cells compared to control (Figure 8).

Proteins which were involved in cell death, positive regulation of biosynthetic process, response to organic substance, glycolysis, anti-apoptosis, and phosphorylation were downregulated in RA treated cells compared to control and CPX treated cells (Figures 6B and 7B).

Analysis of the molecular function of the differently expressed proteins demonstrated a potential involvement of some of these in metal ion binding, mainly iron binding. Cazzola *et al.*^[59] in 1990 established that iron is essential for proliferation, DNA synthesis and repair and mitochondrial electron transport. Therefore, it is assumed that CPX can stop the cell proliferation by regulating the expression of iron binding proteins.

The present study could give some insights into the mode of action of CPX in terms of expression regula-

tion of various proteins. It not only shed light on the previously discussed roles of CPX, but could also provide some further insight into their mechanism. We could identify some potential candidates which can effect the cell proliferation directly or indirectly through other cellular processes. By understanding the mode of action of CPX, this study may provide new aspect that will help in the future strategy to improve therapeutic intervention in the treatment with CPX.

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COMMENTS

Background

Ciclopirox olamine (CPX), a synthetic antifungal agent used in the treatment of fungal and yeast infection of skin or mucosa. Apart from its antimycotic activity, CPX is also effective against both gram-positive and gram-negative bacteria. CPX might also serve as an alternative agent for therapeutic angiogenesis. CPX was also shown to have an antiproliferative effect on stem cells without affecting their pluripotency.

Research frontiers

Although CPX is used as therapeutic for different aspect the mechanism of action is still not clear. In this study, the authors investigated the impact of CPX on stem cell proteome and identified cellular mechanisms that may explain the way of action of CPX. The authors provided evidence that CPX is involved in expression regulation of nucleotide binding proteins resulting in cell cycle arrest.

Innovations and breakthroughs

It is postulated that the CPX works as an inhibitor of the iron-dependent enzymes due to its potential role as a chelator of intracellular iron. The present study could give some insights into the mode of action of CPX in terms of expression regulation of various proteins especially nucleotide-binding proteins. It not only shed light on the previously discussed roles of CPX, but could also provide some further insight into their mechanism. We could also identify some potential candidates, which can effect the cell proliferation directly or indirectly through other cellular processes.

Applications

By understanding the mode of action of CPX, this study may provide new aspects that will be helpful in the future strategy for therapeutic intervention in the treatment with CPX.

Terminology

Multipotent adult germline stem cells (maGSCs) are spermatogonial stem cells isolated from murine testis. CPX, the ethanolamine salt of 6-cyclohexyl-1-hydroxy-4-methylpyridin-2(1H)-one, is a synthetic antifungal agent and is a hypusination inhibitor that controls the second step of the modification, which is catalyzed by deoxyhypusine hydroxylase. The hypusine is the result of a post-translational modification catalyzed by two enzymes: deoxyhypusine synthase and deoxyhypusine hydroxylase.

Peer review

This is a descriptive study in which the authors analyzed the proteome changes of embryonic stem cells and maGSCs accompanying the treatment with CPX and subsequent inhibition of hypusination using classical proteomic techniques like 2-DE, differential in-gel electrophoresis and mass spectrometry. The results are interesting and we could highlight that a treatment with CPX resulted in an alteration of the expression of 56 proteins compared to non-treated cells, and 54 proteins compared to retinoic acid treated cells. The majority of these proteins are involved in nucleotide binding and nucleotide biosynthetic processes, metal binding, DNA binding, and other processes which have been linked to CPX.

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Fetal cardiac mesenchymal stem cells express embryonal markers and exhibit differentiation into cells of all three germ layers

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Abstract

AIM: To study the expression of embryonal markers by fetal cardiac mesenchymal stem cells (fC-MSC) and their differentiation into cells of all the germ layers.

METHODS: Ten independent cultures of rat fC-MSC were set up from cells derived from individual or pooled fetal hearts and studies given below were carried out at passages 3, 6, 15 and 21. The phenotypic markers CD29, CD31, CD34, CD45, CD73, CD90, CD105, CD166 and HLA-DR were analyzed by flow cytometry. The expression of embryonal markers Oct-4, Nanog, Sox-2, SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA 1-81 were studied by immunocytochemistry. The fC-MSC treated with specific induction medium were evaluated for their differentiation into (1) adipocytes

and osteocytes (mesodermal cells) by Oil Red O and Alizarin Red staining, respectively, as well as by expression of lipoprotein lipase, *PPAR γ 2* genes in adipocytes and osteopontin and *RUNX2* genes in osteocytes by reverse-transcription polymerase chain reaction (RT-PCR); (2) neuronal (ectodermal) cells by expression of neuronal Filament-160 and Glial Fibrillar Acidic Protein by RT-PCR and immunocytochemistry; and (3) hepatocytic (endodermal) cells by expression of albumin by RT-PCR and immunocytochemistry, glycogen deposits by Periodic Acid Schiff staining and excretion of urea into the culture supernatant.

RESULTS: The fC-MSC expressed CD29, CD73, CD90, CD105, CD166 but lacked expression of CD31, CD34, CD45 and HLA-DR. They expressed embryonal markers, viz. Oct-4, Nanog, Sox-2, SSEA-1, SSEA-3, SSEA-4, TRA-1-81 but not TRA-1-60. On treatment with specific induction media, they differentiated into adipocytes and osteocytes, neuronal cells and hepatocytic cells.

CONCLUSION: Our results together suggest that fC-MSC are primitive stem cell types with a high degree of plasticity and, in addition to their suitability for cardiovascular regenerative therapy, they may have a wide spectrum of therapeutic applications in regenerative medicine.

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Key words: Fetal cardiac mesenchymal stem cells; Embryonal markers; Multipotent differentiation potential

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INTRODUCTION

The heart is composed of multiple cell types, including cardiomyocytes, endothelial cells, vascular smooth muscle cells, connective tissue and cells of the conduction system; hence, stem cells that have a multipotent differentiation potential with ability to give rise to cells of different germ layers would be the most suitable for efficient regeneration of heart^[1,2]. Although embryonic stem cells are pluripotent, their clinical applications are restricted by their teratogenic and ethical concerns. Hence, different pre- and post-natal tissues are being extensively explored as an alternative source of pluripotent/multipotent stem cells for cardiovascular regenerative therapy^[3-5].

Recently, we have identified a population of mesenchymal stem cells (MSC) from rat fetal heart and termed them as fetal cardiac MSC (fC-MSC)^[6]. These stem cells possessed morphological and phenotypical characteristics of typical bone marrow derived MSC. In addition, they expressed cardiovascular markers and differentiated into all major cells of cardiovascular lineage. These cells exhibited several primitive characteristics, including extended self renewal properties, and expression of OCT-4, Nanog and Sox-2 at gene level and SSEA-1, SSEA-3 and SSEA-4 at protein level^[6]. However, it remains to be determined whether the differentiation of fC-MSC is restricted to cardiovascular or mesodermal lineage or if they possess a multipotent differentiation potential with ability to differentiate into cells of other germ layers as well.

Therefore, the objective of the present study was to evaluate further the primitive characteristics of fC-MSC and we studied the expression of a wide array of embryonic/pluripotency markers by fC-MSC and their capacity to differentiate into mesodermal, ectodermal and endodermal lineages.

MATERIALS AND METHODS

Isolation, culture and characterization of rat fC-MSC

Heart tissues obtained from 16 d gestation age fetuses of female Sprague-Dawley rats were minced and digested with 1 mg/mL collagenase type-IV (Worthington Biochemical, United States) in serum free α -MEM medium for 30 min at 37 °C with intermittent stirring. After washing, the minced tissues were cultured under standard tissue culture conditions in 25 cm² tissue culture flasks (Becton, Dickinson; United States) using complete culture medium consisting of α -MEM medium, 2 mg/mL of Glutamax (Gibco-Invitrogen), 16.5% fetal bovine serum (Hyclone, United States) and bacteriostatic level of penicillin-streptomycin (Gibco-Invitrogen). The semi confluent cultures of cells obtained within 72 h were harvested by trypsinization (0.05% Trypsin-EDTA) (Gibco-Invitrogen) and the cells were expanded in larger flasks up to 30 passages. Ten independent fC-MSC cultures were set up from cells derived from individual hearts or pooled from 2-3 hearts and studies given below were carried out at passages 3, 6, 15 and 21. All the procedures were per-

formed as per guidelines of the Institutional Animal Ethics Committee of Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India.

Flow cytometry

The cells were directly stained with pre conjugated anti-rat monoclonal antibodies to CD44-fluorescein isothiocyanate (FITC), CD90-FITC, CD45-phycoerythrin (PE) and HLA DR-PE. Indirect staining was performed using un-conjugated primary antibodies viz. mouse anti-rabbit CD29 (Abcam, United Kingdom), rabbit anti-mouse CD73 (Becton Dickinson), rabbit anti-mouse CD105 (Santa Cruz, United States), rabbit anti-mouse CD166 (LsBio, United States), rabbit anti-mouse CD34 (Santa Cruz, United States) and rabbit anti-mouse CD31 (Serotec, United Kingdom). Thereafter, cells were stained with FITC/PE conjugated polyclonal rabbit anti-mouse or mouse anti-rabbit secondary antibodies (Abcam) or isotype matched control monoclonal antibodies (Becton Dickinson). Stained cells were analyzed using FCS Express 3.0 in Flow Cytometer (FACS Calibur, Becton, and Dickinson, United States).

Immunocytochemistry

Expression of Oct-4, Nanog, Sox-2, SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 was studied by immunocytochemistry. The fC-MSC was fixed with 4% para-formaldehyde (Sigma Aldrich) in phosphate buffered saline (PBS), pH 7.4, for 1 h at room temperature. The fixed cells were incubated overnight at 4 °C with the following primary antibodies: Oct-4, Nanog and Sox-2, SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 (ES cell characterization kit; Chemicon, United States), diluted 1:50. After washing with PBS, cells were incubated with 1:500 diluted IgG (Fab)₂ FITC as the secondary antibody (Abcam) and stained with Hoechst dye. The pictures were taken using a fluorescent microscope (Nikon 80i, Japan).

Reverse-transcription polymerase chain reaction

Expression of lipo-protein lipase, PPAR γ 2, osteopontin, RUNX2, neuronal filament (NF)-160, glial fibrillar acidic protein (GFAP), albumin and GAPDH was determined by reverse-transcription polymerase chain reaction (RT-PCR). RNA of the cells was extracted using RNeasy Mini RNA isolation kit (Gibco-Invitrogen). One μ g of total RNA was reverse transcribed into cDNA using random hexamers (Gibco-Invitrogen). The gene expression was analyzed for the following genes using primers from MWG Biotech, Germany (Table 1). The amplicons were resolved on 2% agarose gel (Sigma-Aldrich, United States) and pictures acquired using a gel documentation system (Alpha Imager, United States).

Osteogenic differentiation

The cells were treated with osteogenic medium consisting of DMEM medium (Gibco-Invitrogen) containing 10% FBS (Hyclone), 1 mmol/L dexamethasone, 10 mg/mL glyceraldehyde 3-phosphate and 0.1 mmol/L ascorbic

Table 1 Primers used in the present study

Gene	Primer sequence	Product size (bp)	Accession number
Osteopontin	F: TCGGAGGAGAAGGCGCATTACAGC R: TCCTCATGGCTGTGAAATCGTGG	778	AB001382.1
RUNX2	F: TTCGTCAGCGTCCTATCAGTTC R: CTTCCATCAGCGTCAACACC	149	NM_053470.2
PPAR γ 2	F: TTGATTTCTCCAGCATTTTC R: GCTCTACTTTGATCGCACT	125	NM_001145366.1
Lipoprotein Lipase	F: GGGTCGCCTGGTCGAAGT R: AAAGTGCCTCCATTGGGATAAA	450	L03294.1
Neurofilament-160	F: CTCGACTTCAGCCAGTCTCTTCG R: TCTTTGCGCTCTACGGTGATGTGC	550	NM_017029.1
GFAP	F: AGCTGAACCAGCTTCGAGCCAAGG R: GGAAGCAACGTCGTGAGGTCTGC	508	NM_017009.2
Albumin	F: TCGTGACAACCTACGGTGAACCTGGC R: TGTTCGTCTCAGCGAGACACTGG	640	NM_134326.2
GAPDH	R: CCTCTCTCTTGCTCTCAGIAT F: GTATCCGTTGIGGATCTGACA	284	NM_017008.3

GFAP: Glial fibrillar acidic protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

acid (Osteogenesis kit, Chemicon). Control cells were treated with complete medium alone. After 21 d, the experimental and control cells were fixed with 4% para-formaldehyde and stained with Alizarin Red Stain to visualize mineralization.

Adipogenic differentiation

The cells were treated with adipogenic medium consisting of DMEM medium (Gibco-Invitrogen) containing 10% FBS (Hyclone), 500 mmol/L isobutylmethylxanthine, 1 mmol/L dexamethasone, 10 mg/mL insulin and 100 mmol/L indomethacin (Adipogenesis kit, Chemicon). Control cells were treated with complete medium alone. Cells were fixed and stained with Oil Red O Stain to visualize fat droplets in the cells.

Neuronal differentiation

The cells were treated with neurogenic medium consisting of complete culture medium supplemented with 100 ng/mL of basic fibroblast growth factor, 100 ng/mL of noggin, 20 ng/mL of neurotrophin, 10 ng/mL of brain derived growth factor, 10 ng/mL of glial derived growth factor and 20 μ mol/L of retinoic acid (all from (R and D systems, United States) and 1X of B-27 (Gibco-Invitrogen) and 1X of 2-mercaptoethanol (Sigma Aldrich) (experimental cells) or complete culture medium alone (control cells). The cultures were terminated after 21 d and the neuronal nature of the cells was characterized by immunocytochemistry using primary antibodies against NF-160 (ready to use) and GFAP (ready to use) (Biovision Inc., United States). After washing with PBS, cells were incubated with 1:500 diluted IgG (Fab) γ FITC secondary antibody (Abcam) and stained with Hoechst dye (Sigma). The pictures were taken using a fluorescent microscope (Nikon).

Hepatocytic differentiation

The cells were treated with hepatogenic medium con-

sisting of complete culture medium supplemented with 50 ng/mL of hepatocyte growth factor, 50 ng/mL of fibroblast growth factor-4 (R and D systems) (experimental cells) or complete culture medium alone (control cells). Hepatocytic characterization was done by immunocytochemistry using 1:500 diluted primary antibody to albumin (Sigma-Aldrich). After washing with PBS, cells were incubated with 1:500 diluted IgG (Fab) γ FITC as the secondary antibody (Abcam) and stained with Hoechst dye (Sigma). The pictures were taken using a fluorescent microscope (Nikon).

The hepatocytic differentiation was further confirmed by urea assay and Periodic Acid-Schiff assay.

The urea assay was performed by estimating the concentration of urea in the supernatant of experimental and control cells at 11, 14, 17 and 21 d using urea assay kit (Merck diagnostics, India), following the manufacturer's instructions.

In the Periodic Acid-Schiff assay, experimental and control cells after fixation with 4% para-formaldehyde were oxidized in 10 g/L periodic acid for 10 min and rinsed thrice in dH $_2$ O. Thereafter, cells treated with Schiff's reagent for 10 min were rinsed in dH $_2$ O for 10 min and stained with hematoxylin for 2 min to visualize glycogen deposits.

Statistical analysis

Results are presented as mean \pm SE. Statistical significance was defined as $P < 0.05$ by analysis of variance using SPSS 16.0 software.

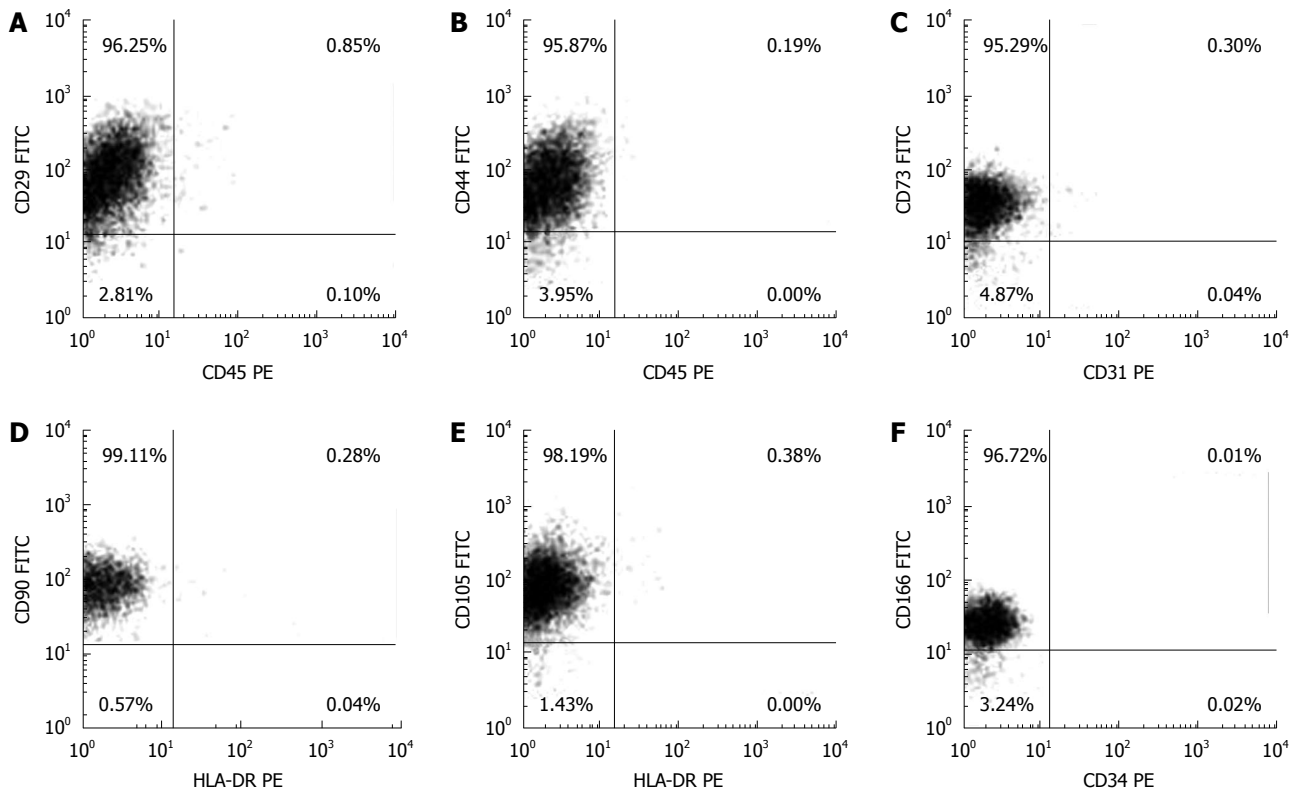
RESULTS

Immunophenotypic characteristics of fC-MSC

Flow cytometric analysis showed a typical mesenchymal phenotype of fC-MSC with expression of CD29, CD44, CD73, CD90, CD105 and CD166 markers and no expression of CD31, CD34, CD45 and MHC-II markers (Figure 1);

Table 2 Immunophenotype of rat fetal cardiac mesenchymal stem cells in primary culture and at passages 3, 6, 15 and 21 (mean \pm SE)

Markers	Primary culture	Passage 3	Passage 6	Passage 15	Passage 21
CD29	96.62 \pm 0.26	96.54 \pm 0.44	96.16 \pm 0.58	96.41 \pm 0.34	95.80 \pm 0.44
CD44	95.80 \pm 0.18	95.96 \pm 0.10	96.00 \pm 0.30	96.50 \pm 0.44	95.66 \pm 0.84
CD73	95.78 \pm 0.12	95.10 \pm 0.16	95.60 \pm 0.24	95.60 \pm 0.30	95.30 \pm 0.32
CD90	98.56 \pm 0.30	98.90 \pm 0.18	98.68 \pm 0.28	98.60 \pm 0.12	98.80 \pm 0.12
CD105	98.40 \pm 0.30	98.00 \pm 0.12	97.68 \pm 0.18	97.60 \pm 0.22	97.40 \pm 0.24
CD31	0.44 \pm 0.08	0.50 \pm 0.02	0.52 \pm 0.02	0.50 \pm 0.02	0.51 \pm 0.01
CD45	0.66 \pm 0.02	0.64 \pm 0.02	0.54 \pm 0.02	0.62 \pm 0.01	0.58 \pm 0.04
HLA-DR	0.38 \pm 0.02	0.30 \pm 0.08	0.28 \pm 0.04	0.34 \pm 0.04	0.32 \pm 0.02

**Figure 1** Representative flow cytometric dot-plots of rat fetal cardiac mesenchymal stem cells showing. A: CD29⁺/CD45⁻; B: CD44⁺/CD45⁻; C: CD73⁺/CD31⁻; D: CD90⁺/HLA-DR⁻; E: CD105⁺/HLA-DR⁻; F: CD166⁺/CD34⁻ phenotype.

this phenotype was maintained over the successive passages (Table 2).

Expression of embryonal markers by fC-MSC

The fC-MSC expressed embryonal markers Oct-4, Nanog, Sox-2, SSEA-1, SSEA-3, SSEA-4, TRA 1-81 but not TRA 1-60, as revealed by immunocytochemistry (Figure 2).

Differentiation of fC-MSC into cells of all three germ layers

Treatment of fC-MSC with adipogenic and osteogenic induction media resulted in their differentiation into adipocytes and osteocytes (mesoderm), as demonstrated by Oil Red O and Alizarin Red staining, as well as expression of lipoprotein lipase, *PPAR γ 2* and osteopontin and *RUNX2* genes by RT-PCR, respectively (Figure 3).

The neurogenic induction medium treated fC-MSC differentiated into neuronal cells (ectoderm), as revealed by expression of NF-160 and GFAP by RT-PCR and im-

munochemistry (Figure 4).

Similarly, on treatment with hepatogenic medium, fC-MSC exhibited differentiation into hepatocytic cells (endoderm), as demonstrated by expression of albumin by RT-PCR and immunocytochemistry, glycogen deposits by Periodic Schiffs staining and excretion of urea in the supernatant (Figure 5).

DISCUSSION

We have recently isolated a population of rat fC-MSC with typical MSC characteristics, including trigonal/spindle shaped morphology, expression of CD29, CD44, CD73, CD90 and CD105, but not of CD31, CD45 and HLA-DR, and potential to differentiate into adipogenic and osteogenic cells^[6]. The fC-MSC exhibited a cardiovascular commitment, as revealed by expression of cardiovascular genes *Isl-1*, *flk-1*, *Nkx2.5* and *GATA-4*, and differentiated

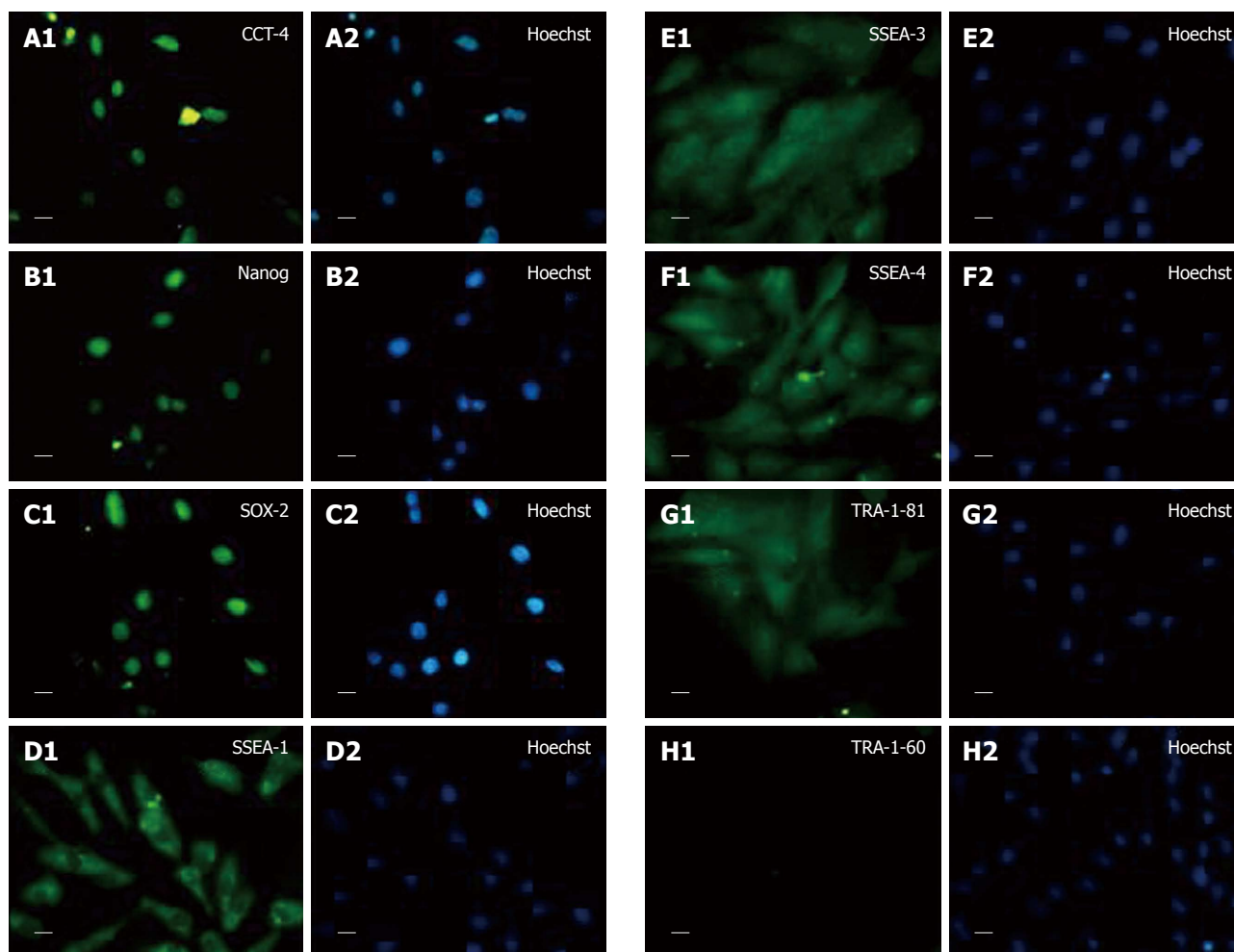


Figure 2 Representative immunocytochemistry photomicrographs (40 ×, 20 μm) of rat fetal cardiac mesenchymal stem cells showing expression. A: OCT-4 (A1: OCT-4 and A2: Hoechst dye); B: Nanog (B1: Nanog and B2: Hoechst dye); C: SOX-2 (C1: SOX-2 and C2: Hoechst dye); D: SSEA-1 (D1: SSEA-1 and D2: Hoechst dye); E: SSEA-3 (E1: SSEA-3 and E2: Hoechst dye); F: SSEA-4 (F1: SSEA-4 and F2: Hoechst dye); G: TRA 1-81 (G1: TRA 1-81 and G2: Hoechst dye); H: TRA1-60 (H1: TRA 1-60 and H2: Hoechst dye).

into all major cardiovascular lineage cells, including cardiomyocytes, endothelial cells and smooth muscle cells. In addition, these cells expressed some embryonal markers and had an extensive expansion potential with continuous expression of telomerase reverse transcriptase and maintenance of a normal karyotype throughout the monitoring period for up to the 21st passage^[6].

These primitive characteristics of fC-MSC prompted us to undertake the present study and we found that, similar to embryonic stem cells^[7], the fC-MSC expressed a wide range of embryonal markers, including Oct-4, Nanog, Sox-2, SSEA-1, SSEA-3, SSEA-4 and TRA-1-81, but not TRA-1-60. In addition, on treatment with lineage specific induction medium, they differentiated into adipocytes and osteocytes (mesoderm), neural cells (ectoderm) and hepatocytic cells (endoderm). To the best of our knowledge, this is the first report showing differentiation of fC-MSC into cells of all three germ layers.

In two studies on stem cells from human fetal heart, the stem cells were demonstrated to express phenotypic markers of MSC, but they were not evaluated for expression of embryonal markers and tri-lineage differentiation

potential^[8,9]. Our observation on embryonic stem cell like characteristics of fC-MSC corroborates with results of a few previous studies on different fetal MSCs of rat and humans. MSC derived from rat and human amniotic membranes has been reported to exhibit expression of pluripotency markers and to differentiate into cells of ectodermal, mesodermal and endodermal lineages^[10,11]. Similarly, human MSC derived from fetal lung are reported to express various embryonal markers, including Oct-4, Nanog, Sox-2, TRA-1-60, TRA-1-81 and SSEA-4, as well as to differentiate into cells of three germ layers^[12]. In another human study, stem cells derived from different fetal organs have been shown to express Oct-4 and could be differentiated into cells of all three germ layers^[13].

In conclusion, our study has shown that fC-MSC are primitive stem cell types that have a high degree of plasticity, as demonstrated by their expression profile of embryonal markers and potential to differentiate into cells of all the three germ layers, suggesting that, in addition to their suitability for cardiovascular regenerative therapy, they may have a wide spectrum of therapeutic applications in regenerative medicine.

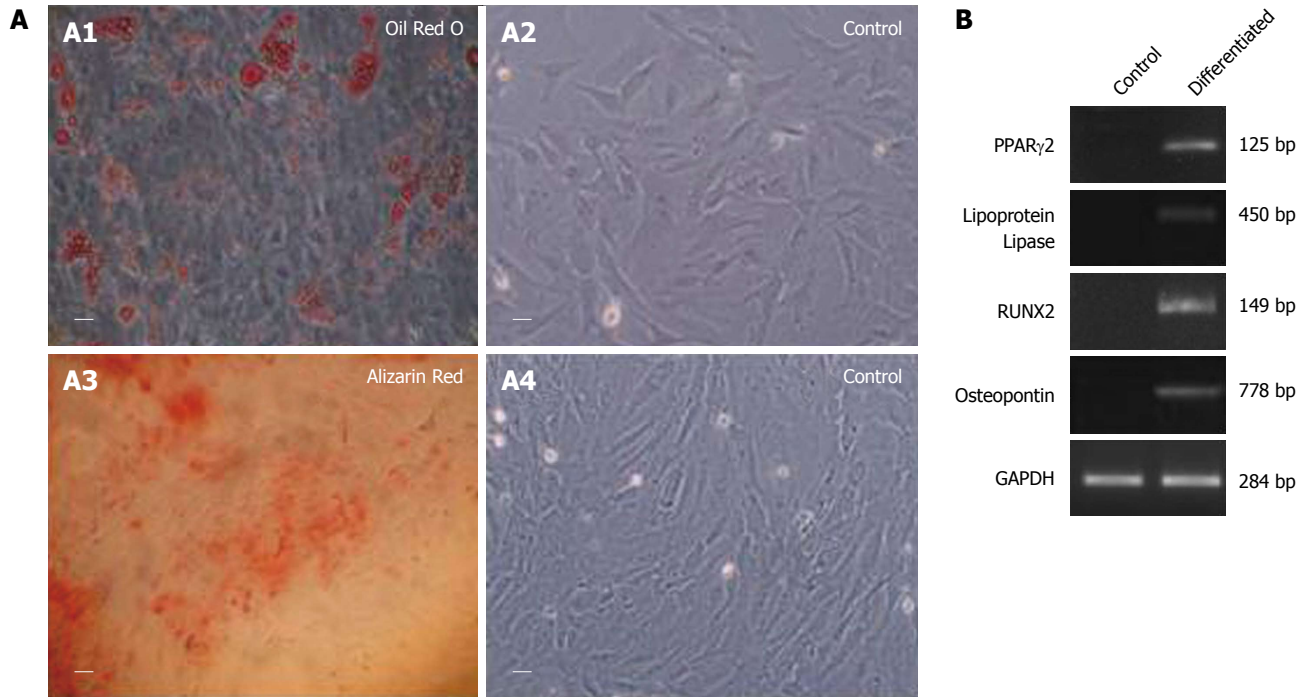


Figure 3 Representative photomicrographs (A) and representative reverse-transcription polymerase chain reaction gel photomicrographs (B). A: Representative photomicrographs (40 \times , 20 μ m) showing differentiation of rat fetal cardiac mesenchymal stem cells into osteocytes (A1: differentiated cells positive for Alizarin Red stain and A2: control cells negative for Alizarin Red stain) and adipocytes (A3: differentiated cells positive for Oil Red O stain and A4: control cells negative for Oil Red O stain); B: Representative reverse-transcription polymerase chain reaction gel photomicrographs showing expression of lipoprotein lipase and PPAR γ 2 by adipocytes and osteopontin and RUNX2 by osteocytes cells, induced from rat fetal cardiac mesenchymal stem cells. Control cells not treated with induction medium did not show expression of above markers.

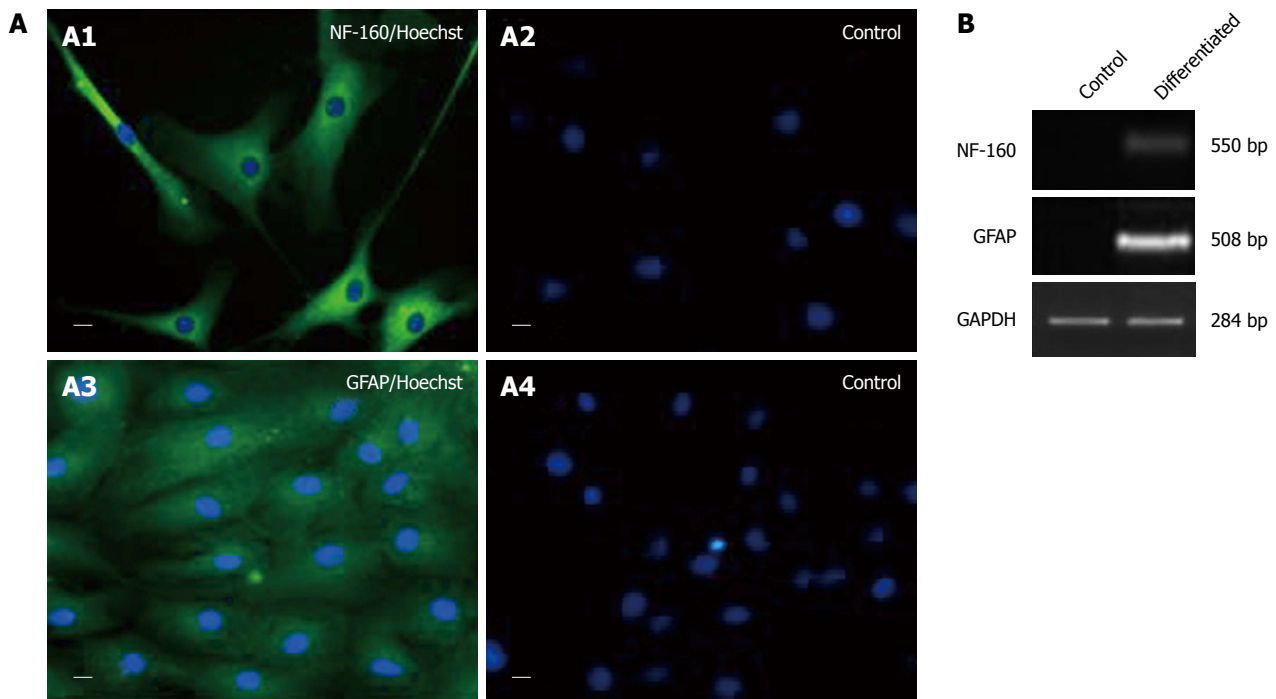


Figure 4 Representative immunocytochemistry photomicrographs (A) and representative reverse-transcription polymerase chain reaction gel photomicrographs (B). A: Representative immunocytochemistry photomicrographs (40 \times , 20 μ m) showing differentiation of rat fetal cardiac mesenchymal stem cells into neuronal cells [A1: Neuronal filament (NF)-160 and Hoechst and A2: no NF-160 only Hoechst dye in control cells]; [A3: Glial fibrillar acidic protein (GFAP) and Hoechst dye and A4: no GFAP only Hoechst dye in control cells]; B: Representative reverse-transcription polymerase chain reaction gel photomicrographs showing expression of NF-160 and GFAP by neuronal cells differentiated from rat fetal cardiac mesenchymal stem cells. Control cells without induction medium did not show expression of above markers.

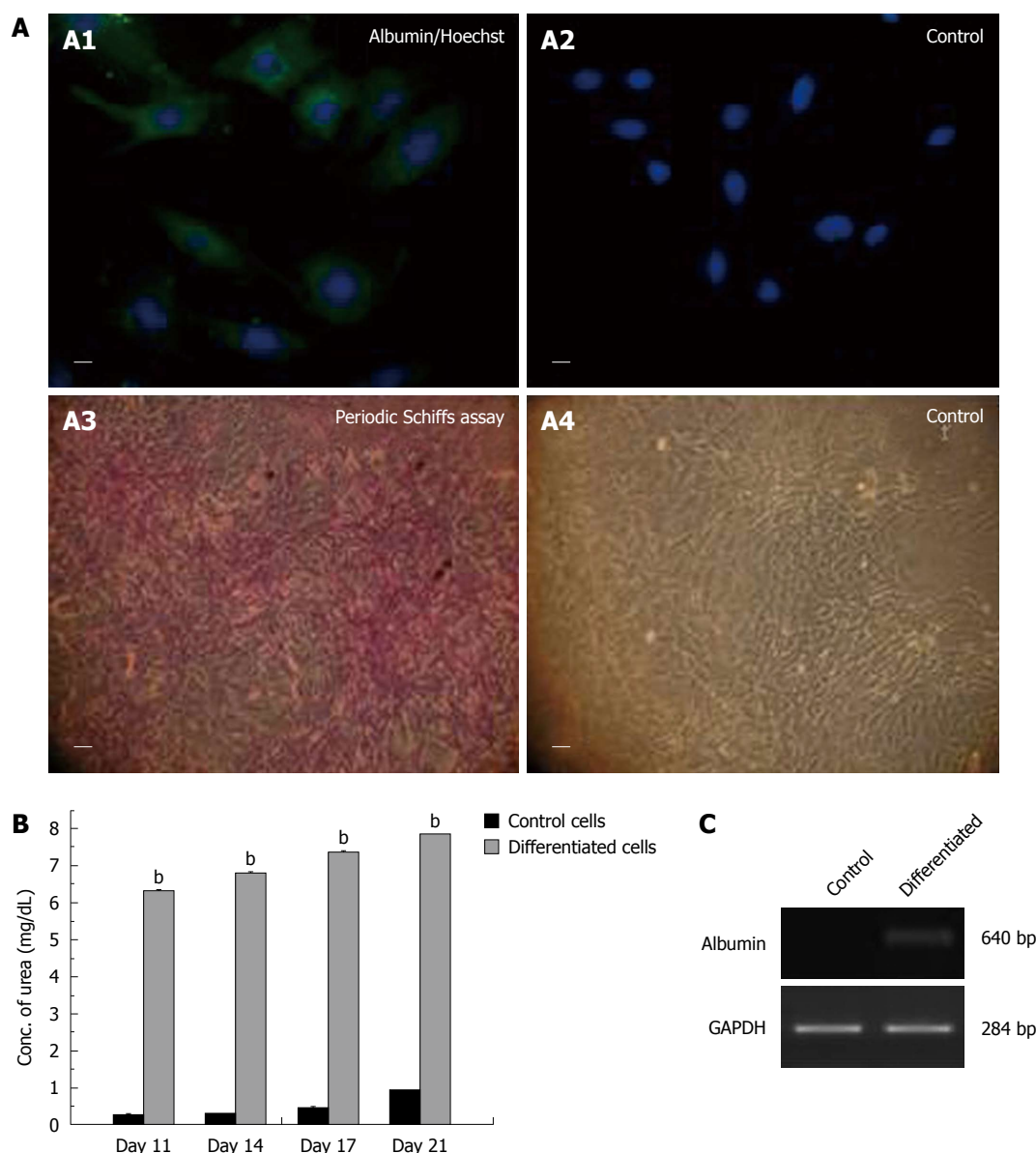


Figure 5 On treatment with hepatogenic medium, fetal cardiac mesenchymal stem cells exhibited differentiation into hepatocytic cells (endoderm), as demonstrated by expression of albumin by reverse-transcription polymerase chain reaction and immunocytochemistry, glycogen deposits by Periodic Schiff's staining and excretion of urea in the supernatant. A: Representative immunocytochemistry photomicrographs (40 ×, 20 μm) showing differentiation of rat fetal cardiac mesenchymal stem cells into hepatocytes (A1: albumin and Hoechst dye and A2: no albumin only Hoechst dye in control cells); (A3: Periodic Acid Schiff stain and A4: control cells negative for Periodic Acid Schiff stain); B: Urea levels in the supernatant of control and differentiated cells at days 11, 14, 17 and 21. Values are mean ± SE of three experiments; ^b*P* < 0.001 for control cells vs differentiated cells; C: Representative reverse-transcription polymerase chain reaction gel photomicrographs showing expression of albumin by hepatocytic cells induced from fetal cardiac mesenchymal stem cells. Control cells without induction medium did not show any expression of albumin.

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COMMENTS

Background

The authors' previous observation of fetal cardiac mesenchymal stem cells (fC-MSC) expressing primitive markers and differentiation ability to cardiovascular or mesodermal lineage led us to determine multipotent differentiation potential with ability to differentiate into cells of other germ layers as well.

Research frontiers

In this study, the authors have demonstrated that fC-MSC are primitive stem cells with capacity to differentiate into cells of all the three germ layers. Future studies on their basic biology and *in vivo* differentiation and function may be important for cell based therapy, generation of artificial tissues and organs and gene therapy.

Innovations and breakthroughs

To the best of our knowledge, this is the first report expressing a wide range of embryonal/pluripotent markers and showing differentiation of fC-MSC into cells of all three germ layers.

Applications

The fC-MSC with primitive stemness and multi-lineage differentiation potential might be novel stem cell types for regeneration/repair of tissues and organs derived from different primary germ layers and thus they may have a wide spectrum of therapeutic applications in regenerative medicine.

Terminology

The fC-MSC are non-hematopoietic stem cells derived from fetal heart that share morphological and phenotypical characteristics of typical bone marrow derived MSC but they are very primitive nature and express cardiovascular markers, pluripotent markers and have potential to differentiate into cells of all the three germ layers.

Peer review

This is an interesting study, wherein the authors have demonstrated that fC-MSC are primitive and highly multipotent stem cells and, in addition to their cardiovascular commitment, they could differentiate into cells of all the three germ layers; thus, they may have a wide spread application in regenerative therapy.

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Immunophenotype and differentiation capacity of bone marrow-derived mesenchymal stem cells from CBA/Ca, ICR and Balb/c mice

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Author contributions: Ooi YY performed the research and analysed the data; Rahmat Z assisted with the differentiation assays; Jose S assisted with the immunophenotyping; Ramasamy R provided technical advice on culture and characterisation of the mesenchymal stem cells; Vidyadaran S conceived the study, designed the experiments and wrote the manuscript.

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marrow of all 3 strains, albeit differences in the temporal expression of certain surface antigens. Their differentiation into osteocytes and adipocytes were also observed. MSC from all 3 mouse strains demonstrated a shift from a haematopoietic phenotype (CD106⁺CD45⁺CD11b⁺Sca-1^{low}) to typical MSC phenotype (CD106⁺CD45⁺CD11b⁺Sca-1^{high}) with increasing passages.

CONCLUSION: Information garnered assists us in the decision of selecting a mouse strain to generate MSC from for downstream experimentation.

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Key words: Mesenchymal stem cells; Mouse bone marrow; CBA/Ca strain; ICR strain; Balb/c strain; Immunophenotyping; Differentiation

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Abstract

AIM: To assess the capacity to isolate and expand mesenchymal stem cells (MSC) from bone marrow of CBA/Ca, ICR and Balb/c mice.

METHODS: Bone marrow of tibia and femur were flushed, cultured and maintained in supplemented Dulbecco's modified Eagle's medium. MSC immunophenotype of cultures were tracked along increasing passages for positivity to CD106, Sca-1 and CD44 and negativity to CD45, CD11b and MHC class II. Differentiation capacity of MSC towards osteogenic and adipogenic lineages were also assessed.

RESULTS: MSC were successfully cultured from bone

INTRODUCTION

Mesenchymal stem cells (MSC) are a great therapeutic interest for tissue regeneration and immunomodulation. To elucidate the role of MSC in these two paradigms, experimentation is often performed with MSC generated from mouse bone marrow. For this, researchers have a choice to make in selecting the mouse strain to generate their MSC from. Here, we provide a report on the comparative description of mouse MSC derived from 3 different strains under the same experimental conditions. The mouse strains assessed were CBA/Ca, ICR and Balb/c.

MSC are stromal cells with self-renewal and differen-

tiation properties^[1]. MSC have been isolated from various tissues including bone marrow, adipose, skin, bone, synovial membrane, placenta and umbilical cord^[2-4] and proliferate *in vitro* as plastic-adherent cells with fibroblast-like morphology^[5]. The long term self-renewal and multilineage differentiation properties of MSC reflect their potential for tissue regeneration and cell/gene therapy-based treatment of diseases^[6] including acute graft-vs-host disease^[7], osteogenesis imperfecta^[8], cardiomyopathy^[9] and Crohn's disease^[10]. Recent findings that MSC have immunosuppressive properties^[11-14] have also increased the need for a suitable supply of MSC for experimental research. The expansion of undifferentiated MSC is a research tool for functional and genetic studies for subsequent development of preclinical protocols to treat a wide range of diseases. Murine sources of MSC are of great use for the extensive preclinical studies that are required within this research area.

Murine MSC is commonly isolated from the bone marrow aspirates of the femur and tibia. Studies demonstrate that isolation of MSC from murine bone marrow using standard methods usually results in a heterogeneous cell population with a high degree of non-mesenchymal contaminants^[2,15-17]. Furthermore, there are reports of preferential growth of MSC from different mouse strains^[17]. MSC isolated from four different inbred mouse strains C57Bl/6J, Balb/c, FVB/N and DBA1 showed great variation in growth rate, differentiation capacity and immunophenotype^[17]. In this study, we compare phenotypic characteristics of MSC derived from bone marrow of 3 strains of mice (CBA/Ca, ICR and Balb/c) to determine a suitable source for these cells for downstream experimentation. As MSC that are cultured lack specific markers^[5], cells were immunophenotyped for a range of surface markers classically associated with MSC expression. The differentiation capacity of MSC confirmed the multipotency of these stem cells. Although there were differences in temporal pattern of surface marker expression and preferential differentiation, bone marrow cultures from all 3 strains of mice tested successfully yielded MSC.

MATERIALS AND METHODS

Isolation and culture of bone marrow-derived MSC

Bone marrow-derived MSC were generated from 3 different strains of mice, namely CBA/Ca, Balb/c and ICR. Mice used were male and between the ages of 6-10 wk. Animals were sacrificed by a CO₂ overdose and bone marrow cells were obtained by flushing femurs and tibias with Dulbecco's modified Eagle's medium (DMEM). Cells were centrifuged at 289 *g* for 10 min, resuspended and seeded into three 25 cm² flasks in DMEM with high glucose supplemented with 10 nmol/mL GlutaMAX™-I Supplement, 100 U/mL penicillin and 100 µg/mL streptomycin, 1 mL/L gentamycin, 250 µg/mL Fungizone (all Invitrogen), 1.5 g/L sodium bicarbonate and 15% Mesenchymal Stem Cell Stimulatory Supplement (STEM-

CELL Technologies, Canada). Cultures were maintained at 37 °C in 95% humidified air and 5% CO₂. After 48 and 72 h, cells were washed gently using 1X PBS and replaced with fresh culture media. Adherent cells were further cultured with a medium change every 3-4 d. At approximately 80% confluency (occasionally localised), cells were harvested by treating with 0.25% trypsin containing 1 mmol/L EDTA for 5 min at 37 °C. Trypsin was neutralised with culture medium and detached cells were replated in a new 25 cm² culture flask. Cell cultures were routinely assessed using an inverted phase contrast microscope and cell viability was determined *via* trypan blue staining.

Flow cytometry analysis of cell surface markers

The following antibodies were obtained from Becton Dickinson: fluorescein isothiocyanate-conjugated anti-mouse CD106 (vascular adhesion molecule-1), CD11b, and MHC I ; phycoerythrin-conjugated anti-mouse CD45, Sca-1, and MHC II and allophycocyanin-conjugated anti-mouse CD44. Cells were trypsinised, washed with 0.1% BSA/PBS and incubated with antibody (1 µL per 10⁶ cells) for 30 min at 4 °C. Cells were then resuspended in 0.1% BSA/PBS and analysed by a FACS Calibur cytometer (BD Biosciences, San Jose, CA) using CellQuest Pro software. Ten thousand gated events were recorded. For each antibody, gating was determined based on appropriate isotype-stained controls.

MSC differentiation

Mouse bone marrow cultures of passages 10-16 were plated at a density of 6×10^4 cells/well in a 24-well plate and incubated at 37 °C in 95% humidified air, 5% CO₂ till confluency was reached. For adipocytic differentiation, the Mesenchymal Stem Cell Adipogenesis Kit (Chemicon; Cat. No. SCR020) was used. Briefly, cells were stimulated with induction medium (DMEM with high glucose supplemented with 10% FBS and 1X penicillin and streptomycin, 1 µmol/L dexamethasone, 0.5 mmol/L IBMX, 10 µg/mL insulin and 50 µmol/L indomethacin) and maintained with maintenance medium (DMEM with high glucose supplemented with 10% FBS and 10 µg/mL insulin) according to kit instructions. For analysis, cells were fixed with 4% paraformaldehyde and stained with 0.36% Oil Red O solution for 50 min. For osteogenic differentiation, the Mesenchymal Stem Cell Osteogenesis Kit (Chemicon; Cat. No. SCR028) was used. Briefly, cover slips were coated with 12 µg/mL vitronectin and 12 µg/mL collagen prior to cell seeding. Cells were then stimulated with induction medium (DMEM with high glucose supplemented with 10% FBS and 1X penicillin and streptomycin, 0.1 µmol/L dexamethasone, 0.2 mmol/L ascorbic acid 2-phosphate, 10 mmol/L glycerol 2-phosphate and 1X glutamine). Cells were replaced with fresh induction medium every 2 d. For analysis, osteocytes were fixed with 70% ethanol and stained with Alizarin Red S. For both differentiation assays, controls were MSC cultures without adipocytic/osteogenic induc-

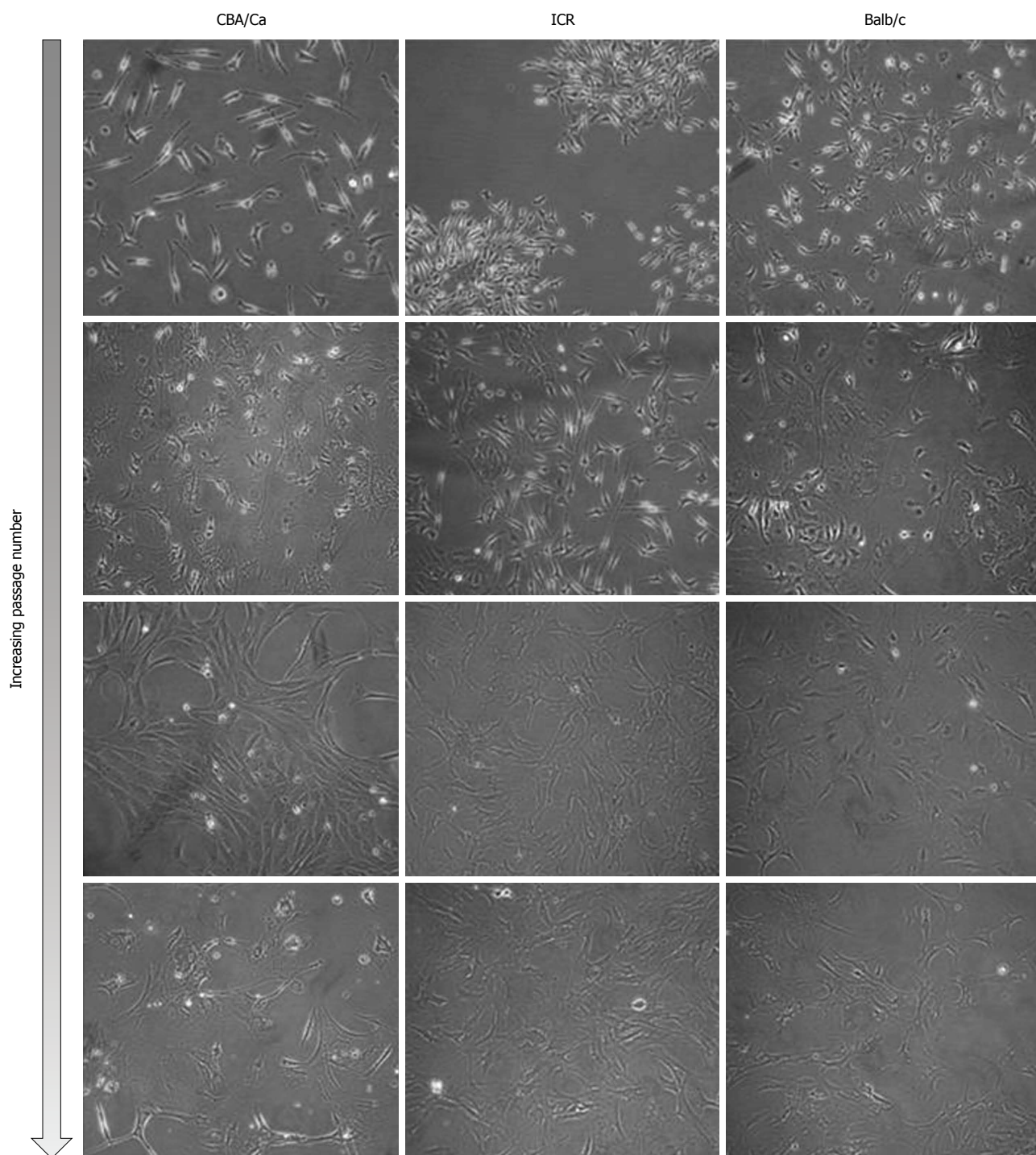


Figure 1 Morphology of mouse bone marrow cultures. Cells shown here represent passages 0-19 of bone marrow cells from CBA/Ca, ICR and Balb/c mice. Original magnification: $\times 200$.

tion medium.

RESULTS

Morphology of bone marrow cell cultures

Bone marrow cells were isolated from the femurs and tibias of a total of 11 mice (4 ICR, 2 CBA/Ca and 5 Balb/c mice) and plated onto culture flasks. Non-adherent cells were removed by replacing media and cultures were ob-

served *via* phase-contrast microscopy for their morphology. Initially, cells of all 3 strains of mice were small and exhibited spindle-shaped morphology (Figure 1, first and second row). Cells also tended to be locally confluent, growing in distinct colonies (as seen for ICR mice, first row; Balb/c mice, second row). A small number of round and flattened cells were observed within the colonies. As cells approached confluency within culture flasks, they were trypsinised and replated into new flasks. Bone mar-

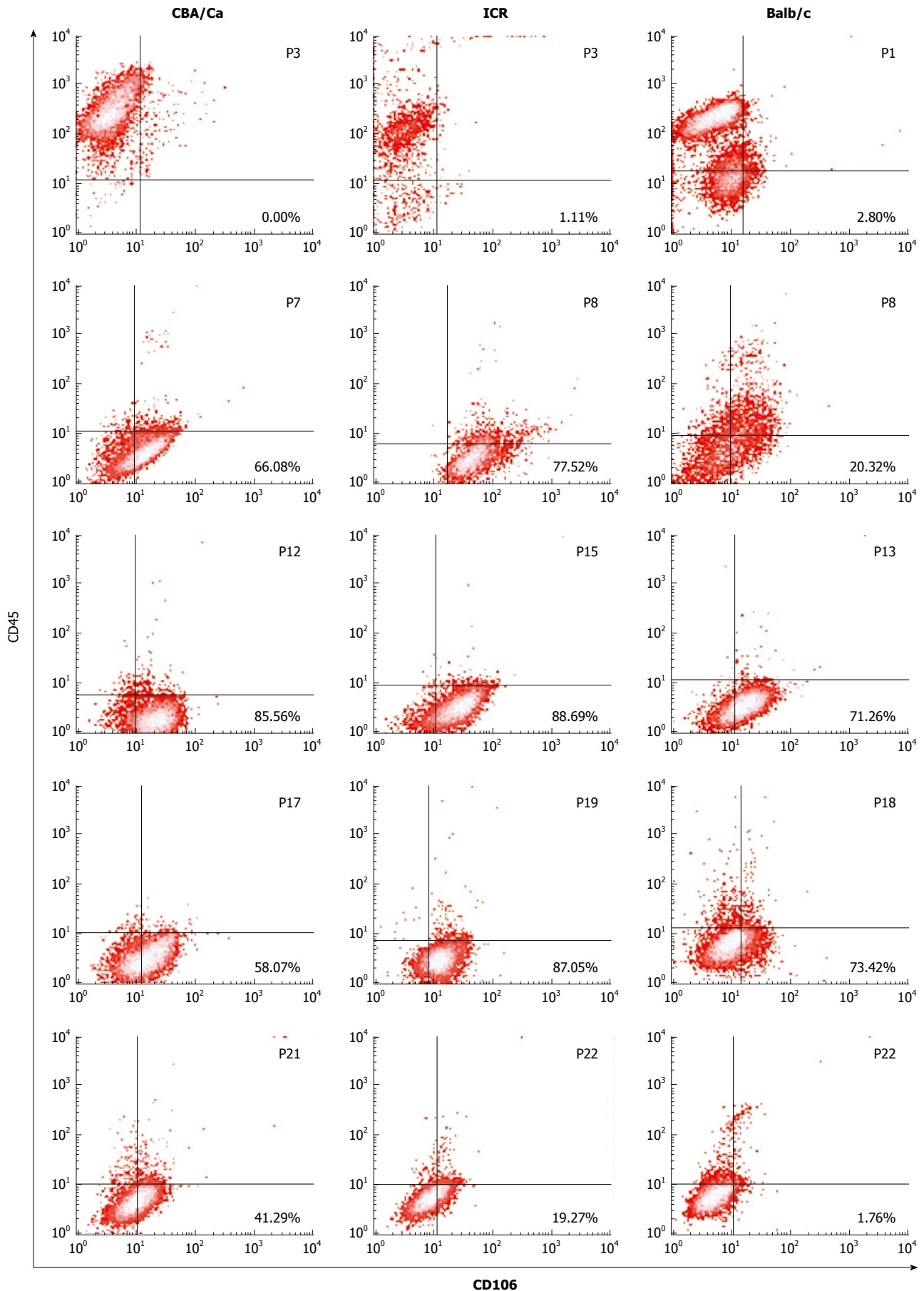


Figure 2 Bone marrow cultures acquire CD106⁺CD45⁻ immunophenotype at later passages. Bottom right quadrants show percentage of CD106⁺CD45⁻ cells for mesenchymal stem cells of CBA/Ca, ICR and Balb/c mice. Numbers in the upper right region indicate passage number.

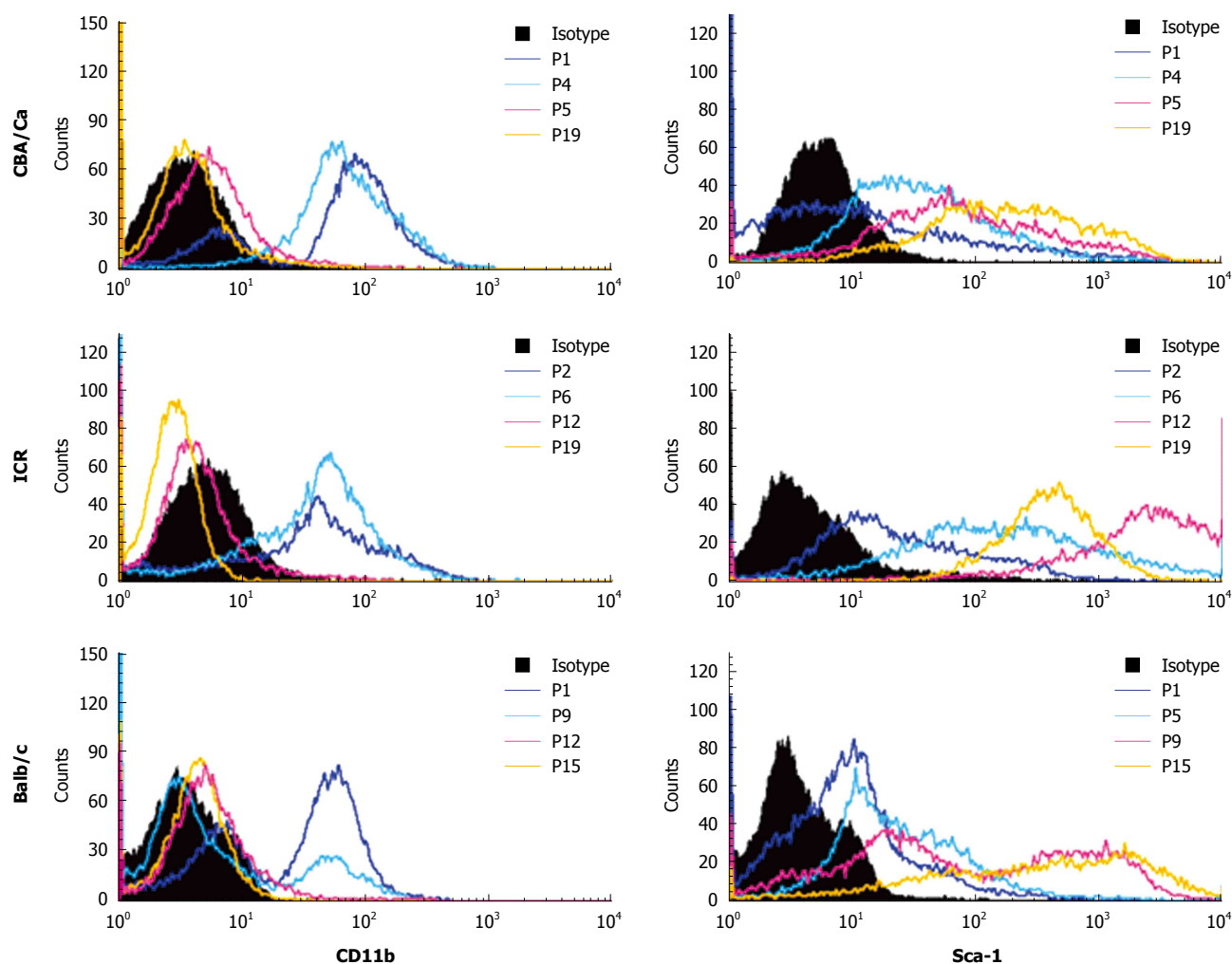


Figure 3 Bone marrow cultures show decreased CD11b and increased Sca-1 expression at later passages. Histograms show the shift of CD11b⁺ at earlier passages to CD11b⁻ at later passages for each strain of mice. Expression of Sca-1 is increased with mesenchymal stem cells passages.

row cultures became increasingly homogeneous, with cells displaying a fibroblast-shape (Figure 1, third and last row). At this point within the culture protocol, cells from all 3 mouse strains took 3-4 d to reach confluency within a 25 cm² flask. Bone marrow cells continued to proliferate in culture beyond passage 35.

Immunophenotypic profile of bone marrow cultures

Immunophenotyping of bone marrow cultures derived from the 3 mouse strains were performed at various passages throughout the culture period. Presence of MSC were confirmed by positivity to CD106, CD44, Sca-1 and MHC I markers and negativity to CD45, CD11b, and MHC II by flow cytometry.

Flow cytometric analyses revealed that MSC demonstrated a shift from a haematopoietic phenotype to typical MSC phenotype with increasing passages. MSC of all 3 strains of mice showed this shift in phenotype from CD106⁺CD45⁺ cells at earlier passages to CD106⁺CD45⁻ at later passages (Figure 2). ICR mice maintained their CD106 positivity for longer, remaining > 77% from passage 8 to 19. For CBA/Ca and Balb/c mice, CD106 ex-

pression began reverting after passage 17 and passage 18 respectively. Expression of CD45 (a common leukocyte antigen) was strong (70%-99%) at early passages for all 3 strains but decreased with increasing passages to < 9% positivity. Cultures derived from Balb/c mice took longer to lose their CD45 positivity compared to CBA/Ca and ICR strains (Figure 2).

Similar to CD45, expression of haematopoietic marker CD11b also revealed a shift from CD11b⁺ to CD11b⁻ with increasing passages (Figure 3). MSC cultures from all 3 strains of mice showed high positivity to CD11b (69%-99%) at early passages. Positivity for CD11b decreased with later passages. Cultures from CBA mice achieved CD11b⁻ phenotype earlier than the other strains, beginning from passage 5. For the MSC marker Sca-1, cultures showed lower positivity to Sca-1 at early passages with only 24%-50% cells positive (Figure 3). Sca-1 expression increased to a higher percentage after passage 5 for CBA/Ca mice, passage 6 for ICR mice and passage 9 for Balb/c mice. At late passages, MSC from all 3 mouse strains showed strong positivity for Sca-1 at 84%-100%. ICR mice demonstrated a distinctly positive (96%-100%)

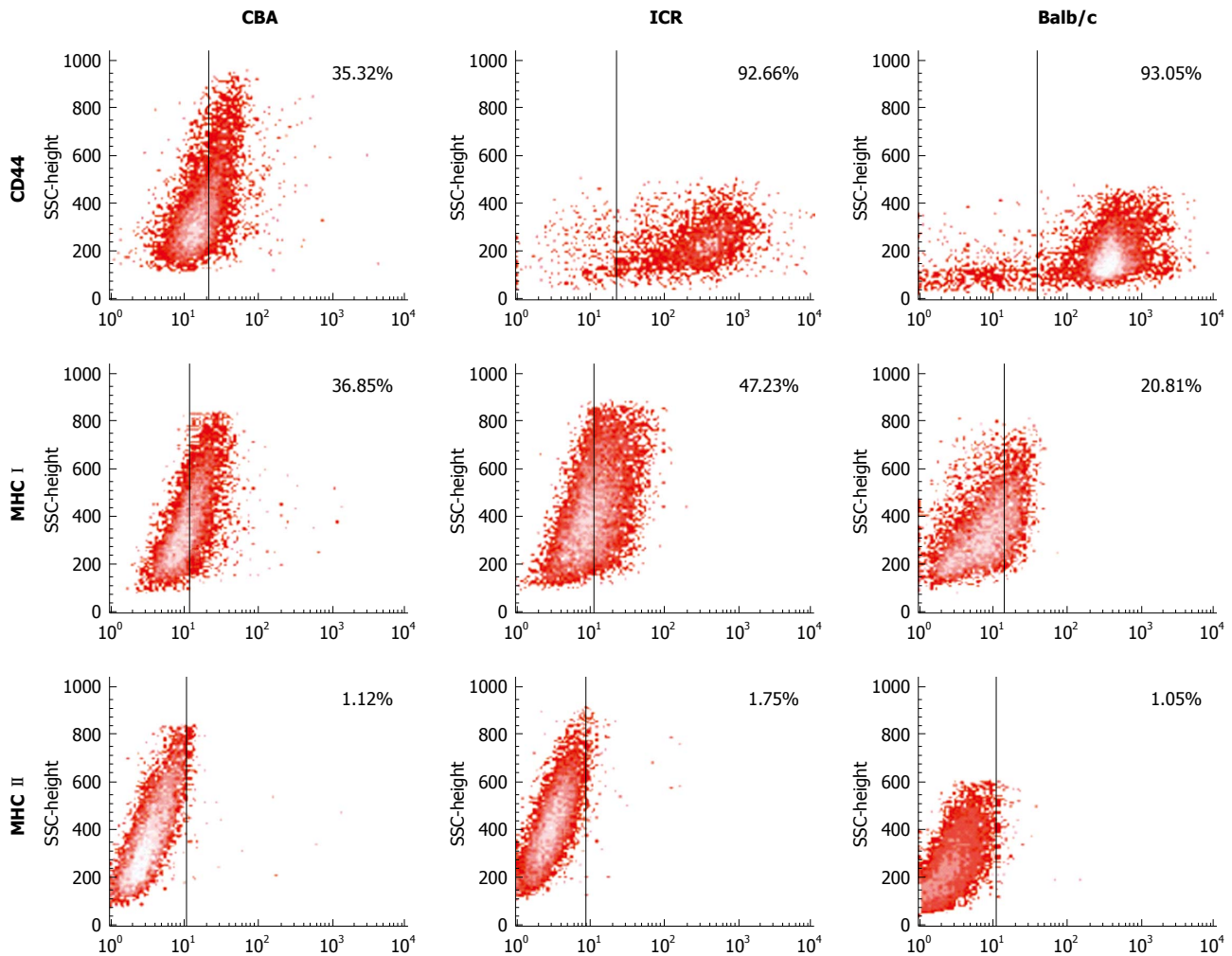


Figure 4 Mesenchymal stem cells cultures from all 3 strains of mice show positivity to CD44 and MHC I but negativity to MHC II. Numbers within plots indicate the percentage of positivity to each marker.

population for Sca-1 after passage 6, while expression in CBA/Ca and Balb/c mice were 91%-97% after passage 15 and 84%-99% after passage 12 respectively.

Next, CD44, MHC I and MHC II expression was examined in bone marrow cultures with CD106⁺CD45⁻ phenotype. For CD44 expression, cells from ICR and Balb/c mice had the highest expression (77%-99% and 79%-99% accordingly), compared to CBA/Ca mice that had varying CD44 expression ranging from 13%-90% (Figure 4). All 3 strains of mice consistently demonstrated positivity to MHC I but not MHC II (< 2%) (Figure 4).

Results from MSC immunophenotyping shown above suggest that bone marrow cultures from all 3 strains of mice acquire an MSC phenotype (CD106⁺CD44⁺Sca-1⁺MHC I⁺CD45⁻CD11b⁻MHC II⁻) after multiple rounds of passaging. Also, amongst the 3 mouse strains tested, bone marrow cells for ICR and Balb/c maintained MSC phenotype for longer compared to CBA/Ca.

Differentiation assay

Bone marrow cultures from all 3 strains of mice differentiated into adipocytes and osteocytes when stimulated

with adipocytic and osteogenic induction medium respectively (Figure 5). Three weeks of exposure to an adipocytic induction medium resulted in formation of lipid vacuoles in cultured MSC that could be observed with phase-contrast microscope. When cultured MSC were exposed to osteogenic induction medium, they aggregated and formed calcium deposits after 2 wk. Both of these cell differentiations were confirmed with cell-specific stains - Oil Red O for adipocytes and Alizarin Red S for osteocytes. Oil Red O-stained lipid vacuoles appeared bright red whereas Alizarin Red S stained precipitated calcium deposits dark red (Figure 5).

DISCUSSION

Isolation of mouse MSC is generally more difficult than human and rat MSC due to a high number of contaminating haematopoietic lineage cells and slow cell growth^[2,15,17]. Studies have also demonstrated mouse strain-dependent variability of MSC in terms of growth kinetics, surface markers and potential for differentiation^[16-18]. With the protocol described here for bone

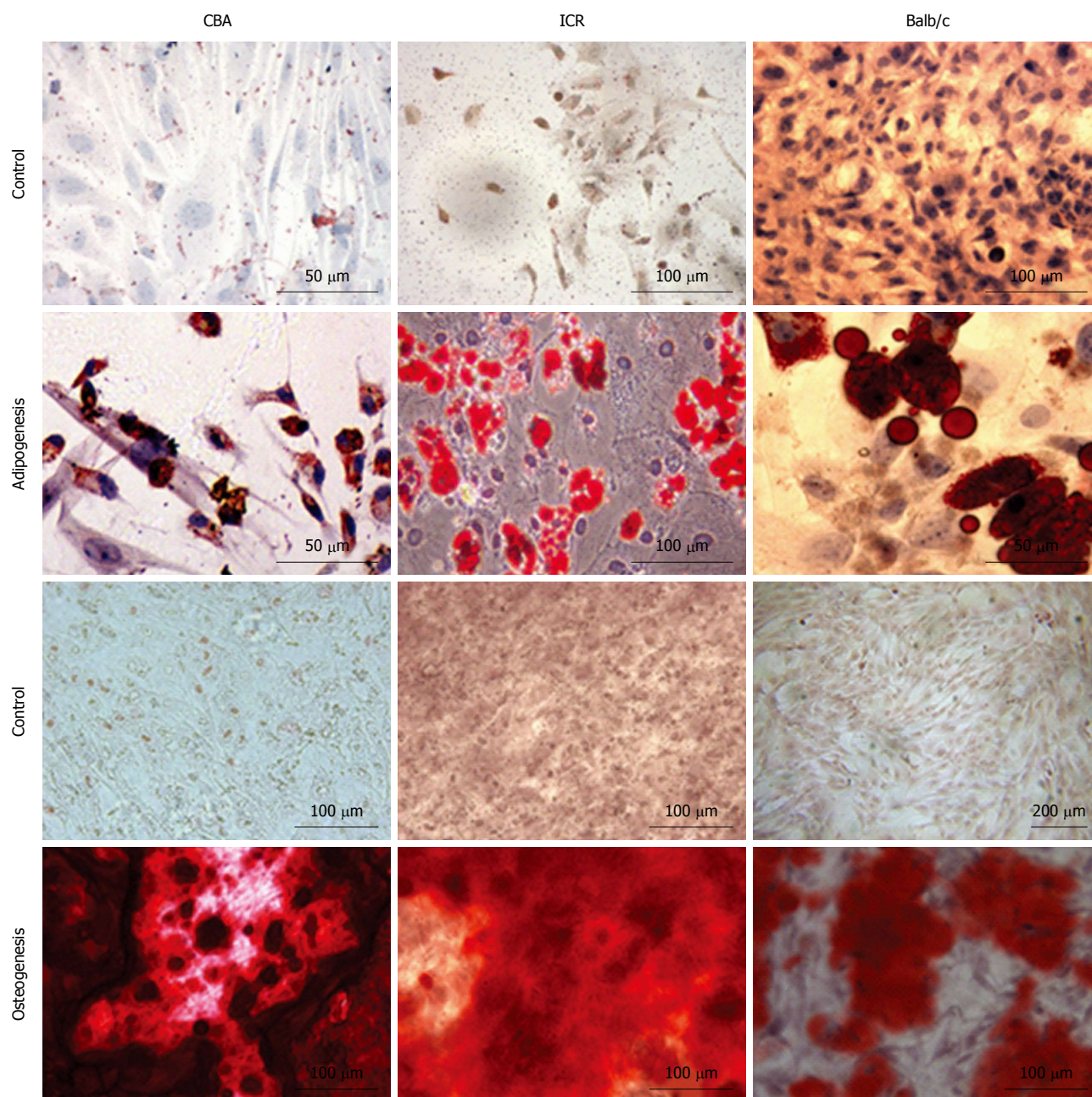


Figure 5 Mesenchymal stem cells from all 3 strains of mice differentiate into adipocytes and osteocytes. First and second row: images of non-induced mesenchymal stem cells (MSC) control and MSC differentiated into adipocytes, stained with Oil Red O. Third and fourth row: images of non-induced MSC control and MSC differentiated into osteocytes, stained with Alizarin Red S. Differences in quality of images are because they were taken using different camera/microscope systems.

marrow-derived MSC expansion, we provide descriptive data that MSC can be successfully generated from 3 different mouse strains, namely CBA/Ca, ICR and Balb/c. Bone marrow cells were cultured and each culture was characterised for their morphology, immunophenotype and differentiation potential. MSC from all strains tested showed expression of relevant surface markers and differentiation potential into osteogenic and adipocytic cells, albeit some differences in terms of time to achieve MSC immunophenotype.

Isolation of MSC from mouse bone marrow usually results in a highly heterogenous cell population with a high degree of haematopoietic contaminants such as

macrophages and fibroblasts^[2,16]. Immunophenotyping analysis revealed early passages of our bone marrow cultures to be positive for haematopoietic markers CD45 and CD11b (a macrophage-specific marker). Continuous change of cell culture medium removes non-adherent haematopoietic populations from the cultures^[3]. Passaging bone marrow cultures also helps eliminate haematopoietic contaminants and yield purer MSC cultures^[17]. With these approaches, we accordingly observed the MSC cultures become increasingly homogenous at later passages with minimal CD45 expression profile by P7-P8, although it took a little longer for cells from Balb/c mice. Better quality MSC cultures were also obtained by dis-

carding cells that remained attached to flasks following trypsinisation. These firmly adherent cells are probably contaminating fibroblasts and have been reported by other laboratories^[17,19]. Expression of stem cell marker Sca-1 also increased at later passages. Sca-1 is a marker associated with MSC and also haematopoietic and endothelial progenitors^[20]. MSC also expressed positivity to CD44 and CD106 and negativity to MHC II. To further encourage MSC growth and colony formation, we used 15% of a mouse mesenchymal supplement (MesenCult®, STEMCELL Technologies) instead of the routine 10% FBS to obtain enriched MSC cultures.

In addition to morphology and phenotype, the characterisation of MSC is complete with demonstration of their capacity for multilineage mesenchymal differentiation^[15,18,19,21]. All 3 mouse strains exhibited multilineage potential *in vitro*, differentiating into adipocytes and osteocyte when stimulated with appropriate induction media. MSC from Balb/c mice more readily differentiate into adipocytes than the other strains, also shown by Peister and colleagues who found Balb/c more readily differentiating into adipose cells than C57Bl/6J and DBA 1 mice^[17]. Conversely, MSC from Balb/c took longer to differentiate into osteocytes compared to the other 2 strains tested in our study. In view of the duration that MSC cultures remained in suitable phenotype and their ease to differentiation, our group has primarily utilised ICR mice for downstream experimentation where we show these stem cells to have immunomodulatory properties^[13].

MSC from all 3 different strains of mice tested were suitable sources for bone marrow-derived MSC as they showed typical morphology, immunophenotype and differentiation capacities of MSC. However, bone marrow cultures from Balb/c mice took longer than the other strains to achieve MSC immunophenotype.

COMMENTS

Background

Experimental research concerning mesenchymal stem cells (MSC) relies heavily on cells isolated from animals. In this paper, we sought to determine whether MSC could be successfully generated from three different mouse strains. The ICR, CBA/Ca and Balb/c mice strains were available to us in the laboratory, and are strains commonly used in research. However, there is no literature stating the feasibility of each of these strains to yield bone marrow-derived MSC. It is important to determine the strain to use, as strain-dependent variability has already been reported by Peister and colleagues.

Research frontiers

The therapeutic relevance for MSC is not limited to their regenerative properties, but also their capacity for modulation of immune responses. Beyond the efficacy of MSC to ameliorate disease, it is essential to test the immunogenicity and tumorigenicity of these cells. The use of murine-derived MSC for preclinical research is indisputable and their applicability in experimental settings should be determined for researchers to be able to accurately assess their use.

Innovations and breakthroughs

In this article, the authors show that bone marrow MSC can be derived from ICR, CBA/Ca and Balb/c mice strains with the protocol described. Similar information for these three mouse strains was previously unavailable. With passaging, bone marrow cultures achieved MSC immunophenotype and displayed multipotency by differentiating into osteocytes and adipocytes.

Applications

This study demonstrates that ICR, CBA/Ca and Balb/c mice strains can yield

bone marrow-derived MSC for downstream experimentation. Based on the protocol described for culture of MSC, the authors describe the passage range of bone marrow cultures for each mouse strain that yields MSC (based on immunophenotyping data). This information provides data that can assist researchers faced with a choice of setting up mouse bone marrow-derived MSC cultures.

Peer review

This article assessed bone marrow-derived MSC cultivated among three different mouse strains. The authors found some differences between strains with regards to the expression of certain surface antigens and differentiation capacity. These results will be useful for the selection of mouse for preclinical studies concerning bone marrow MSC.

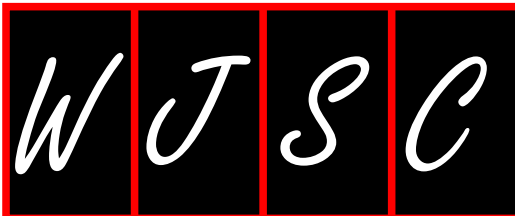
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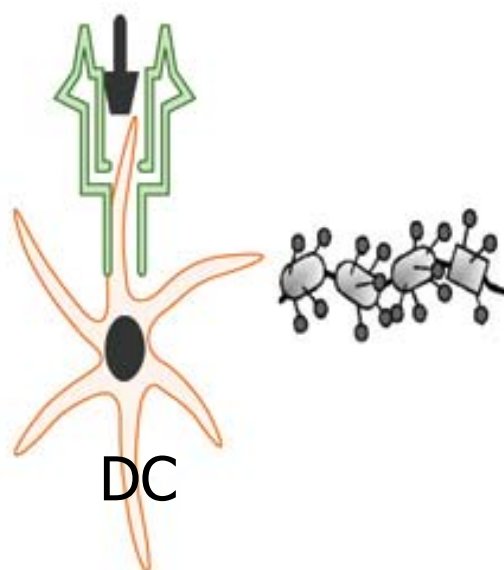
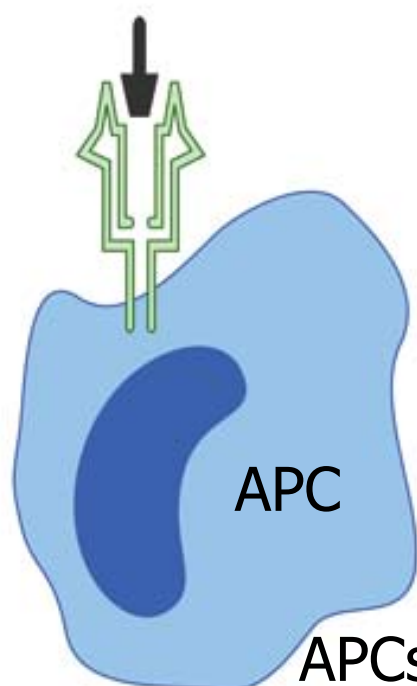
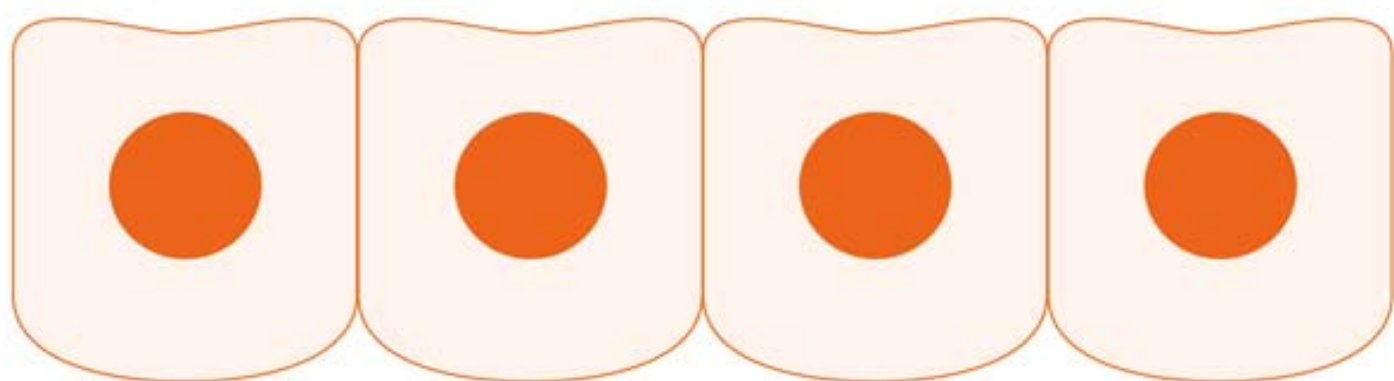
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In utero transplantation: Disparate ramifications

Pixley JS, Zanjani ED

BRIEF ARTICLE

53

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Singh SP, Tripathy NK, Nityanand S

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In utero transplantation: Disparate ramifications

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INTRODUCTION

A variety of genetic disorders of the hematolymphoid system have been treated successfully by transplanting hematopoietic stem cell (HSC) into pediatric and adult patients. However, this procedure's applicability is limited by compatible donor availability, the use of immunosuppression/cytoablation and the potential development of graft-*vs*-host disease (GVHD). Further, for some pediatric diseases (*i.e.*, the glycogen storage diseases, thalassemia, *etc.*) significant disease progression begins during fetal development such that even at birth the patient is irreversibly compromised. In these conditions, postnatal bone marrow transplantation can ameliorate disease progression but is unable to correct the existing damage^[1].

Successful allogeneic HSC transplantation is accomplished by donor reconstitution of hematopoiesis (usually after depletion of defective or undesirable host cells) following the infusion of donor HSC in anticipation of their homing to appropriate hematopoietic tissues. This requires overcoming the immune barrier of the host, limiting the ability of donor immune cells to mediate GVHD and facilitation of homing and engraftment of donor HSC where the inductive milieu will promote regulated reconstitution of hematopoiesis. In the clinical setting many of the complications of postnatal HSC transplantation are the result of procedures such as ionizing radiation and chemotherapy used to limit host immune rejection or deplete host marrow to allow adequate donor engraftment/expression.

In theory, intrauterine stem cell transplantation (IUSCT) presents significant advantages in comparison to postnatal stem cell (SC) transplantation, which if optimized, could present less toxic treatment protocols for many genetic/developmental diseases. Advances in molec-

Abstract

In utero stem cell transplantation, which promises treatment for a host of genetic disorders early in gestation before disease effect stems from Ray Owen's seminal observation that self-tolerance, is acquired during gestation. To date, in utero transplantation (IUT) has proved useful in characterizing the hematopoietic stem cell. Recent observations support its use as an in vivo method to further understanding of self-tolerance. Preclinical development continues for its application as a treatment for childhood hematolymphoid diseases. In addition, IUT may offer therapeutic options in the treatment of diabetes among other diseases. Thus IUT serves as a technique or system important in both a basic and applied format. This review summarizes these findings.

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Key words: Self non-self discrimination; Immune ontogeny; Stem cell transplantation

ular biological techniques and improvements in obstetric procedures such as chorionic villous sampling permit the collection of fetal material to diagnose these conditions early enough in gestation to allow the use of IUSCT for treatment prior to the onset of irreversible organ damage. This enthusiasm is founded on the concept that establishment of graft tolerance without cytoablation and without GVHD can be accomplished in a cost effective manner. Unfortunately, at present, the only clinical applications for which IUSCT has proven successful are in diseases associated with host immunodeficiency^[2,3].

As we reported in our earlier review, intrauterine transplantation (IUT) as a method emanated from attempts in the first half of the 20th century to understand self-tolerance and accept the concept of autoimmunity^[1,4]. While these observations ruled out genetic determinism and identified fetal developmental events as responsible, it became clear in the latter half of the last century lead by the experiments of Le Douarin in birds, that how cellular deletion and cellular suppression (the main mechanisms underlying self-tolerance formation and maintenance) were formed during development were and are poorly understood^[5]. Indeed, only recently have we identified the importance of and probably of more interest, why gestational timing is crucial to utilizing IUT both clinically and experimentally^[6].

Nonetheless, once the concept that introduction of foreign antigens (*i.e.*, HSCs) could be introduced into the fetus and persist, clinicians were encouraged that this breakthrough might offer a path to cure. Supporting evidence that IUSCT could work comes from the observations on naturally occurring chimerism in dizygotic twins in both large animals and humans. Here, the mixing of HSC through the placental circulation throughout gestation may result in enhanced sibling cell expression (> 30%)^[7]. Natural chimerism is common^[8]. In some veterinary diseases amelioration of disease phenotype has been attributed to sibling cell expression^[9-11]. Experimental work transplanting a variety of HSC into large and small animal fetuses was supportive^[2].

Clinical experience has been disappointing. In part this may be due to inadequate understanding of immune ontogeny and the importance of time in rendering tolerance. As often, procedures were performed after our proposed gestational tolerance window (approximately weeks 12-16 gestation) closes. However there are well-documented examples of proper timing and administration of an adequate SC dosage to treat a disease that theoretically should benefit for which there is no engraftment or if engrafted no clinically meaningful expression is seen^[12,13]. What we hope to show is given recent experimental observations; a successful protocol for treatment of even non-immunodeficiency diseases is within our grasp.

In this report, we would like to expand both experimentalists' and clinicians' appreciation of the power of this procedure (ramifications). Our observations on immune ontogeny reveal IUT's utility in unlocking events fundamental to understanding self-tolerance. Also, we

believe it possible that this fetal tolerance phase in a large animal may allow *in vivo* investigation of biologic systems that may permit the development of alternative solutions to a number of clinical problems. Herein, we review its experimental background, propose the antigen exposure model to explain developmental acquisition of self-tolerance, examine impediments and promising areas for optimizing the procedure for treatment of childhood diseases and finally speculate regarding this technique's utility as *in vivo* platform for therapeutics.

BACKGROUND

The discovery of common placental circulation between dizygotic twins as the explanation for the freemartin coupled with the development of erythrocyte antigen profiling in cattle allowed Ray Owen to determine that dizygotic twins were chimeric with their sibling's blood cells after birth. Thus, he concluded that self-tolerance is acquired during fetal development and not innate^[1,14]. Subsequent experiments in mice, sheep and cattle confirm that imprinting (Rapid time-dependent irreversible behavioral learning that occurs during development originally described by Karl Lorenz in young geese.) during fetal development is responsible for immune tolerance in adult life^[15-17]. In direct experiments, fetal transplantation with allogeneic HSC, xenogeneic HSC or RTV (Gene expression following retroviral vector transfer in utero follows similar kinetics to that observed after cellular transplantation. We believe this is due to the establishment of recipient transplantation tolerance to the gene product hence we use transplantation rather than transfer.) in sheep reveals a gestational window of receptivity to engraftment mirroring the acquisition of self-tolerance^[6,18]. The SC xenografts are highly expandable and are associated with extensive differentiation. Indeed, besides normal hematopoietic lineages, differentiated cardiac, gastrointestinal, liver and pancreatic islet cell activity can be demonstrated years after transplantation^[19,20]. This window occurs in mice later in gestation but successful long-term engraftment and expression of both allogeneic and xenogeneic HSC has been realized^[1,21-23]. For example, in Figure 1 we note bi-lineage human chimerism in a mouse following IUT at the proper gestational age; significant expression required graft stimulation with human growth factors (see below). In summary, self non-self discrimination is relative and time dependent.

We have found performing allogeneic and xenogeneic IUT in sheep (a large animal) useful in the study of the HSC^[24,25]. Formal study using timed gestational sheep identified the developmental event permissive for long-term engraftment receptivity as the period immediately following thymic demarcation (The timing of thymic demarcation into cortex and medulla varies with the size of the animal. In mice demarcation occurs at 66%, sheep 35% and humans 31% expiration of gestation. It is thought that the medulla is primarily responsible for deletion and cellular tolerance.). This phase is finite last-

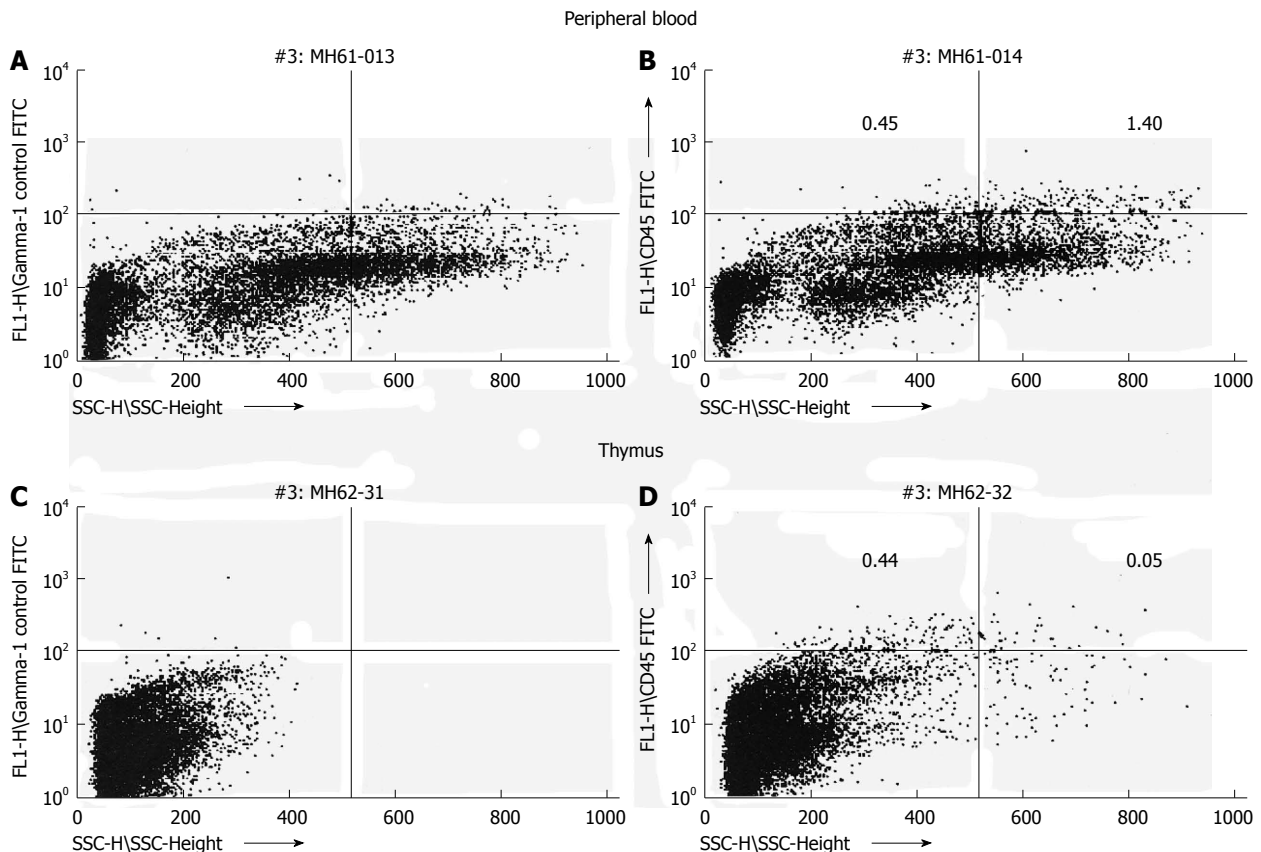


Figure 1 Transplantation of human hematopoietic stem cell (CD34+) during the murine engraftment window results in bi-lineage expression and relevant cell migration of the differentiated human progeny 6 mo after transplantation. Human CD45 cells exhibit different side scatter characteristics (low side scatter: lymphocytes; high side scatter: granulocytes) dependent on organ tested (A, C: Control mouse; B, D: Experimental mouse)^[23].

ing no more than 30 d in sheep (term gestation 145 d) or 2 d in mice (term gestation 21 d). The ability to determine exactly when in gestation engraftment receptivity occurs permitted parallel experiments on lymphocyte ontogeny in sheep. These experiments identified the thymus as the site of immune activity (CD45 differentiation) during the transplant receptivity window. CD45 isoform differentiation occurring only in the thymus included all identifiable lineages: T cell, B cell and antigen presenting cell (APC). Unfortunately, ovine natural killer (NK) cell specific reagents were not available to track NK cell development. Evidence for thymic deletion of T and B cells is seen. It is important to note that these observations suggest B cell tolerogenesis does not occur in spleen, bone marrow or Peyer's patches^[6,26-30]. Studies using retroviral vector (RTV) transplantation in sheep demonstrate preferential thymic epithelial cell (TEC) expression of the gene product during this period with the development of specific lymphocyte unresponsiveness to the gene product and inability to generate viral specific antibody after birth^[31,32]. Thus, B cell deletion (perhaps mediated by the developing "deletional TEC matrix") is likely formed in the thymus during the window. Further, expression of the RTV gene product in the TECs in Hassall's corpuscles (the proposed site Treg formation) and our phenotype experiments during this period (JSP, unpublished observations) suggest that, the onset of Treg formation occurs simul-

taneous with or near the conclusion of the tolerance window^[31]. Reports by McCune and colleagues support this conclusion, as Tregs in humans are a predominant T cell phenotype after thymic demarcation. They further propose that fetal HSC may provide additional stimulus to this thymic differentiation cascade^[33,34].

RAMIFICATIONS: DISSECTION OF SELF-TOLERANCE

These observations support a model for fetal tolerance induction where antigens (either autologous, allogeneic or xenogeneic) in the extracellular compartment of the developing thymus during this temporal phase are processed by the differentiating TEC progenitors and APC component of the thymic stroma and presented to developing immunocompetent cells (identifiable by changes in CD45 isoform expression) to form the tolerance repertoire. This two-stage antigen exposure model is presented in Figure 2. Presentation of surface antigens on cells intercalating the developing thymus during this finite period might also contribute. Endogenous gene derived presentation (to explain solid organ tolerance) is not consistent with our observations and unresponsiveness in the NK lineage must also be acquired during this period^[35-42]. The extracellular antigen exposure

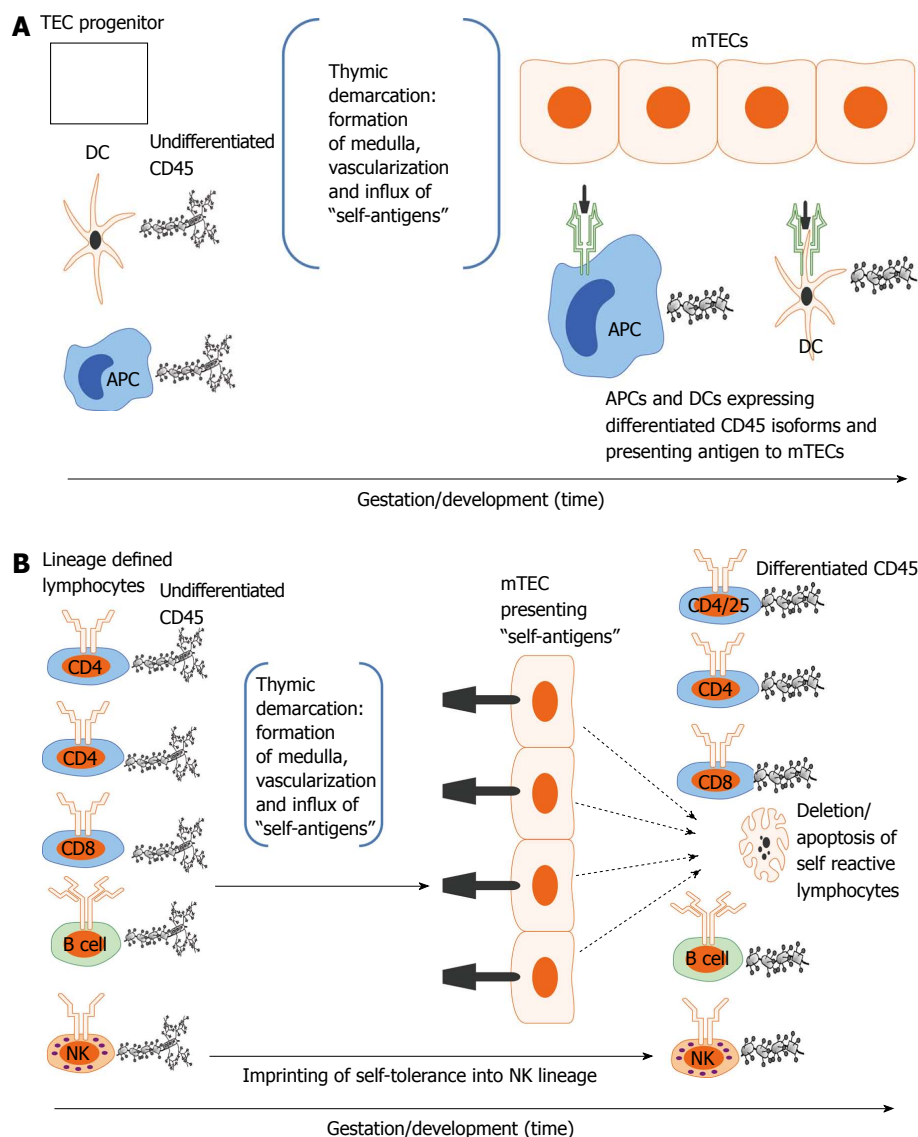


Figure 2 Developmental acquisition of self-tolerance: Antigen exposure model. A: Thymic epithelial (TEC) progenitors mature in intimate contact with dendritic cells (DCs) and likely other antigen presenting cells (APCs) thus forming the thymic medulla (*i.e.*, thymic demarcation). With vascularization, self-antigens (*i.e.*, antigens present during the finite tolerance window) from the periphery are processed and presented to medullary thymic epithelial cells (mTECs). The developing mTEC matrix is only receptive to antigen presentation during the tolerance window (time dependence) and subsequently exhibits on its surface the self-antigen repertoire (see 3. Ramifications: Dissection of immune tolerance); B: All lymphoid compartments [T, B and natural killer (NK) cell] enter the thymus expressing undifferentiated CD45 but are lineage defined. They then migrate through the thymus encountering mTECs expressing the self-antigen repertoire. CD4, CD8 and B cells reactive to self-antigens are deleted. CD4/CD25 cells reactive to self-antigens are rendered suppressive (Treg) in Hassall's corpuscles. NK cells are imprinted in an undefined fashion (likely based on major histocompatibility complex exposure) to become tolerant to self. All differentiated lymphocytes (*i.e.*, cells that have undergone CD45 isoform maturation and are not self-reactive or are tolerogenic (*i.e.*, Treg) then migrate to the spleen or other secondary lymphoid organs (see 3. Ramifications: Dissection of self-tolerance)^[6,21,31-37,42].

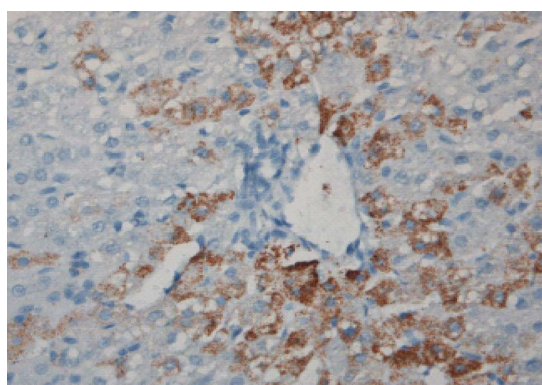


Figure 3 Induction of sheep tolerance to human hepatocytes by transplantation of human hematopoietic stem cells during the temporal engraftment window (note absence of sheep round cell infiltration). Sheep liver stained for human specific antibody 11 mo following transplantation^[49].

model would explain the absence of rejection of both hematolymphoid and cellular components of solid organ xenografts and the marked expandability of grafts following transplantation^[43,44]. It suggests that the NK cell

compartment undergoes thymic imprinting during this critical phase to explain the absence of NK cell mediated rejection. Evidence supporting thymic processing and adaptability of NK cells is reported^[42,45-47]. It seems unlikely that Treg(s) could account for the complete absence of NK cell mediated rejection (Figure 3)^[48,49]. Thus these observations demonstrate the value of IUT as a method to explore mechanisms underlying induction of self non-self discrimination.

RAMIFICATIONS: TREATMENT OF CHILDHOOD HEMATOLYMPHOID DISEASES

Xenotransplantation of human HSC following IUT in sheep and mice results in human cell engraftment and differentiation. In sheep this includes cardiac, liver, intestinal and pancreatic cells. Differentiated cellular function manifested by identification of circulating human proteins in transplanted sheep and mice is presented in Table 1^[23,49-52]. The absence of rapid circulatory clearance

Table 1 Human proteins detected in circulation of animals transplanted in utero with human stem cells	
Animal transplanted	Human protein detected
Sheep	IgM ¹
Sheep	Albumin ^[47,48]
Sheep	Factor VIII ¹
Sheep	C-peptide ^[49,50]
Sheep	α -fetoprotein ¹
Mouse	IgM ^[23]

¹Unpublished observation by Esmail D Zanjani. IgM: Immunoglobulin M.

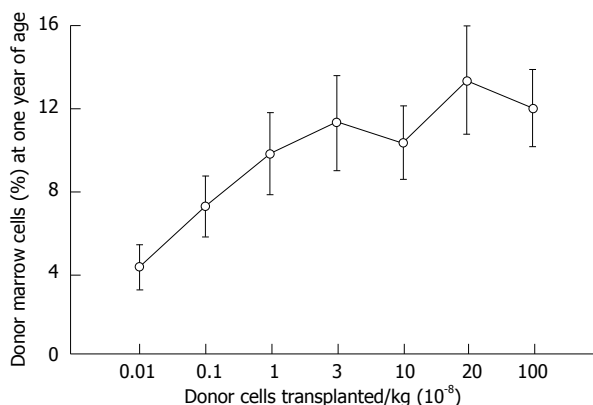


Figure 4 Levels of allogeneic engraftment plateau with transplantation of log-fold increases in donor cells in sheep. This is most consistent with a model of saturation kinetics with engraftment limited by available receptive sites. An identical curve is seen in the xenogeneic human-sheep model^[2].

speaks to the profound ability of IUT to alter the self non-self paradigm. IUSCT is thought to provide a number of therapeutic advantages over postnatal transplantation besides tolerance including expanding bone marrow space (or niche), sterile environment, proliferative environment and preempting clinical disease. Diseases thought amenable to IUT include hemoglobinopathies, immunodeficiency states and inborn errors of metabolism leading to storage diseases (mucopolysaccharidosis and mucopolipidosis)^[11].

As Figures 4 and 5 reveal maximizing cell dosage and serial cell administrations can improve donor engraftment yet, there remain a set of problems in achieving adequate donor proliferation to ameliorate and/or cure these diseases^[2]. The parameters proposed that may be limiting successful clinical application include: (1) failure to induce tolerance and/or an inadequate engraftment frequency (chimeric incidence); and (2) limited graft expression: gestational developmental impediments, maternal derived inhibition, lack of selective advantage (*i.e.*, inadequate donor proliferation).

Tolerance/engraftment following IUT

Both natural and experimentally derived hematopoietic chimeras demonstrate immune tolerance using skin graft acceptance and mixed lymphocyte reactions. Deletional and cellular tolerance has been reported^[16,43,53-55]. Mar-

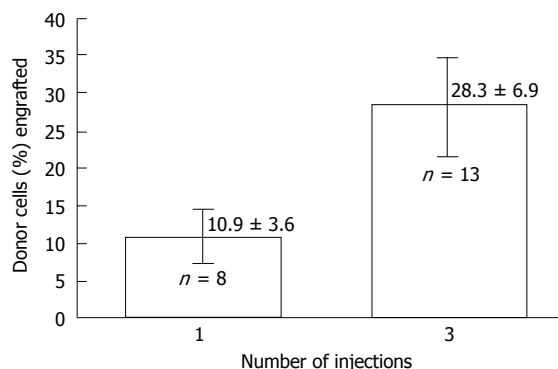


Figure 5 The effect of dividing cell dose on engraftment in sheep. Transplantation of the same total number of cells as a single injection vs a series of three injections given 1 wk apart is shown. The engraftment with divided doses is significantly higher supporting the concept that engraftment is limited at any particular time by the number of available receptive sites^[2].

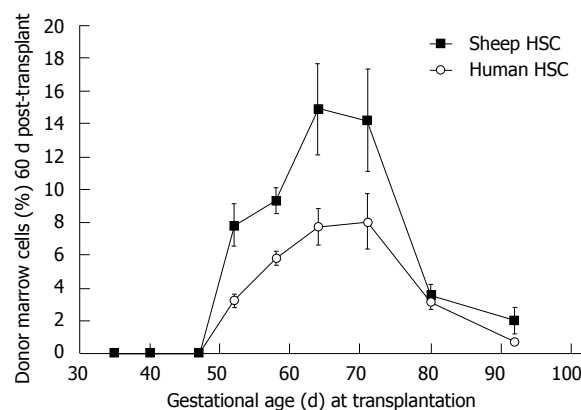


Figure 6 Allo- or xenogeneic stem cell bone marrow engraftment in sheep following transplantation during the temporal window when self non-self discrimination occurs (note parallel kinetics). Engraftment receptivity is gestational age-dependent^[6].

row engraftment levels 60 d after HSC transplantation in sheep using xenogeneic or allogeneic HSC reveals a parallel kinetic profile with a peak level when transplantation is performed between days 65-70 gestation (Figure 6). This suggests accurate gestational timing may be critical to accessing the full panoply of immune tolerance mechanisms (*i.e.*, deletion, regulatory cell formation and anergy among all immune competent lineages) forming during the window^[56]. Transplantation before the onset of the window and maternal derived histoincompatibility may limit engraftment or expression but in general tolerance is achieved^[6,54,55,57-60]. The short tolerance induction period in mice may contribute to occasional microchimerism and NK cell mediated rejection^[21,57,58]. In sheep and mice, engraftment and/or expression can be augmented long after birth (*i.e.*, further supporting the achievement of long-term immune tolerance and memory). In Table 2, groups of 6 sheep were assessed for tolerance to allogeneic HSC and then re-transplanted after birth with same donor HSC resulting in marked improvement in the level of engraftment. Flake has demonstrated similar findings

Table 2 Groups of 6 sheep were assessed for tolerance to allogeneic human stem cells and then re-transplanted after birth with same donor human stem cells

Allogeneic sheep HSC render recipient sheep ¹ tolerant following in utero transplantation		
Stimulator	Responder	Stimulation index ²
Donor	Donor	0
Recipient	Recipient	0
Donor	Recipient	0-8
Recipient	Donor	58 ± 11
Pooled	Donor	69 ± 12
Pooled	Recipient	78 ± 12
Postnatal infusion of allogeneic same donor HSC augments engraftment in tolerant sheep ³		
% donor cells at birth	<i>n</i>	% increase ⁴
6-10	4	86 ± 29
11-15	5	63 ± 22
> 15	4	21 ± 11

¹Representative sample of 6 chimeric lambs; ²Variation of mixed lymphocyte reaction (MLR) previously reported^[32]; ³Tolerance determined *via* MLR^[32]; ⁴Assessed 6 mo after postnatal stem cell infusion (3×10^5 cells/kg) in thirteen chimeric lambs rendered tolerant. HSC: Human stem cell.

in the allogeneic mouse model^[43]. In a similar vein, mixed lymphocyte reaction unresponsiveness has been detected following xenogeneic in utero transplantation (unpublished observations).

As noted above, if performed during the proper phase in gestation, the SC engraftment incidence is high in both large and small animal models. Mouse models are hampered by the short induction period and multiple small fetuses to inject resulting in a diminished engraftment frequency. In large animals, the engraftment frequency is considerably higher. For example, less than exhaustive examination of the pancreas in sheep revealed a chimeric incidence of 79%^[51].

Limited graft expression following IUT

Gestational developmental impediments: The course of hematopoietic ontogeny is associated with an orderly and predictable switch in primary sites of hematopoiesis. Mammalian hematopoietic sites are gestational age dependent starting in the yolk sac migrating to liver and spleen then finally lodging in the bone marrow. Yet, the marrow contributes little to circulating peripheral blood until just prior to birth. The mechanisms underlying this migration to marrow reflect bone and marrow ontogeny including formation of the osteoblastic niche. With regard to transplanted HSC, we reported a similar engraftment gradient dependent on bone and marrow maturity with little peripheral blood expression^[61-63]. This osteoblastic niche (site of primitive HSC engraftment) matures by day 65 and may in part influence the peak in marrow engraftment seen following transplantation (Figures 6 and 7). But again, the liver/spleen remains the main site of hematopoiesis until just prior to birth. These represent serious homing and maturational impediments to achieving therapeutic levels of donor cell expression to treat diseases that are clinically evident during gestation.

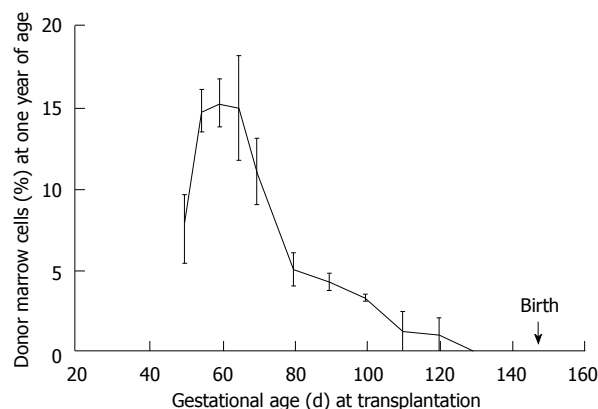


Figure 7 Maximal donor cell engraftment in sheep occurs during the mid-to-later stages of the tolerance induction period. This period follows thymic demarcation and formation of the osteoblastic niche. Further, engraftment is durable as the engraftment kinetics are similar to that noted at 60 d post transplant supporting our contention that the graft is viewed by the recipient as self (see Figure 6)^[2].

Maternal-derived inhibition: Maternal derived inhibition either through histoincompatible maternal lymphocyte microchimerism or maternal alloantibodies may limit engraftment/expression. To address this issue, we observed that maternal histocompatible donor grafts from matched cord blood improved peripheral blood donor expression (2.75% *vs* 0.93%) in comparison to relatively mismatched grafts^[62].

Inadequate competitive advantage to promote donor expression: Allogeneic SCs can repair genetic hematologic defects due allograft responsiveness to endogenous growth factors^[22,64-66]. Donor-species-specific growth factors improve graft expression in xenografts confirming establishment of tolerance and importance of donor stimulation in either an allogeneic or xenogeneic environment following IUT^[1,44]. A promising approach that needs further examination is the use of autologous stroma. In our hands, co-transplantation improved graft expression (donor hemoglobin) during gestation suggesting this may be a useful tack to treat diseases overtly manifest during gestation (Figure 8)^[67].

The end of the tolerance induction phase in large animals is followed by exponential growth^[6]. Thus, augmentation of donor engraftment/expression with transplantation of high dose donor SC and/or autologous stroma during this exponential growth phase to compete with endogenous/recipient SC differentiation and expansion should prove fruitful. Late retransplantation would mimic the mixing of hematopoietic cells in natural chimera that occurs through the placental circulation during and after closure of the gestational tolerance window and can result in the enhanced expression of sibling cells. The sheep is the maternal/fetal system most stable to test this hypothesis^[68]. Studies in mice support this notion as endogenous marrow ablation after birth with retransplantation of donor HSC can essentially replace recipient cells^[69].

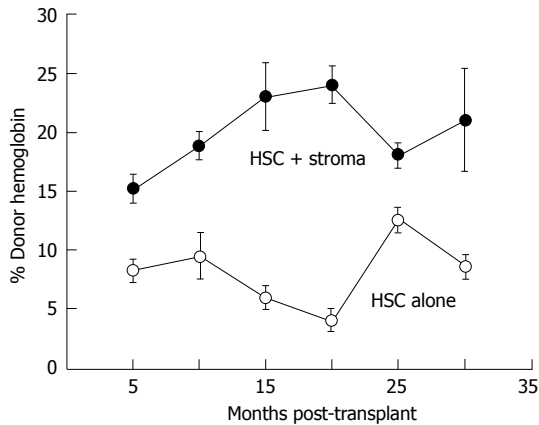


Figure 8 Co-transplantation with autologous adult sheep stromal cells improves allogeneic donor hematopoietic stem cell expression. Donor expression (circulating hemoglobin) persists more than 30 mo after transplantation. Donor hemoglobin levels in the peripheral blood of sheep co-transplanted with autologous adult bone marrow stroma ($n = 4$) are significantly higher ($P < 0.01$) than those transplanted with adult T-depleted bone marrow mononuclear cells alone ($n = 3$)^[67]. HSC: Human stem cell.

Clinical experience and roadmap for implementation

Therapeutic success following IUT in fetuses diagnosed with a variety of genetic diseases has been disappointing. In repair of the severe combined immune deficiency (SCID) defect, the transplanted cells have a competitive advantage and a poorly populated functional organ in which differentiation may occur^[3]. Yet, there are well-documented examples of proper timing and administration of an adequate SC dosage to treat a disease that theoretically should benefit for which limited graft expression is seen^[12,13]. Moving forward will require a more coordinated approach, a clinical trial in an ideal candidate disease.

This coordinated approach should identify a candidate disease that will provide “proof principle” that our theories are correct in a clinical setting. While at first glance one might conjecture that transplantation in X-linked SCID was successful because the fetus is immunodeficient, an alternative and more likely explanation is that an under populated developing thymus provided important stimulus for expansion and expression of the donor graft (a proliferative stimulus similar to Mintz’s demonstration in anemic mice)^[22]. For this reason thalassemia is a poor disease with which to test the feasibility of IUT. Here there is often marked over growth of endogenous marrow cells limiting donor cell engraftment during development^[1]. In a similar fashion, failure to observe enhanced expression in mismatched allogeneic grafts in sheep suggest that either maternal derived inhibition limits donor expression or autologous stromal factors are required to augment graft expression. Likely mechanisms would be through either stromal major histocompatibility complex matching to preferentially nurture the developing autologous SC or autocrine or paracrine factors which specifically stimulate autologous SC. Thus histocompatibility is an important factor not due to potential recipient immune inhibition (*i.e.*, failure of tolerance induction) but by providing a graft that is

not prone to maternal immune inhibition or providing (as yet undefined) autologous factors supporting graft expression (evidenced by our report demonstrating improved peripheral blood expression during development in allogeneic grafts co-transplanted with autologous stroma, Figure 8)^[67].

In summary, IUT recapitulates normal acquisition of self-tolerance. Experimentally, tolerance and engraftment are readily achieved. The problem remains identifying a disease that will promote graft expansion, limit maternal interference or selectively promote the graft through autologous stimulation. This likely requires retransplantation either late in gestation or shortly after birth (during exponential growth) to provide a dosing advantage to allow adequate numbers of donor HSC to compete with recipient HSC to ameliorate disease in this tolerant environment. Risks such as procedural, the possible need for multiple IUT procedures, infection and GVHD likely are not prohibitive^[2]. Since the majority of the genetic disorders correctable with HSCT can be diagnosed early in gestation to allow for IUHSC, it is important that attempts be made to correct these deficiencies before birth. Use of compatible donors, determining the optimal gestational age and co-transplantation of donor-derived stroma/mesenchymal SCs may help achieve this end.

Ramifications: In vivo platform for therapeutics

The formation of differentiated human cells/organs (without evidence for donor/recipient cell fusion) and functional human proteins following transplantation allows speculation as to the feasibility of IUT in applied therapeutics in an autologous or allogeneic setting. This would necessitate resolving problems such as cell/protein separation and isolation, as well as contamination with and transmission of infectious agents. Yet, development of effective methods to mitigate these impediments could unlock uncharted therapeutic possibilities. The finding of differentiated human functional proteins and evidence that engraftment/expression can be expanded before or after birth offers the possibility that IUT in a large animal could provide a source of autologous human proteins. In a similar fashion, effective cell isolation (hematopoietic cells seem the most feasible) might offer the possibility of autologous cell transfusion for clinical support.

While the above possible applications of IUT may be better served using *ex vivo* bioreactors or systems, *in vivo* use of sheep or an alternative animal of similar size to humans may be useful with regard to development of organs for transplantation. Our laboratory has been investigating this with regard to the liver and endocrine pancreas^[49-52]. Our studies involving the endocrine pancreas have demonstrated long-term circulating human insulin following SC transplantation. The ease with which islets can be isolated for transplantation makes this an ideal organ to determine feasibility of IUT as a therapeutic modality for human organ transplantation. For both systems, the critical determinant will be the role of a single SC (or as Owen described them embryonal ancestral cells) in or-

gan repair^[1,70].

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The representations of CD45 used in Figure 2 were obtained from Annual Review of Immunology 21 (2003) 107-37. CD45: a critical regulator of signaling thresholds in immune cells, Hermiston ML, Xu Z, Weiss A, authors; Sanford H Barsky for review of Figure 3; Richard Cacciato, Librarian, VA Sierra Nevada Health Care System.

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Comparison of phenotypic markers and neural differentiation potential of multipotent adult progenitor cells and mesenchymal stem cells

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Abstract

AIM: To compare the phenotypic and neural differentiation potential of human bone marrow derived multipotent adult progenitor cells (MAPC) and mesenchymal stem cells (MSC).

METHODS: Cultures of MAPC and MSC were established in parallel from same samples of human bone marrow ($n = 5$). Both stem cell types were evaluated for expression of pluripotency markers including Oct-4 and Nanog by immunocytochemistry and reverse-transcription polymerase chain reaction (RT-PCR) and expression of standard mesenchymal markers including CD14, CD34, CD44, CD45, CD73, CD90, CD105 and

human leukocyte antigen (HLA)-ABC by flow cytometry. After treatment with neural induction medium both MAPC and MSC were evaluated for expression of neural proteins [neuronal filament-200 (NF-200) and glial fibrillar acidic protein (GFAP)] by immunocytochemistry and Western blotting and neural genes [NF-200, GFAP, Tau, microtubule-associated protein (MAP)-1B, MAP-2, neuron-specific enolase (NSE) and oligodendrocyte-1 (Olig-1)] by quantitative real-time-PCR.

RESULTS: MAPC had small trigonal shaped while MSC had elongated spindle-shaped morphology. The MAPC expressed Oct-4 and Nanog both at gene and protein levels, whereas MSC were negative for these pluripotent markers. MAPC were negative for HLA-ABC while MSC had high expression of HLA-ABC. In addition, MAPC as compared to MSC had significantly lower expression of CD44 ($36.56\% \pm 1.92\%$ vs $98.23\% \pm 0.51\%$), CD73 ($15.11\% \pm 2.24\%$ vs $98.53\% \pm 2.22\%$) and CD105 ($13.81\% \pm 3.82\%$ vs $95.12\% \pm 5.65\%$) ($P < 0.001$, for all) MAPC cultures compared to MSC cultures treated with neural induction medium had significantly higher fold change expression of NF-200 (0.64), GFAP (0.52), Tau (0.59), MAP-2 (0.72), Olig-1 (0.18) and NSE (0.29) proteins ($P < 0.01$ for Olig-1 and $P < 0.001$ for rest) as well as higher fold change expression of genes of NF-200 (1.34), GFAP (1.12), Tau (1.08), MAP-1B (0.92), MAP-2 (1.14) and NSE (0.4) ($P < 0.001$ for all).

CONCLUSION: MAPC can be differentially characterized from MSC as Oct-4 and Nanog positive stem cells with no expression of HLA-ABC and low expression of mesenchymal markers CD44, CD73 and CD105 and when compared to MSC they possess greater predilection for differentiation into neuro-ectodermal lineage.

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Key words: Bone marrow; Human multipotent adult progenitor cells; Human mesenchymal; Stem cells; Pheno-

typic markers; Neural differentiation

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INTRODUCTION

Multipotent adult progenitor cells (MAPC) and mesenchymal stem cells (MSC) are two predominant non-hematopoietic stem cell types of the bone marrow stroma, that have enormous therapeutic properties including regenerative therapy for neurodegenerative disorders^[1,2]. The MAPC are pluripotent stem cells with capacity to differentiate into cells of all the three germ layers^[3-5]. They have been variously described in literature as multipotent adult stem cells (MASC)^[6], mesodermal/multipotent progenitor cells (MPC)^[7], marrow-isolated multi-lineage inducible cells^[8], adult pluripotent stem cells^[9] and embryonic like stem cells (ELSC)^[10] by different groups. The expression of pluripotent and neural markers in MAPC and their increased mobilization in patients with neurodegenerative diseases like stroke show potential role of these stem cells in neurogenesis^[11-13]. The MSC are mesodermal progenitors that are committed to differentiate into cells of mesodermal lineage^[14]. However, some studies have shown that in addition to their mesodermal commitment they also differentiate into cells of neuro-ectodermal lineage, claiming their role in neurogenesis^[15,16]. However, it is not known whether MAPC or MSC possess superior neurogenic potential and very less information is available on phenotypic differences between MAPC from MSC so it is also difficult to distinguish them from each other.

Therefore, the aim of the present study was to evaluate the expression of pluripotency and mesenchymal markers and to carry out a parallel comparison of neural differentiation potential of MAPC and MSC derived from the same samples of human bone marrow.

MATERIALS AND METHODS

Isolation, culture and characterization of MAPC and MSC

Subjects included in the study ($n = 5$) consisted of 2 healthy donors for bone marrow transplant patients and 3 patients with suspected iron deficiency anemia where bone marrow (BM) was done to look for iron stores, who otherwise had a normal BM. After informed consent, 5mL of BM aspirate was collected from each individual for this study, and it was divided into two equal parts for growing MAPC and MSC from the same sample in parallel.

The MAPC were cultured using Verfaillie's protocol^[3]. Briefly, bone marrow mononuclear cells (BMNC) of the marrow aspirates were depleted of CD45 and GlyA positive cells were cultured in growth medium consist-

ing of 53.8% 1.5 mg/mL bovine serum albumin (BSA) mixed Dulbecco's modified Eagle's medium (DMEM)-low glucose medium (Gibco, www.invitrogen.com), 40% MCDB-201 (Sigma, www.sigmaaldrich.com), 2% fetal bovine serum (FBS) (Hyclone, www.thermoscientific.com), 1% ITS+1 Supplement (Sigma), 0.5 μ mol/L dexamethasone (Sigma), 0.1 mmol/L L-ascorbic acid (Sigma), 1% LA-BSA (Sigma), 1% penicillin/streptomycin (Gibco), 10 ng/mL each of platelet-derived growth factor-BB (R and D, www.rndsystems.com) and epidermal growth factor (R and D) under hypoxic condition. The sub-confluent cultures were trypsinized and further expanded under same culture conditions to get optimal number of cells. The MAPC were characterized by expression of Pluripotency markers Oct-4 and Nanog and their differentiation into cells of three germ layers viz. neuronal (ectodermal cells), endothelial (mesodermal cells) and hepatocytes (endodermal cells).

The culture of MSC was carried out using Prockop's protocol^[17]. Briefly, BMNC were cultured in complete medium consisting 88% of α -MEM Medium, 10% of FBS, 2 mmol/L of L-Glutamine and 100 units/mL of pen-strep (all from Gibco) under normoxic condition. The MSC were characterized by expression of conventional mesenchymal markers and their differentiation into mesodermal cell including bone and fat cells.

Flow-cytometry

The phenotypes of MAPC and MSC were analyzed by two color flow cytometry at 3rd passage using human leukocyte antigen (HLA)-ABC [fluorescein isothiocyanate (FITC)]/CD44 [phycoerythrin (PE)], CD34 (FITC)/CD73 (PE), CD14 (FITC)/CD105 (PE) and CD45 (FITC)/CD90 (PE) (all from Serotec, www.abdserotec.com). The flow-cytometer used was FACS-calibur (Becton Dickinson) and data analysis was done using FACS express software.

Reverse-transcription polymerase chain reaction

Expression of *Oct-4* and *Nanog* genes was done by reverse-transcription polymerase chain reaction (RT-PCR). Total RNA of the cells was extracted using RNeasy mini RNA isolation kit (Invitrogen). Two μ g of total RNA was reverse transcribed into cDNA using random hexamers (Invitrogen). The cDNA was normalized by amplification of β -actin. The PCR conditions included denaturation at 94 °C for 4 min, amplification cycles 35 and elongation temperature 72 °C for 30 s with annealing. The amplicons were resolved on 2% agarose gel (Sigma-Aldrich) and pictures acquired using gel documentation system (Alpha Imager, www.proteinsimple.com).

Immunocytochemistry

The expression of pluripotency genes *Oct-4* and *Nanog* on MAPC and MSC was analyzed by immunocytochemistry. The cells were fixed with 4% para-formaldehyde (Sigma Aldrich) in PBS for 1 h at room temperature. The fixed cells were incubated overnight at 4 °C with following

Table 1 Sequence of primers used in reverse-transcription polymerase chain reaction and real time-polymerase chain reaction

No.	Primer	Sequence	Accession number
1	4-Oct	f: 5'-CGTGAAGCTGGAGAAGGAGAAGCTG-3' r: 5'-CAAGGGCCGACGTTACACATGTTTC-3'	NM_002701.4
2	Nanog	f: 5'-GCCGAAGAATAGCAATGGTGTG-3' r: 5'-CCAGGACTGGATGTTCTGGGTC-3'	NM_024865.2
3	NF-200	f: 5'-CAGAGCTGGAGGCACTGAA-3' r: 5'-CATCTCCCACTTGGTGTTCC-3'	NM_021076.3
4	GFAP	f: 5'-GAGTACCAGGACCTGCTCAA-3' r: 5'-TTCACCAAGATGTTCTCTT-3'	NM_002055.4
5	MAP-1B	f: 5'-GCGGAGACAGTACCTTCGGAG-3' r: 5'-CCGACGACCACCAGCAAGTAG-3'	NM_005909.3
6	MAP-2	f: 5'-TCAGAGCCAATTCGAGAG-3' r: 5'-TGTTGTC TGTTGATCCGATTTT-3'	NM_002374.3
7	Tau	f: 5'-TCATTAGGCAACATCCATCATA-3' r: 5'-CACCTCGTCAGCTAGCGT-3'	NM_001203252.1
8	NSE	f: 5'-TCTGCAGTCCCAGATCCCAGC-3' r: 5'-CTGATGAGGGCTGGCGGAT-3'	NM_001975.2
9	Olig-1	f: 5'-GCCCAACCAAGTACCTGTCTC-3' r: 5'-GGGACCAGATGCGGGAAC-3'	NM_138983.2
10	β -actin	f: 5'-GCTCGTCGTCGACAACGGCTC-3' r: 5'-CAAACATGATCTGGTCATCTTCTC-3'	NM_001101.3
11	GAPDH	f: 5'-GATTGTGCTCGTATTGGG-3' r: 5'-TCCACGACGTACTCAGC-3'	NM_002046.3

NF-200: Neuronal filament-200; GFAP: Glial fibrillar acidic protein; Olig-1: Oligodendrocyte-1; NSE: Neuron-specific enolase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MAP-1B: Microtubule-associated protein-1B; MAP-2: Microtubule-associated protein-2.

primary antibodies: Nanog and OCT-4 (1:200 dilution) (Chemicon, www.millipore.com) After washing with PBS, cells were incubated with 1:500 diluted goat anti-mouse immunoglobulin G (IgG) (Fab)₂ FITC (Abcam, www.abcam.com) as secondary antibody and stained with Hoechst dye. The pictures were acquired by fluorescent microscope (Nikon 80i, Japan).

Differentiation into neuro-ectodermal cells

We used following protocol for differentiation of MAPC and MSC into neuronal cells. The cells were plated into 12-well plates at a density of 2000-2500 cells/cm² and incubated in neuro-ectodermal induction medium consisting of 98% basal medium (57% DMEM low glucose, 40% FBS, 1% Pen-strip, 1% ITS+1, 0.1 mmol/L L-ascorbic acid, 0.5 μ mol/L dexamethasone), 100 ng/mL basic fibroblast growth factor (R and D systems), 100 ng/mL Noggin (R and D systems), 20 ng/mL NT-3 (R and D systems), 10 ng/mL brain-derived neurotrophic factor (R and D systems), 10 ng/mL glial cell line-derived neurotrophic factor (R and D systems), 20 μ mol/L RA (Sigma), 1X B-27 supplement (Gibco), 1X 2-ME (Gibco). The differentiated cells were characterized as neuronal cells by immunocytochemical detection of neuronal filament-200 (NF-200), microtubule-associated protein 2 (MAP-2) and glial fibrillary acidic protein (GFAP) on the cells using MAP-2 (Abcam), NF-200 and GFAP primary antibodies (Biovision). Goat anti-mouse IgG (Fab)₂ FITC (Abcam) as secondary antibody and Hoechst dye staining as described above.

Western blotting

Western blotting was done using primary antibody NF-200 (200 kDa), GFAP (51-52 kDa) (Biovision), Tau (52 kDa), MAP-2 (280 kDa), Olig-1 (28 kDa) and neuron-specific enolase (NSE) (47 kDa) (Abcam) and β -actin (42 kDa) (Abcam) and horseradish peroxidase conjugated corresponding secondary antibodies. The signals were detected using an enhanced chemiluminescence detection system (Amersham Biosciences, www.gelifesciences.com).

Real-time PCR

The quantification of neuronal gene expression in MAPC and MSC was carried out by real time PCR. Total mRNA was isolated from the undifferentiated and neuro-ectodermal differentiated cells following single step mRNA isolation method using RNA isolation kit (Invitrogen). Total mRNA (2 μ g) was reverse transcribed to cDNA using RT-PCR kit (Applied Biosystems) following manufacturer's instructions. Real time analysis for NF-200, GFAP, MAP-2, MAP-1B, Tau, oligodendrocyte-1 (Olig-1), NSE and normalizing gene glyceraldehyde 3-phosphate dehydrogenase (Table 1) was performed using Sybr Green Master mix as per the manufacturer's instruction (Applied Biosystems), analysis were done on Light-cycler 480 (Roche) and fold changes in gene expression was calculated using $2^{-\Delta\Delta CT}$ method.

Statistical analysis

The results were calculated as mean \pm SE. The statistical significance between MAPC and MSC comparisons was determined by using one-way analysis of variance test. *P* values < 0.05 were considered to be statistically significant.

RESULTS

Morphology and phenotypes

The MAPC and MSC both grew as adherent cells in culture but they were morphologically distinct from each other. The MAPC had small trigonal morphology while the MSC were large cells having elongated spindle shaped morphology (Figure 1A).

The MAPC had no expression of HLA-ABC (0%) while MSC had high expression of HLA-ABC (93.32% \pm 2.58%). The MAPC compared to MSC had significantly lower expression of CD44 (36.56% \pm 1.92 % *vs* 98.23% \pm 0.51%), CD73 (15.11% \pm 2.24% *vs* 98.53% \pm 2.22%), CD105 (13.81% \pm 3.82% *vs* 95.12% \pm 5.65%) (*P* < 0.001 for all). Both MAPC and MSC had high expression of CD90 (99.80% \pm 0.14% *vs* 99.47% \pm 0.44%; *P* > 0.5) and no expression of CD14, CD34 and CD45 (Figure 1B).

Expression of pluripotency markers

The MAPC expressed pluripotency markers Oct-4 and Nanog at gene and proteins levels while MSC expressed none of these markers either at gene or protein levels (Figure 2).

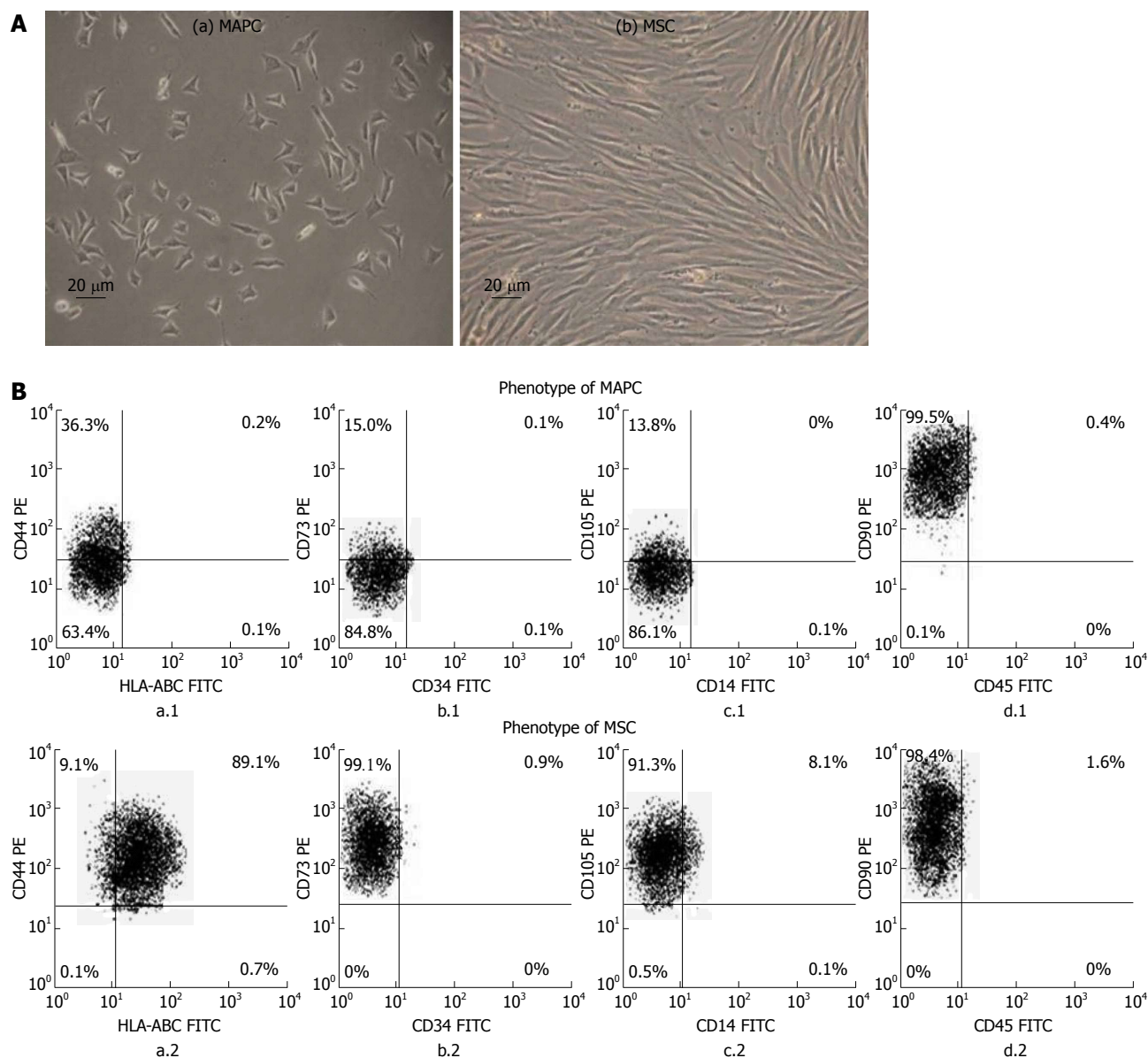


Figure 1 Morphology and phenotypic markers of multipotent adult progenitor cells and mesenchymal stem cells. A: Representative photomicrographs ($\times 10$, 20 μ m) of primary cultures showing (a) small trigonal morphology of multipotent adult progenitor cells (MAPC) and (b) large spindle shaped morphology of mesenchymal stem cells (MSC); B: Representative flow-cytometric dot-plots showing expression of (a.1 and a.2) HLA-ABC (FITC)/CD44 (PE), (b.1 and b.2) CD34 (FITC)/CD73 (PE), (c.1 and c.2) CD14 (FITC)/CD105 (PE), and (d.1 and d.2) CD45 (FITC)/CD90 (PE) on MAPC and MSC, respectively. FITC: Fluorescein isothiocyanate; PE: Phycoerythrin.

Neuro-ectodermal differentiation efficiency

Following treatment with neurogenic induction medium, the cells which differentiated from both MAPC and MSC, had morphological characteristics of neuronal like cells as revealed by their bipolar elliptical shape and/or multiple branching points and neuritis (Figure 3A) and they expressed NF-200, MAP-2 and GFAP as revealed by immunocytochemistry (Figure 3B). The MAPC derived neuronal cells compared to those derived from MSC, showed a significantly higher fold change expression of NF-200 (0.64), GFAP (0.52), Tau (0.59), MAP-2 (0.72), Olig-1 (0.18) and NSE (0.29) ($P < 0.01$ for Olig-1 and $P < 0.001$ for rest) (Figure 3C). Similarly the fold change expression of NF-200 (1.34), GFAP (1.12), Tau (1.08), MAP-1B (0.92), MAP-2 (1.14) and NSE (0.4) genes were

significantly higher in MAPC derived neuronal cells compared to those derived from MSC ($P < 0.001$ for all) but there was no difference in the fold change expression of Olig-1 gene (0.08) in neuronal cells derived from both stem cell types ($P > 0.5$) as revealed by real time quantitative PCR (Figure 3D).

DISCUSSION

Our study shows that MAPC and MSC differ from each other in terms of morphology, phenotypic and pluripotency markers, and their neuro-ectodermal differentiation potential. Morphologically, MAPC are small trigonal cells while MSC are elongated spindle shaped cells. Phenotypically MAPC have no expression of HLA-ABC and

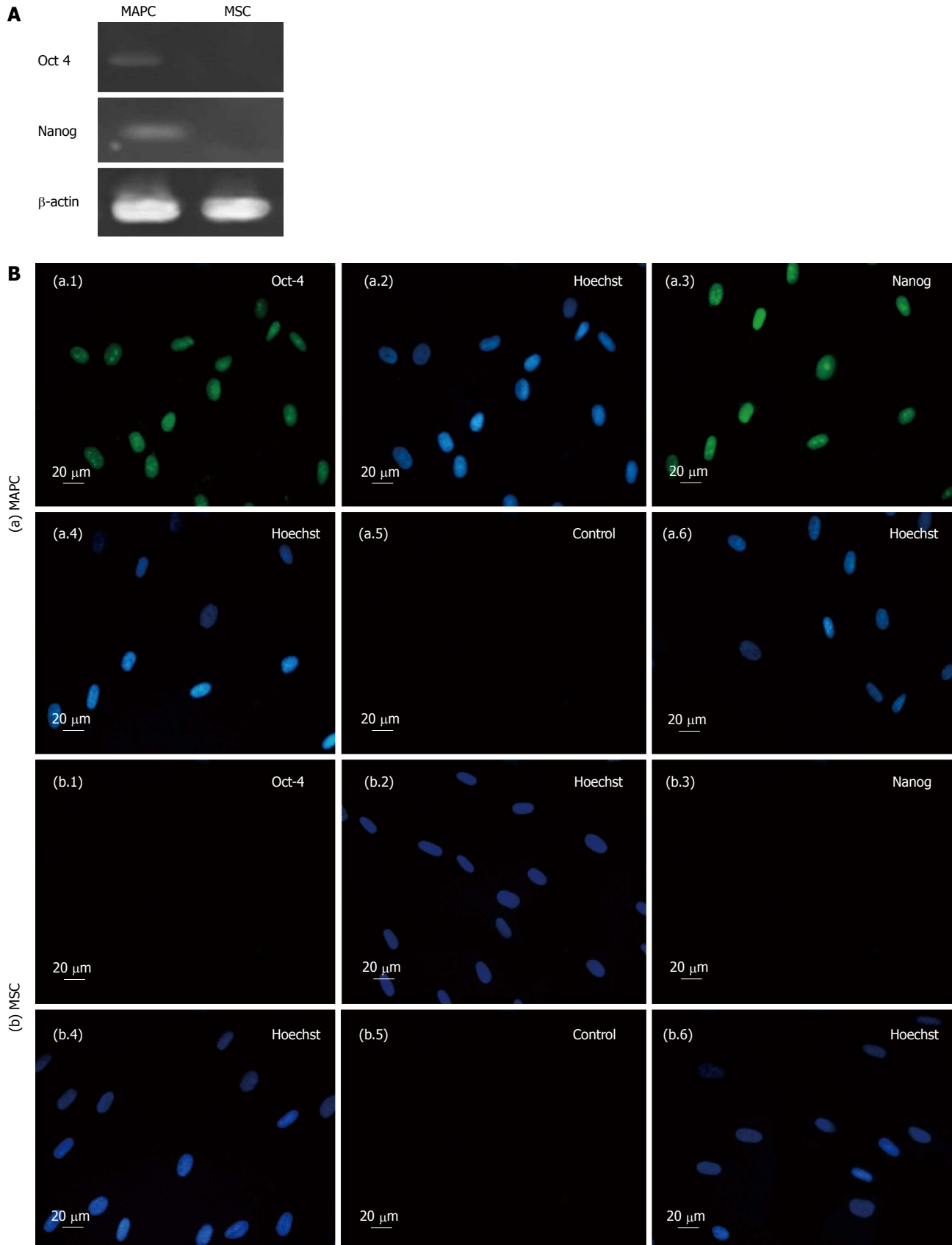


Figure 2 Expression of embryonic markers by multipotent adult progenitor cells and by mesenchymal stem cells. A: Expression of *Oct-4* and *Nanog* genes in multipotent adult progenitor cells (MAPC) and no expression of these genes in mesenchymal stem cells (MSC) as revealed by reverse-transcription polymerase chain reaction. β -actin represents the house keeping gene; B: Representative Immunocytochemistry photomicrographs ($\times 40$, 20 μm) of (a) MAPC showing fluorescein isothiocyanate (FITC) and Hoechst staining for Oct-4 (a.1 and a.2, respectively), Nanog (a.3 and a.4, respectively) and of controls, i.e., cells with no primary antibody (a.5 and a.6, respectively); and (b) MSC showing FITC and Hoechst staining for Oct-4 (b.1 and b.2, respectively) and Nanog (b.3 and b.4, respectively) and of controls, i.e., cells with no primary antibody (b.5 and b.6, respectively).

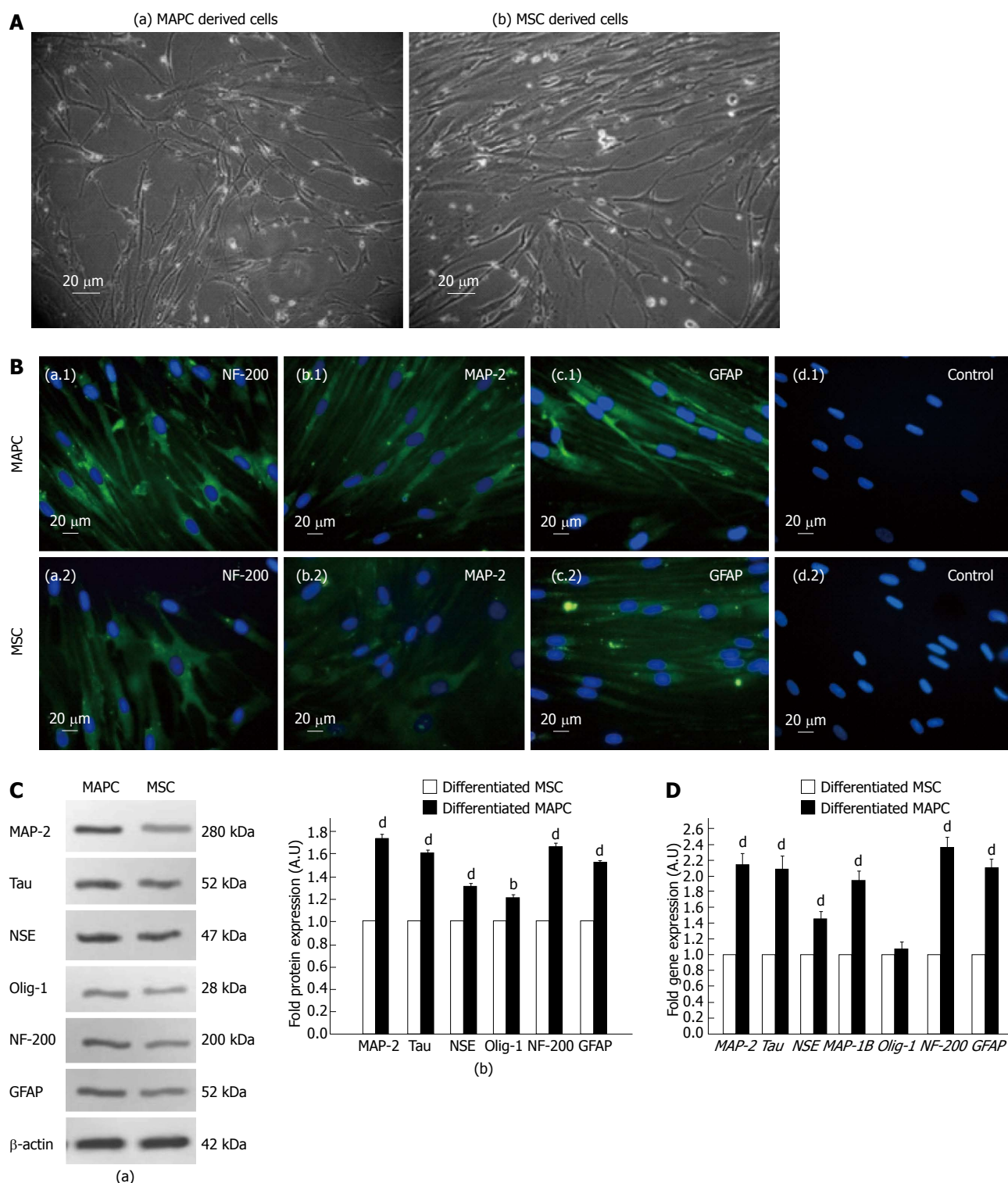


Figure 3 Neural differentiation potential of multipotent adult progenitor cells and of mesenchymal stem cell. A: Representative photomicrographs ($\times 10$, $20\ \mu\text{m}$) of neuronal cells differentiated from (a) multipotent adult progenitor cells (MAPC), and (b) mesenchymal stem cell (MSC); B: Representative immunocytochemical photomicrographs ($\times 40$, $20\ \mu\text{m}$) showing an overlay of fluorescein isothiocyanate (FITC) and Hoechst staining for (a.1 and a.2) neuronal filament-200 (NF-200), (b.1 and b.2) microtubule-associated protein 2 (MAP-2), and (c.1 and c.2) fibrillar acidic protein (GFAP) expression in neuronal cells differentiated from MAPC and MSC, respectively. Negative control represents cells with no treatment with neurogenic medium (d.1) MAPC and (d.2) MSC; C: Immunoblots analysis of neural proteins in MAPC and MSC derived neuronal cells; (a) Representative immunoblots of MAP-2, Tau, neuron-specific enolase (NSE), oligodendrocyte-1 (Olig-1), NF-200 and GFAP proteins in MAPC and MSC derived neuronal cells; (b) Error-bar diagrams showing fold change expression of proteins in MAPC (solid bars) and MSC (open bars) derived neuronal cells as revealed by densitometric quantification of immunoblots. ^b $P < 0.01$, ^d $P < 0.001$ vs MSC group; D: Error-bar diagrams showing expression of MAP-2, Tau, NSE, MAP-1B, Olig-1, NF-200 and GFAP genes in MAPC (solid bars) and MSC (open bars) derived neuronal cells as revealed by real-time quantitative polymerase chain reaction. The values of MSC and MAPC treated with neurogenic medium and their untreated counterparts were normalized to housekeeping glyceraldehyde-3-phosphate dehydrogenase and the fold increase values of MAPC compared to MSC have been expressed as mean \pm SE, ^b $P < 0.001$ vs MSC group.

low expression of CD44, CD73 and CD105 while MSC possess high expression of all these markers. In addition, MAPC express Oct-4 and Nanog both at gene and protein levels but MSC lack expression of these markers. The MAPC have higher neuro-ectodermal differentiation potential than MSC as revealed by their significantly higher expression of NF-200, GFAP, Tau, MAP-2, Olig-1 and NSE proteins and *NF-200*, *GFAP*, *MAP-2*, *MAP-1B*, *Tau* and *NSE* genes. To the best of our knowledge this is the first study showing a parallel comparison of phenotype, pluripotency markers and neuro-ectodermal differentiation potential of MAPC and MSC isolated from the same samples of human bone marrow.

The existence of MAPC in the stroma of adult bone marrow has been described previously^[1,3-5] by different groups and most of these studies support our observation of small triangular morphology of MAPC. However despite this morphological difference, there are no well defined phenotypic markers distinguishing MAPC from MSC. We have observed that MAPC have low expression of CD44, CD73, and CD105 and no expression of HLA-ABC, while MSC have high expression of these markers.

Human embryonic stem cells also have no or negligibly low expressions of HLA-ABC highlighting that MAPC have properties similar to embryonic stem cells^[18]. We found that MAPC express pluripotency markers Oct-4 and Nanog both at gene and protein levels but MSC entirely lacked expression of these pluripotency markers. The expression of Oct-4 and Nanog on MAPC corroborates with expression of these and other pluripotency markers in ELSC^[10], MASC^[6] and MPC^[7]. A few studies have reported that Oct-4, Nanog and other pluripotency markers are also expressed in MSC derived from bone marrow and other adult tissues^[19] and one study has shown that MSC express Nanog but not OCT-4^[20]. In another study, it has been shown that culture conditions of low serum content, induce expression of Oct-4, Nanog and other pluripotency markers on MSC^[21]. We have cultured MSC under standard serum conditions, and thus the difference in expression of pluripotency markers between our and these studies may be due to difference in culture conditions which either have induced expression of Oct-4 and Nanog on MSC or promoted the growth of a population of MAPC in the cultures. Similar to our observation in MAPC, the expression of Oct-4, Nanog and other pluripotency markers has been shown in fetal MSC, but not in adult MSC^[22]. Thus lower expression of the conventional mesenchymal markers, no expression of HLA-ABC and expression of Oct-4 and Nanog, may be used as suitable markers to distinguish MAPC from MSC.

Bone marrow derived MSC have been reported to exhibit trans-differentiation into cells of neuronal lineage, thereby claiming for a role of these stem cells in therapy for neurological disorders^[15,16]. More recently, MAPC have been shown to differentiate into neuronal cells and promote neuronal regeneration^[12,13]. However, no data exists on comparative analysis of neural differentiation

potential of MAPC and MSC. In the present study, we have carried out a parallel comparison of neuro-ectodermal potential of MAPC and MSC at protein and gene levels. We studied both stem cell types for gene and protein expression of markers of axons (NF-200 and Tau), astrocytes (GFAP), dendrocytes (MAP-1B, MAP-2 and Olig-1) and neurons (NSE) and observed that MAPC show significantly higher expression of *NF-200*, *Tau*, *GFAP*, *MAP-1B*, *MAP-2* and *NSE* genes in comparison to MSC. Moreover, we compared the protein expression of NF-200, Tau, GFAP, Olig-1, MAP-2 and NSE, and similar to gene expression, we found significantly increased expression of these proteins in MAPC compared to MSC. Thus MAPC appear to have a greater predilection for neural differentiation, which needs to be therapeutically evaluated *in vivo* in pre-clinical animal models of neurological disorders.

In conclusion, our study showed that MAPC can be differentially characterized from MSC as Oct-4 and Nanog positive stem cells with no expression of HLA-ABC and low expression of mesenchymal markers CD44, CD73 and CD105 and they possess higher neuro-ectodermal differentiation potential than MSC indicating that MAPC may be more suitable cell type than MSC for cell based therapy for neurodegenerative disorders. Future studies directed towards the *in vivo* evaluation of the therapeutic potential of MAPC in pre-clinical models would lead to development MAPC based therapies for neurological diseases.

COMMENTS

Background

Multipotent adult progenitor cells (MAPC) and mesenchymal stem cells (MSC), the two predominant stem cell types of the bone marrow stroma, are currently being explored for cellular therapy of neurodegenerative disorders. However, there is no data on their phenotypic difference and it is also not yet known that which of these two possess a greater potential for neural differentiation and thus would be more suitable for therapeutic use.

Research frontiers

On the basis of a parallel comparison of the two stem cell types derived from the same sample of the bone marrow, this study reports that MAPC can be differentially characterized from MSC as Oct-4 and Nanog positive stem cells with no expression of human leukocyte antigen-ABC and low expression of other mesenchymal markers and with a greater predilection for differentiation into neuro-ectodermal lineage. Future studies comparing their *in vivo* therapeutic efficacy in pre-clinical animal models of neurodegenerative disorders would be important to confirm superiority of MAPC over MSC for therapeutic application in these diseases.

Innovations and breakthroughs

This is the first study reporting a parallel comparison of MAPC and MSC and demonstrating phenotypic differences between two stem cells types and a greater potential of MAPC than MSC towards neuro-ectodermal lineage.

Applications

This study offers a foundation for comparative studies on MAPC and MSC in experimental models of neurodegenerative disorders that in turn may lead to initiation of clinical studies on MAPC in these disease states.

Terminology

MAPC represent a primitive population of non-hematopoietic stem cells present in the bone marrow and other tissues. Their phenotype is not yet well defined but they express embryonal markers and give rise to cells of all the three germ layers.

Peer review

This is a great manuscript that compares MAPC with MSC. The presented data are significant to the field of research and adds to the knowledge about these stem cell types and their potential.

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Perspectives of gene combinations in phenotype presentation

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Abstract

Cells exhibit a variety of phenotypes in different stages and diseases. Although several markers for cellular phenotypes have been identified, gene combinations denoting cellular phenotypes have not been completely elucidated. Recent advances in gene analysis have revealed that various gene expression patterns are observed in each cell species and status. In this review, the perspectives of gene combinations in cellular phenotype presentation are discussed. Gene expression profiles change during cellular processes, such as cell proliferation, cell differentiation, and cell death. In addition, epigenetic regulation increases the complexity of the gene expression profile. The role of gene combinations and panels of gene combinations in each cellular condition are also discussed.

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Key words: Gene combination; Cellular phenotype; Stem cell; Cancer stem cell; Gene expression

Core tip: This article discusses the effects of gene combination on cellular phenotype, demonstrating different capacities for proliferation and differentiation. Recent advances in genome technology have revealed that each cell type exhibits different gene expression pat-

terns, suggesting that the combination of genes might represent different cellular features or phenotypes. Because cellular diversity is derived from the regulation of gene expression, it is important to investigate the genes in various cells, including differences between cell types or the processes of cell development. Moreover, because stem cells are used as therapeutics, these gene combinations should be evaluated for the assembly of cells in medicinal use.

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INTRODUCTION

Recent advances in genome technology have revealed several perspectives associated with gene regulatory networks^[1]. Databases, such as encyclopedia of DNA elements (ENCODE), use chromatin immunoprecipitation and high-throughput sequencing to describe human transcription factors associated with cell type differences^[1]. Recently, stem cells, such as induced pluripotent stem (iPS) cells, have been developed for medical application. For safe application, researchers must address concerns about the ability of stem cells to switch the cellular phenotype to cancer or other cellular phenotypes different from those originally expected. There are also arguments concerning the immunogenicity of iPS cells^[2-4]. Thus, the clarification of stem cell characteristics is required for the safe application of iPS cells. There is little evidence indicating that mutations do not frequently occur during reprogramming^[5]. In this review, gene combinations associated with cellular phenotype, particularly in stem cells, are discussed to address the possibility of the detection of cell features using gene combinations to facilitate the safe application of stem cells without tumorigenesis.

From the viewpoint of the risk of tumorigenesis associated with stem cells and the origin of cancer, genes regulated in cancer stem cells are also discussed.

VARIETY OF GENE REGULATION

Genes involved in self-renewal and pluripotency

Cell self renewal is defined as cellular duplication, whereas pluripotency is defined as differentiation into any cell type, including their own, a process in which genes, such as *Pou5f1* (also known as *Oct4* or *Oct3/4*), *Sox2*, *Klf4*, or *Nanog*, play an important role. A previous study indicated that combination of Oct4, Sall4, and Nanog is involved in transport, metabolism, and development in mouse embryos^[6]. *Pou5f1* (*Oct4*) is a key factor in the maintenance of embryonic stem cells. In addition to *Pou5f1* (*Oct4*), several gene combinations and coordinated network gene regulation are essential to stem cell development. DNA methyltransferase 3B (*Dnmt3b*) has also been implicated as a developmental stage determinant working in conjunction with other pluripotency factors^[6].

Cell cycle control and long-term self-renewal have been linked, and the cyclin-dependent kinase inhibitor 1A (P21) (*Cdkn1a*) has been shown to regulate *Sox2* gene expression and adult neural stem cell expansion^[7]. The combination of p21, Sox2, and p53 is important for the regulation of neural stem cells, during which p21 binds to Sox2 enhancers and negatively regulates the gene expression of *Sox2*, and increased expression of Sox2 in p21-null neural stem cells induces p53-dependent growth arrest^[7]. Reprogramming clonally expanded antigen-specific CD8⁺ T cells obtained from an human immunodeficiency virus (HIV)-1-infected patient to pluripotency using the gene combination of *POU5F1* (*OCT3/4*), *SOX2*, *KLF4*, and *c-MYC* resulted in the expression of the pluripotency markers SSEA-4, Tra-1-60, and Tra-1-81, and the re-differentiation of these cells into CD8⁺ T cells resulted in T-cell functionality and antigen specificity, as observed in the original cytotoxic T lymphocyte clone^[8]. A study demonstrated that lactic acid bacteria-incorporated human dermal fibroblast cell clusters were positive for the multipotency markers *NANOG*, *POU5F1* (*OCT3/4*), *SOX2*, and SSEA-4, and these cells were converted into multipotent cells that differentiate into multiple lineages^[9]. Interestingly, not all pluripotent markers were expressed in the lactic acid bacteria-incorporated cells^[9]. A minimum gene combination might be sufficient for the acquisition of multipotency. A recent report has revealed that protein kinase C, located downstream of fibroblast growth factor-2 (FGF-2), regulates the self-renewal of human pluripotent stem cells^[10].

Genes involved in proliferation

The tumor suppressor p53 is associated with metabolism and senescence^[11]. p53 suppresses the gene expression of the tricarboxylic acid cycle-associated malic enzymes, including NADP⁺-dependent, cytosolic malic enzyme 1 (*ME1*) and *ME2*, leading to the regulation of cell metabolism and proliferation^[11]. The combination of p53 and malic enzymes

has been implicated in cellular senescence. Another finding demonstrated that the fifth component (CSN5) of the mammalian COP9 signalosome complex binds to CDK2 and controls senescence via cytoplasmic cyclin E^[12].

Genes involved in differentiation and stem cell activity

The combination of force and the scleraxis (*SCX*) gene promotes the commitment of mesenchymal stem cells derived from embryonic stem cells to tenocytes^[13].

The differentiation of T cells is regulated through various genes, including the regulation of Th17 cell differentiation through sphingosine 1-phosphate receptor 4 (*S1PR4*), an endothelial differentiation, G-protein-coupled (*EDG*) receptor^[14]. Glycerophosphodiester phosphodiesterase 2 (*GDE2*) induces spinal motor neuron differentiation^[15]. *GDE2* also inactivates the Notch activator RECK (reversion-inducing cysteine-rich protein with kazal motifs) through a glycosylphosphatidylinositol anchor cleavage and inhibits the Notch pathway, which promotes neurogenesis^[15]. The cyclin-dependent kinase inhibitor p27^{Kip1} has been implicated in the regulation of cellular processes, such as T cell differentiation^[16]. Moreover, the regulation of CD8 T cell memory through p27^{Kip1} has also been proposed^[16].

WNT signaling is one of the major signaling pathways involved in the regulation of bone homeostasis^[17]. WNT- β -catenin signaling is essential for the commitment of bone marrow-derived cells to the osteoblast lineage, and this signal inhibits adipogenic and chondrogenic differentiation^[17]. Molecules, such as low-density lipoprotein receptor-related protein 5 (*LRP5*), frizzled (*FZD*), adenomatous polyposis (*APC*), and GSK-3 β , are involved in WNT signaling^[17].

The endodermal differentiation of human embryonic stem cells is regulated through interactions between integrin receptors and extracellular matrix proteins^[18]. In addition to growth factors, such as FGF, TGF- β , and WNT, extracellular matrix proteins, such as fibronectin (*FN*) and vitronectin (*VTN*), are involved in the differentiation of the embryonic stem cell population^[18]. The combination of extracellular matrix proteins and integrins might affect the phenotype of stem cells during development.

It has recently been shown that fatty acid synthase (*Fasn*) is involved in the proliferative activities of adult neural stem and progenitor cells^[19]. *Fasn* is a key enzyme involved in *de novo* lipogenesis, and the deletion of *Fasn* in mouse neural stem and progenitor cells impairs adult neurogenesis^[19]. A recent study has revealed that Notch inhibition induces cochlear hair cell regeneration^[20].

MOLECULES AND GENES INVOLVED IN STEM AND IPS CELL DEVELOPMENT

Molecules and genes involved in stem cell development

Various sources of autologous stem cells are used for the application of cell-based therapies. Mesenchymal stem cells (MSCs) differentiate into various cells, such as osteogenic cells, adipocytes, and chondrocytes. The markers

commonly expressed in human adult MSCs include alanyl (membrane) aminopeptidase (ANPEP, CD13), integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12) (ITGB1, CD29), CD44 molecule (Indian blood group) (CD44), intercellular adhesion molecule 1 (ICAM1, CD54), 5'-nucleotidase, ecto (CD73) (NT5E), Thy-1 cell surface antigen (THY1, CD90), endoglin (ENG, CD105), activated leukocyte cell adhesion molecule (ALCAM, CD166), and HLA-DR^[21]. Proteins, such as CD34 molecule or vascular cell adhesion molecule 1 (VCAM1, CD106), are either positive or negative markers in MSCs depending on the conditions^[21,22]. The combination patterns of these molecules might be different in each MSC phenotype, reflecting the cell source or developmental stage. The gene expression profile and differentiation or proliferation capacity is altered at each stage of MSC culture^[23]. The gene expression of EPH receptor A5 (*EPHA5*) and nephroblastoma overexpressed gene (*NOV*) is upregulated, whereas the expression of necdin homolog (mouse) (*NDN*) and runt-related transcription factor 2 (*RUNX2*) is downregulated during late-stage MSC culture compared with early-stage culture^[23]. The MSCs in different environments might exhibit unique phenotype, as demonstrated through the capacity of these cells for differentiation or proliferation.

The leucine-rich repeat-containing G-protein-coupled receptor 5 (*Lgr5*) is a Wnt target gene and a stem cell marker for the small intestine, colon, stomach, and hair follicles^[24]. The regeneration of *Lgr5*⁺ liver stem cells is mediated through Wnt signaling^[24]. *LGR5* might become a good marker for self-renewing stem cells in combination with Wnt signaling pathway genes.

High-throughput screening of small molecules that inhibit human pluripotent stem cells has identified 15 compounds that specifically inhibit stem cell development^[25]. This approach contributes to the safe application of stem cells in cell therapy, as undifferentiated cells have a risk of forming tumors, and these molecules might play a role in the safety of cell therapy in regenerative medicine.

Genes involved in iPS cell development

Recent advances in technology have been developed to investigate iPS cells. A study showed that iPS cells successfully differentiate into the intermediate mesoderm^[26], and pluripotent stem cells can be transformed into Odd-skipped related 1 (*OSR1*)-expressing intermediate mesoderm^[26]. These *OSR1*-positive intermediate mesoderm cells form nephrogenic tissue cells^[26]. Marker genes are typically used to detect stem cell properties for the characterization of pluripotent stem cells^[27]. Alkaline phosphatase (AP), Pou5f1 (Oct4), Nanog, Sox2, Tra-1-60, Tra-1-81, SSEA3, and SSEA4 are markers for the undifferentiated state of human embryonic stem cells^[27].

iPS cells are reprogrammed through four factors [Pou5f1 (Oct3/4), Sox2, Klf4, Myc (c-Myc)]^[28-30]. A recent study has shown that the absence of *Cdkn1b* (*p27*) enables cellular reprogramming through only two factors, *Oct4* and *Klf4*^[31]. Considering that CDKN1B is a tumor

suppressor, and that the absence of *Cdkn1b* increases *Sox2* expression, reprogramming might be associated with proliferation, and the presence of factors associated with cancer progression or the absence of factors associated with cancer repression are important for inducing the reprogramming of iPS cells. Recent studies have revealed that the AMP-activated protein kinase (AMPK) agonist metformin (Imidodicarbonimidic diamide, *N,N*-dimethyl-) regulates the expression of cancer stem cell-specific genes and inhibits the tumorigenicity of iPS cells^[32]. This effect is mediated through the metformin-induced death of cancer stem cells^[33,34].

An association between threonine and S-adenosyl-methionine metabolism has been demonstrated in pluripotent stem cells, resulting in the regulation of histone H3 lysine 4 trimethylation^[35]. Upon the epithelial differentiation of iPS cells from fibroblasts obtained from ectodermal dysplasia-related patients with mutations in the DNA-binding domain of the *p63* gene, APR-246 (*PRIMA-1^{MET}*), a small compound that restores the function of mutant P53 in human tumor cells, rescues the differentiation potential of these cells^[36,37]. Recent studies using patient-specific iPS cells have shown that the induction of adult-like metabolic energetics from peroxisome proliferator-activated receptor gamma (*PPAR-γ*) activation is involved in the pathogenesis of arrhythmogenic right ventricular dysplasia/cardiomyopathy^[38]. In this model, gene mutations in plakophilin-2 (*PKP2*) are important factors for the regulation of plakoglobin nuclear translocation and β -catenin activity^[38]. A mathematical model for tumor evolution has revealed that somatic mutations in the cancers of self-renewing tissues exist before tumor initiation, which might provide an interesting interpretation of the relationship between stem cells and cancer initiation^[39].

MicroRNA involvement in cell development

It has been recently shown that microRNAs play important roles in cell development. For example, the correlation between microRNA expression profiles and cancer survival has been investigated^[40]. The involvement of the miR-200/ZEB2 pathway in Oct4/Sox2-induced mesenchymal-to-epithelial transition and iPS cell generation has also been shown^[41].

MOLECULES AND GENES INVOLVED IN CANCER DEVELOPMENT

Genes involved in cancer development

MDM2 oncogene, E3 ubiquitin protein ligase (MDM2) and Mdm4 p53 binding protein homolog (mouse) (MDM4) (MDMX) proteins are deregulated in human cancers and induce cancer progression through inhibition of the tumor suppressor p53 (encoded by *TP53*)^[42]. The *Mdm2*^{-/-} or *Mdmx*^{-/-} mice are embryonically lethal, whereas conditional knockout mice exhibit apoptosis in the central nervous system, intestine, or heart. MDM2 overexpression induces carcinoma; however, the expres-

sion of MDM2 does not accelerate the onset of cancer progression in transformation-related protein 53 (*Trp53*)-null mice^[42]. The combination of MDM2, MDMX, and p53 signaling is one of the important pathways for cancer development. Wnt and β -catenin signaling is activated in cancer cells^[43]. EMT and Hedgehog signaling is also important for cancer cell phenotypes, such as differentiation states or malignancies^[44-46]. Notch signaling is also involved in cancer, and Notch signaling genes have been associated with cancer patient survival rates^[47]. Cyclin plays important role in cell proliferation and cancer development^[48-52], and cyclin D1 expression might also be involved in cell growth in gastric cancer^[53]. The reduced expression of claudin in breast cancer mediates the development of cyclin D1^{low}/ID1^{high} tumors^[54]. Peroxisome proliferator-activated receptor (PPAR) also plays an important role in cancer^[55]. Thus, the origin of cancer from stem cells suggests the importance of investigating molecules expressed in stem cells in cancer.

Genes involved in cancer stem cells

Several studies have been conducted to identify cancer-associated genes, and evaluations of sets of cancer-associated genes have revealed a variety of biological functions for these genes^[56]. Cancer genes have been analyzed from several viewpoints, such as common Pfam domain analysis in Cancer Gene Census, which demonstrated that the protein kinase domain is the most common cancer gene-encoding Pfam domain (<http://www.sanger.ac.uk/genetics/CGP/Census/analysis.shtml>). This annotation scheme and the cancer gene assessment project showed that transcription factors and regulators are the largest functional classes^[56]. Many genes involved in stem cell development are classified as transcription factors, and the genes activated in cancer stem cells might be included in these Cancer Gene Census analyses. Many studies have been conducted to reveal the origin of cancer cells^[57]. It has been shown that SmoM2, KRas, or p53 is involved in epithelial skin cancer^[57]. Intestinal stem cells expressing Bmi1 stabilize β -catenin expression and form intestinal adenomas, whereas *APC* deletion in transit-amplifying cells induces microadenomas^[57]. Cancer stem cell markers include CD44, EpCAM, EZ112, Notch-1, Nanog, and Oct4^[58]. Recent studies have shown that the dual expression of Lgr5 and Dclk1 is observed in cancer stem cells^[59,60]. The Jun dimerization protein 2 (*Jdp2*) regulates the *Trp53* promoter and negatively regulates *Trp53* expression^[61]. As previously demonstrated, the loss of *Trp53* promotes tumorigenesis, and common insertion sites were identified in *Trp53*^{+/+}, *Trp53*^{+/-}, and *Trp53*^{-/-} mice^[61]. Tumors retaining a wild-type copy of *Trp53* exhibit common insertion sites in several genes, including *Jdp2*^[61]. Tumor growth is not always defined through the expression of a single common cancer stem cell marker, highlighting the importance of gene combinations in clarifying cancer stem cell phenotypes. These insights were derived from a study indicating that cells do not express previously

identified CSC markers in each cancer (CD34 for acute myeloid leukemia, CD44 for breast cancer, CD133 for glioblastoma and colon cancer, CD271 for melanoma) and have the capacity for tumorigenesis^[62].

The epithelial-mesenchymal transition (EMT) is one of the important factors in cancer metastasis^[63]. Recent studies have shown a close association between the origin of cancer stem cells and EMT^[64,65]. The induction of EMT through Twist, Snail, and TGF- β exhibits a similar phenotype as cancer stem cells, and EMT and cancer stem cell signatures share the same cell population in nonresponders^[66]. Factors promoting EMT in complex with Smad, include Snail1, Zeb1, Zeb2, Lef1/TCF, β -catenin, AP-1, SP1, and HMGA2^[66]. The homeobox factor *PRRX1* is an EMT inducer and a biomarker associated with patient survival and the lack of metastasis^[67]. The upregulation of *Prrx1* is required for the initiation of metastasis, whereas the downregulation of *Prrx1* is important for secondary tumor colonization^[67]. Another study indicated that the substitution of Twist1 is essential for tumor metastases^[68]. Twist1 expression induces EMT, and the downregulation of Twist1 might be important for secondary tumor formation by circulating cells^[68]. Twist signaling associates EMT with stemness^[69-71]. Twist1 interacts with Bmi1, a member of polycomb-repressive complex 1 (PRC1), which maintains chromatin silencing^[72,73]. *BMI1* is involved in the self-renewal of neuronal, hematopoietic, and intestinal cells through the repression of the *INK4A-ARF* locus^[72,73]. During EMT, various networks are regulated, including the interaction of SNAI with the *CDH1* promoter (which encodes E-cadherin)^[74]. SNAI2, ZEB1 and ZEB2, E47, KLF8, and Brachyury are involved in EMT, which represses E-cadherin and other junction proteins, such as claudins and desmosomes^[74]. Moreover, TWIST1, forkhead box protein C2 (FOXC2), goosecoid, E2-2 (TCF4), homeobox protein SLX1, and paired mesoderm homeobox protein 1 (PRRX1) are also involved in EMT, characterized by reduced E-cadherin expression and increased vimentin expression^[74].

CONCLUSION

In summary, gene combinations are useful for distinguishing cell populations or evaluating cellular characteristics. The cellular phenotype is regulated through genes and epigenetic modifications. A deep understanding of cellular features is important for the safe medicinal application of cells, including pluripotent stem cells. A panel of gene combinations to evaluate and predict precise cell phenotypes is essential for the future development of the cell field. Thus, systems or databases to provide the context of the panel of gene combinations might be of great value for the appropriate assessment of these cells.

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Fifteen years of preclinical and clinical experiences about biotherapy treatment of lesions induced by accidental irradiation and radiotherapy

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Abstract

High dose radiation exposures involving medical treatments or accidental irradiation may lead to extended damage to the irradiated tissue. Alleviation or even eradication of irradiation induced adverse events is therefore crucial. Because developments in cell therapy have brought some hope for the treatment of tissues damages induced by irradiation, the Institute for Radiation and Nuclear Safety contributed to establish the clinical guidelines for the management of accidentally irradiated victims and to provide the best supportive care to patients all over the world. In the past 15 years, we contributed to develop and test cell therapy for protection against radiation side effects in several animal



Biography

Alain Chapel, PhD, scientific investigator at Institute of Nuclear Safety and Radioprotection (IRSN), is team leader in Laboratory of Radiopathologie and Experimental therapies. Since 20 years, he developed gene and cell therapy gene of non-human primate, immune-tolerant mice and rats to protect against side effects of radiation. He has developed representative experimental models of SAI to investigate the effect of radiation on both the radiosensitive hematopoietic cells and their bone marrow microenvironment. He collaborates with clinicians to develop new strategies for treatment of patients after radiation accidents or radiotherapy overexposures. In compassionate trials, he has participated to the first to establishment a proof of concept of therapeutic efficacy of mesenchymal stem cells (MSCs) for the treatment of hematopoietic deficit, radiodermatitis and the over dosages of radiotherapy. In collaboration with Saint-Antoine Hospital (Paris, France), he has contributed to the first reported correction of deficient hematopoiesis in patients (graft failure and Aplastic Anemia) thanks to the intravenous injection of MSCs which restored bone marrow micro-environment, mandatory to sustain hematopoiesis after total body irradiation. Currently his work focuses on the development of radio-induced bone marrow aplasia using human hematopoietic stem cell derived from human IPS. He is member of various learned national and international societies: European Bone Marrow Transplantation Group (EBMT), American Society for Hematology; International Society of Stem Cell Research, member of Société Française de Greffe moelle et de thérapie cellulaire. He is associate editor of 5 international reviews: *World Journal of Stem Cells*, *World Journal of Gastrointestinal Surgery*, *World Journal of Radiology*, *The Open Gene Therapy Journal*, *Journal of Clinical Rehabilitative Tissue Engineering Research*. He has participated to scientific organization of international conference of the European group for Blood and Marrow Transplantation, EBMT Paris 2011.

models, and we proposed mechanisms to explain the benefit brought by this new therapeutic approach. We established the proof of concept that mesenchymal stem cells (MSCs) migrate to damaged tissues in the nonobese diabetic/severe combined immunodeficiency immunotolerant mice model and in non-human primate after radiation exposure. We showed that the intravenous injection of MSCs sustains hematopoiesis after total body irradiation, improves wound healing after radiodermatitis and protects gut function from irradiation damages. Thanks to a tight collaboration with clinicians from several French hospitals, we report successful treatments of therapeutic/accidental radiation damages in several victims with MSC infusions for hematopoiesis correction, radio-induced burns, gastrointestinal disorders and protection homeostatic functions of gut management after radio-therapy.

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Key words: Mesenchymal stem cells; Radiotherapy; Cell therapy; Stem cells; Healthy tissue; Tumor; Radiation

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INTRODUCTION

Radiation therapy, the primary treatment of many cancers, induces lesions to the healthy tissues on the short and long term. About 1.5 million patients undergo external radiotherapy each year in Europe. Acute adverse effects are present in 80% of them including late adverse effects in 5%-10%. About 90000 patients a year receive abdominal/pelvic radiation therapy. Five percent to 10% of patients develop late side effects, the more severe pathologies being hemorrhages, fistula, and fibro-necrosis, all recognized as “pelvic radiation diseases”. Infrequently, as in the Epinal accident (with Recto-vesical fistulas) in 2005 these complications can lead to death. Alleviation or even eradication of radiation induced adverse events is therefore crucial. Accidental radiation exposure, such as seen during the last events at Fukushima in 2011, reminded and emphasized that the “zero risk” level in nuclear industries does not exist, and that research and development of new therapies should be absolutely reinforced. Novel therapies are needed to answer the major risk of radiation crises, in part by proposing efficient medical counter measures in cases of external exposure typical for a major nuclear accident^[1,2].

PREVENTION AND TREATMENT OF IRRADIATION VICTIMS

The Institute for Radiation and Nuclear Safety is the

French National Agency responsible for prevention and treatment of radiation victims. Institute manages a reference network to support and treat patients with radiation induced lesions resulting from radiation therapy or accidental radiation exposure. This platform is based on the development of innovative clinical protocols using mesenchymal stem cells (MSCs) from human bone marrow. It will also explore other sources of stem cells such as pluripotent adult stem cells (IPS) to develop and offer new protocols.

Research and clinical platform

This research and clinical platform is a network composed of different research groups to allow for a multidisciplinary approach. These research groups include research teams from the University Pierre and Marie Curie and radiobiology experts collaborating with the UPMC (IRSN/Department of Human Radioprotection-DRPH), as well as clinical research teams at Saint Antoine Hospital (Department of Clinical Hematology) and Henri Mondor Hospital [Parisian Section of the French Institute of Blood (EFS IdF), Department of Cell Therapy] part of the Parisian Health and Hospital Network (APHP).

This network gathers complementary expertise for the biotherapeutic treatment of radiation therapy side effects and accidental radiation exposure. This therapeutic platform for irradiated patients handles upstream investigations to clinical protocol trials and benefits from the following competences: (1) fundamental research on pluri- and multi-potent cells; and (2) research and development: production of innovative cell therapy products, and R and D of cell therapy products and the creation of a stem cell (IPS) bank for grafting purposes; preclinical animal trials of therapeutic efficiency and study of tissue lesions repair mechanisms following stem cell transplantations, support, trials and intervention protocols, renowned for its expertise in the treatment of overdosed radiation therapy patients, *i.e.*, Epinal cases) and Blood Center Transfusion of Army (CTSA, Percy Hospital, Clamart, France) renowned for its expertise in the treatment of radiodermatitis. Since this partnership has been established several accidents have occurred, in Belgium, Chile, Peru, Japan and also in France at Epinal where a first cohort of 22 patients with prostate cancer has received a dose of irradiation 20% higher on irradiation fields 20% larger than initially planned^[3,4].

Preclinical treatment of radio-induced damages

We have proposed innovative cell therapies for treatment of patients. We have developed, tested and proven that using of cell therapy allows the regenerate damaged tissue after radiation therapy. We established that MSCs migrate to damaged tissues in immunotolerant mice model and in non-human primates after radiation exposure^[5-8]. In immunotolerant mice, we showed that the intravenous injection of MSCs sustains hematopoiesis after total body irradiation^[6], improves wound healing after radio

dermatitis^[9,10] and protects gut function^[11]. MSCs restore gut functions after radiation, through regulation of endogenous epithelial cell homeostasis^[12]. We showed that MSC treatment increases and accelerates the recovery of the small intestine with reversible alterations and extends the life of animals developing irreversible gastrointestinal alterations. Histopathological evaluations provided initial insight into the cellular targets of therapy. MSCs effects are a consequence of their ability to enhance or maintain the re-epithelization process of small intestinal epithelium. To our knowledge, this is the first demonstration that MSC therapeutic effects on small intestinal damage lead to the re-establishment of cellular homeostasis by both increasing endogenous proliferation processes and inhibiting apoptosis of radiation induced intestinal epithelial cells. Furthermore, we demonstrate that MSCs have distant sustained effects^[13]. We found that the MSCs regenerated the small intestinal epithelium, which in turn restored the enterohepatic recirculation pathway initially damaged by irradiation. The consequence was a sustained hepatic protection without engraftment of MSCs in liver. Another mechanism that should be considered is the role of cytokines and growth factors released by the MSCs that are homing to other organs, the paracrine biofactors in MSCs-mediated enhanced wound healing. We previously reported that MSCs act mainly by immunomodulation mechanisms^[14-19]. Cell therapy combining different sources of adult stem cells is under investigation and is being tested in preclinical models of radio induced damage^[20,21]. In parallel, we started analyzing potential side effects of MSCs injections^[22].

TREATMENTS OF THERAPEUTIC/ACCIDENTAL RADIATION DAMAGES

Thanks to a tight collaboration with clinicians, to the best of our knowledge our group is the first to report successful treatments of therapeutic/accidental radiation damages in several victims with MSCs injections in: (1) radio-induced burns: cutaneous reactions are major actors in radiation accidents and a limitation for radiotherapy. In collaboration with Percy hospital (Clamart, France), we have shown for the first time the efficiency of MSCs therapy in seven patients with acute cutaneous and muscle damages following accidental irradiation delivered at doses and to fields higher than initially planned^[23]; (2) gastrointestinal disorder management: we are the first to treat patients over irradiated in Epinal with infusion of MSCs, following a specific mission from the French ministry of health. In 2008, three overdosed Epinal patients presenting serious intestinal radiation induced lesions, compassionately received MSCs treatment. For all three patients, the systematic administration of MSCs was well tolerated; efficient analgesic and anti-inflammatory effects as well as hemorrhage reduction were observed. A fourth patient was successfully treated in 2012^[24]. Compassionate trials demonstrated the feasibility of cell therapy by

MSCs for patients overdosed during radiation therapy of prostate cancer in Epinal Medical Center. A new protocol will be performed in 2013 for the treatment of late severe damages of abdominal radiotherapy. Furthermore, in collaboration with APHP and UPMC, the IRSN is currently participating in a surveillance protocol of a cohort of patients overdosed during radiation therapy for prostate cancer at Epinal Medical Center; and (3) hematopoiesis correction: in collaboration with Saint-Antoine Hospital (Paris, France), we are the first to report the hematopoiesis recovery in two patients with Bone Marrow failure (graft failure post grafting and Aplastic Anemia) after intravenous injection of MSCs which restored the BM micro-environment, mandatory to sustain hematopoiesis after total body irradiation^[15,25]. In case of severe accidental radiation exposure, the primary life-threatening symptom that can occur is medullar aplasia. The field of stem cell research has been profoundly impacted for the long term by the recent technology of adult cells re-programming. UMRS-938 and IRSN are developing an alternative, innovative therapy to treat this hematologic syndrome by revisiting allogeneic transplantation, thus far inconceivable for accidentally irradiated individuals. The innovation is to generate stem cells from IPS originating from healthy, extra-hematopoietic tissues preserved from the radiations to restore a functional hematopoiesis in these patients.

CONCLUSION

Radiotherapy is associated with a high incidence of undesirable acute and/or chronic complications that can affect the patient's quality of life and/or may be life threatening. The lack of curative treatment at present and the potential severity for the disorder highlight the importance of novel and effective therapeutic strategies after radiation exposure. Stem cells can be used to treat toxic side effects induced by irradiation on healthy tissue. As demonstrated in a preclinical model, MSCs may offer a novel strategy to treat radiation diseases. There is interest in using these adult stem cells in critical illness, however, the safety profile of these cells is not well known. Lessons from clinical trials must be taken into account; since the first reported trial in 1995, cultured MSCs have been used in 125 registered clinical trials. In the past 15 years, we contributed to develop and test cell therapy for protection against radiation side effects in several animal models. We report successful treatments of therapeutic/accidental radiation damages in several victims with MSCs infusions for hematopoiesis correction, radio-induced burns and gastrointestinal disorder management after radio-therapy. Concerning gastrointestinal disorder, new protocol will be proposed for the treatment of late severe damages of abdominal radiotherapy. With regard the hematopoiesis, we will generate stem cells from IPS originating from healthy extra-hematopoietic tissues to restore a functional hematopoiesis in patients with acute hematopoietic syndrome.

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Epigenetics and chromatin plasticity in embryonic stem cells

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Abstract

The study of embryonic stem cells is in the spotlight in many laboratories that study the structure and function of chromatin and epigenetic processes. The key properties of embryonic stem cells are their capacity for self-renewal and their pluripotency. Pluripotent stem cells are able to differentiate into the cells of all three germ layers, and because of this property they represent a promising therapeutic tool in the treatment of diseases such as Parkinson's disease and diabetes, or in the healing of lesions after heart attack. As the basic nuclear unit, chromatin is responsible for the regulation of the functional status of cells, including pluripotency and differentiation. Therefore, in this review we discuss the functional changes in chromatin during differentiation and the correlation between epigenetics events and the differentiation potential of embryonic stem cells. In particular we focus on post-translational histone modification,

DNA methylation and the heterochromatin protein HP1 and its unique function in mouse and human embryonic stem cells.

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Key words: Chromatin; Epigenetics; Embryonic stem cells; Nucleus; Pluripotency; Differentiation

Core tip: Here, we provided a summary on epigenetics and chromatin structure in pluripotent embryonic stem cells (ESCs) and their differentiated counterpart. We especially aim at histone signature, function of heterochromatin protein 1. Moreover, we summarized published data on nuclear architecture; we especially addressed arrangement of chromosome territories and genes in pluripotent ESCs and after induced differentiation.

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INTRODUCTION

Biological properties of embryonic stem cells

Human embryonic stem cells (hESCs) were first isolated by the American biologist Thomson *et al*^[1]. Revolutionary breakthrough in stem cell biology represents the first isolation of induced pluripotent stem cells in 2006-2007^[2-5]. Due to their unique properties, stem cells have become the subject of research for many teams that work on the treatment of several serious diseases. Human and mouse ESCs can be grown *in vitro* as clearly visible colonies (Figure 1A-D) and effective methods have been developed for the generation of cardiomyocytes (Figure 1E), hepatocytes, melanocytes, osteoblasts, pancreatic β -cells, or

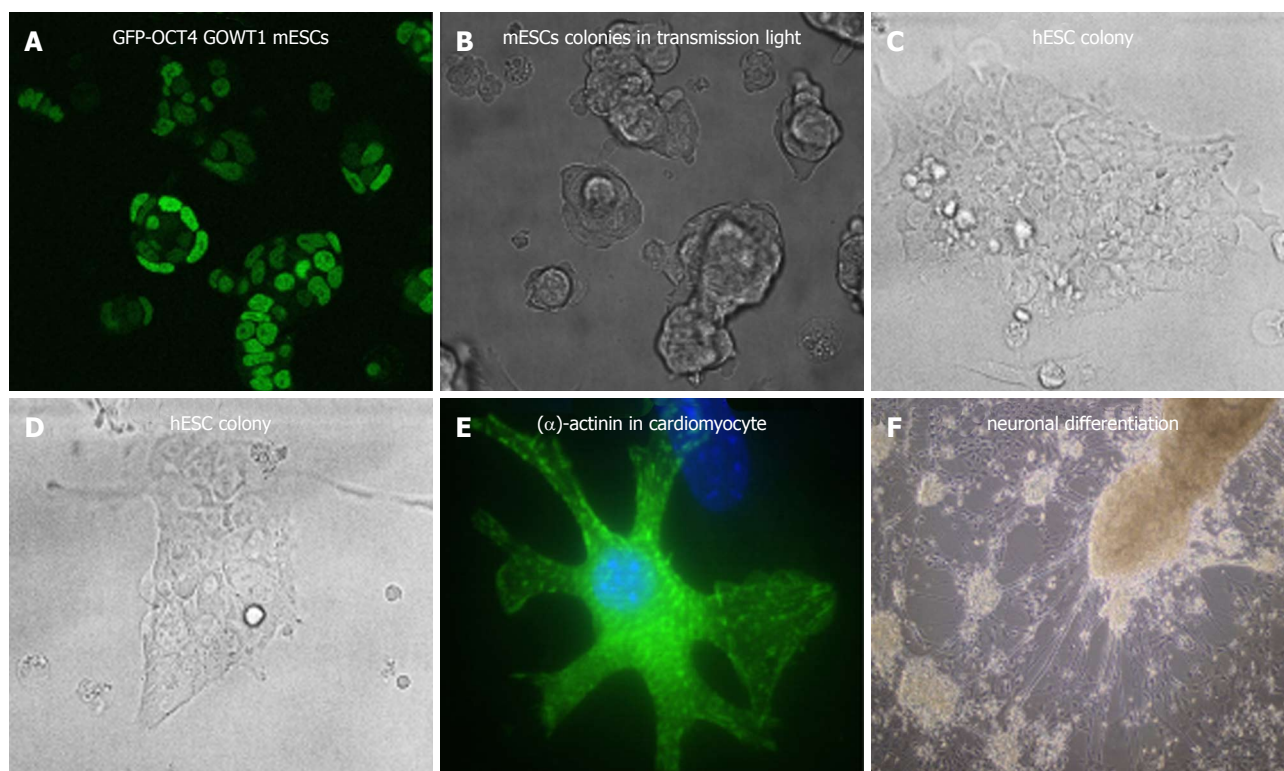


Figure 1 Morphology of a mouse embryonic stem cells colonies. A, B: Confocal microscopy of mouse embryonic stem cells (mESC) colonies (line GOWT1) stably expressing GFP-OCT4 was performed using a Leica SPX-5 microscope. GFP fluorescence is shown as green and the cell colonies were additionally visualized in transmission light (gray); C, D: Morphology of human embryonic stem cells (hESC) colonies studied by transmission light; E, F: mESCs (line D3) were differentiated into cardiomyocytes (gray) (E) according to report of Veselá *et al.*^[113] and α -actinin morphology (green) was studied using the appropriate antibody. Nuclei were visualized by DAPI staining (blue). Cardiomyocytes were characterized by their specific strip-like morphology of α -actinin. Morphology of mouse ESCs differentiated into neuronal pathway^[114] is also showed (F).

neural cells (Figure 1F) from ESCs. Induction of pluripotency in terminally differentiated cells is also considered a key tool for regenerative medicine. Thus, the discovery of induced pluripotent stem cells (iPS) has great potential for the treatment of degenerative diseases without the problems associated with immunogenicity and ethical issues related to the isolation of hESCs from human embryos.

Self-renewal and pluripotency of ESCs

An important characteristic associated with the self-renewal and pluripotency of ESCs is their almost unlimited replication potential, which allows constant proliferation. This is ensured by activation of telomerase, the enzyme that prevents telomere shortening during cell division^[1]. The self-renewal of ESCs is linked to their pluripotency and can be manifested as symmetric or asymmetric cell division. Symmetric division generates two identical sister cells whereas during asymmetric division one cell preserves the original phenotype of the mother cell and the second cell acquires a new phenotype. Asymmetric division is the basic mechanism for maintaining cell diversity, but both types of cell division ensure that physiological and morphological properties of the parental cells are transmitted to the next cell generation^[6]. In this regard, it is important to understand the extent to which the nu-

clear pattern, especially that of chromatin is transmitted through mitosis. This issue has been addressed by several authors, although mostly in somatic cells^[7-12].

In addition to self-renewal, a major feature of ESCs is their pluripotency. The single cell (zygote) formed after fertilization becomes totipotent and can produce all differentiated cells of an organism, including cells of extra-embryonic tissues. This extremely favorable feature is lost after division into the 8-cell embryonic stage^[13]. However, ESCs in the inner cell mass of the blastocyst become pluripotent, and retain the potential to differentiate into cells of all three germ layers: endoderm (cells of the gastrointestinal tract and lungs), mesoderm (muscle cells, bone, blood), and ectoderm (epidermal tissues and nervous system cells) (summarized by Yamanaka and Ralston^[14]). *In vivo* evidence of this differentiation potential is provided by injection of ESCs into immuno-deficient mice, which leads to the generation of teratocarcinomas formed by cells from all three germ layers. Moreover, *in vitro* evidence of ESC differentiation potential is provided by the formation of highly specific embryonic bodies (EBs)^[15].

Transcription factors responsible for pluripotency

The greatest progress in understanding the pluripotency of ESCs came with the identification of three key transcription factors: Oct4, Sox2, and Nanog^[14,16]. These fac-

tors have been shown to be essential for pluripotency in both *in vivo* and *in vitro* conditions^[17].

Several groups of scientists have used the method of immunoprecipitation (IP) to identify the target genes of these transcription factors in mouse and human ESCs. Their data showed that transcription of many genes was regulated by the combination of these three transcription factors in hESCs^[16] and that expression of Oct4 and Nanog is fundamental for the pluripotency of mouse embryonic stem cells (mESCs)^[18]. Furthermore, these studies also showed that Oct4 and Nanog function as mutual regulators and self-regulators. The effect of these transcription factors on target genes seems to be significantly different between mouse and human ESCs. Nonetheless, Oct4, Sox2, and Nanog are general factors required for the maintenance of pluripotency in both organisms and other proteins probably cooperate with them to control the expression of individual genes.

The central regulator of pluripotency is Oct4, which belongs to the Pit-Oct-Unc (POU) protein family^[17]. Oct4 prevents spontaneous differentiation of ESCs and artificial suppression of its expression results in the differentiation of cells into trophoectoderm^[19] due to increased activity of caudal-type homeobox transcription factor 2. Moreover, in some circumstances markedly increased expression of Oct4 can also lead to differentiation, which is the main reason why negative regulators of Oct4 expression are required. For example, liver receptor homolog is believed to be a positive regulator of Oct4, whereas germ cell nuclear factor is a potential negative regulator^[20].

The pluripotency regulator Sox2 is expressed in the inner cell mass of blastocysts as well as in early mesoderm^[21]. In mESCs, increased expression of Sox2 induces neural differentiation and subsequent cell death. Repression of Sox2 leads to differentiation into trophoectoderm in mESCs, and in hESCs induces differentiation towards an endoderm-like pathway. However, although the above role was established in several experimental models, the main function of Sox2 remains co-operation with Oct4 to activate related target genes^[22].

The other gene involved in safeguarding the pluripotent state is Nanog, which belongs to the class of NK-2 transcription factors. In mESCs, increased expression of the Nanog gene helps to maintain pluripotency even in the absence of leukemia inhibitory factor (LIF). However, hESCs can be cultured on a mouse embryonic fibroblast feeder layer, even in the presence of a high level of Nanog. If Nanog is not expressed, ESC morphology is markedly similar to that of cells induced by factor Gata6, which are characterized by the morphology of primitive endoderm^[23]. Although stimulation of Nanog expression inhibits cell differentiation into endoderm, there is no direct evidence that Gata6 activity is inhibited by Nanog^[24]. Nanog also inhibits neural cell differentiation induced by removal of LIF and BMP factor from the culture^[25] and exerts an inhibitory effect on mesodermal differentiation. In conclusion, Nanog is able to inhibit endodermal,

neural, and mesodermal differentiation under different cultivation conditions (summarized by Yamanaka and Ralston^[14]).

Polycomb group proteins and their function in ESCs

Polycomb group proteins (PcGs) were first identified in *Drosophila melanogaster* as molecules that selectively inhibit the expression of many regulatory genes during embryonic development. Polycomb proteins form complexes with gene-suppressor activity. The mechanism of PcG function is based on gene silencing mediated by chromatin modifications, including tri-methylation of histone H3 at the position of lysine 27 (H3K27me3). These changes can help to maintain the undifferentiated state of stem cells^[26,27]. Despite the high transcriptional activity and high degree of chromatin relaxation in ESCs, non-differentiated hESCs contain the low level of repressive chromatin marker H3K27me3. During hESC differentiation there is pronounced accumulation of H3K27me3, mostly in the inactive X chromosome^[28]. PcG proteins form two main, but distinct complexes, polycomb repressive complex (PRC)1 and PRC2. Human cells contain an additional PRC3 complex, consisting of EED, EZH2, SUZ12, and RBBP subunits^[29].

The PRC1 complex contains several subunits, including HPH, RING, CBX, MEL18 and BMI1. This complex is responsible for maintaining the stability of chromatin, repressed by PcG proteins. Specific factor from the PRC1 complex is also responsible for monoubiquitination of histone H2AK119^[30]. The second complex, PRC2, is composed of SUZ12, EED, EZH2, and RbAP48 and is essential for the initiation of gene silencing. EZH2 functions as a histone methyltransferase that mediates H3K27me3, which acts as a binding landscape for the PRC1 protein complex. PRC2 also plays an important role in the inactivation of the X chromosome, which represents a form of facultative heterochromatin^[27]. Chromatin that is modified in this way looks like dense clusters of nucleosomes and this chromatin arrangement is associated with transcriptional repression.

PcG proteins have repressive effects on the expression of many genes, including homeobox (Hox) genes that represent regulatory elements responsible for morphological changes during embryogenesis. PcG proteins maintain repression of these genes as a result of chromatin remodeling and subsequent prevention of transcription factor binding. With regard to nuclear distribution, proteins forming PcG bodies are mainly accumulated in proximity to clusters of pericentric heterochromatin. Moreover, some authors showed on ultrastructural levels PcG bodies located in the nuclear interchromatin regions. Recently, PcG bodies were revealed as as distinct and locally accumulated nuclear domains that are enriched in separated heterochromatin regions^[31].

EPIGENETICS OF ESCS

In the first half of the twentieth century, the scientific fields of developmental biology and genetics were still

considered to be strictly separate. However, the British professor of genetics Conrad Hal Waddington considered this artificial division needless. In 1942, he introduced the concept of epigenetics, representing a combination of these two disciplines that can be summed up by the CH Waddington equation: epigenesis + genetics = epigenetics^[32]. In those days it was still just speculation that each gene affects a number of different processes. It is now well known that genes cooperate to form a specific network and a few decades later epigenetic mechanisms are being widely discussed in the context of DNA and histone modifications.

Nowadays, the term epigenetics represents a science that deals with reversible changes in chromatin structure that are not caused by a change in the nucleotide sequence^[33]. Nowadays, an increasing incidence of serious human diseases is considered one of the biggest health problems and it is assumed that epigenetic changes in the human genome are responsible for their pathophysiological states. Thus, practical application of knowledge on the epigenetics and physiology of ESCs has potential implications for further progress in medicine.

Specific histone signatures in ESCs

Epigenetic modifications of histones can be described as biochemical marks within histones that result in changes to chromatin conformation and thus affect chromatin accessibility to transcription factors.

The N-terminal domains of core histones are susceptible to many post-translational modifications, including acetylation (Ac), methylation (Me), phosphorylation (Ph), ubiquitination (Ub), or SUMOylation (su)^[34,35]. Because histones contain more than 60 residues that can be modified, this translates into countless variations of epigenetic modification. Histones are highly evolutionary conserved, but it is possible to establish general rules for each modification that together form so-called histone code or histone signature^[36,37]. In general, histone post-translational modifications fall into two categories, those that support transcription and those that inhibit transcription^[38]. Typical examples of modifications, characterized for transcriptionally active chromatin, are DNA hypomethylation, histones H3 and H4 acetylation, and methylation of H3 on Lys 4 (H3K4), H3K36, and H3K79. However, it depends which genomic regions (promoters, coding regions or enhancers) are enriched in these histone marks. Moreover, combination of specific histone marks can dictate gene transcription activity/inactivity. For repressive chromatin state is characterized hypoacetylation of H3 and H4, and methylation of H3 on Lys 9 (H3K9), H4K20, H3K27, or H4K20^[39]. However, whether a given modification is gene activating or repressing may also dictate co-interacting factors. Ribosomal genes represent such an example, which transcriptional activity is regulated by ATP-dependent nucleosome remodeling events. In this case repressive H3K9me2, mediated by G9a histone methyltransferase, in a complex with other factors, including Cockayne syndrome protein B and transcription

factor TTF-I, promotes transcription elongation^[40].

One well-characterized post-translational modification of histones is acetylation of lysine residues, which is catalyzed by histone acetyltransferases (HATs). Histone acetyltransferases always transfer an acetyl group from acetyl-CoA to histones, thereby neutralizing their positive charge^[37]. The result is a reduced ability of DNA to bind to histones, which leads to chromatin relaxation and subsequent transcriptional activation^[35]. The reverse event is deacetylation, in which the acetyl group is removed in the presence of histone deacetylases (HDACs). As this increases the positive charge of histones, the chromatin becomes more condensed and transcription is repressed^[37]. For example, H3K9 deacetylation was observed after induced differentiation of human ESCs^[41], and was accompanied by global condensation of chromatin and restriction of global transcription^[42]. Interestingly, levels of other histone modifications that characterize the active chromatin state, including H3K14ac, H3K4me3, and H3K36me2/me3, are also increased in pluripotent ESCs compared with differentiated neuronal progenitor cells (summarized by Mattout and Meshorer^[43]).

Histone methylation is a covalent modification mediated by highly conserved histone methyltransferases (HMTs). This modification occurs through a biochemical reaction responsible for adding one, two, or three methyl groups to the nitrogen atoms of lysine, arginine, or histidine. The fundamental histone methylation is H3K9me2/me3 which regulates the processes of general heterochromatinization and gene silencing that primarily occur during cell differentiation. Regulation of transcription is based on the formation of specific binding sites in the genome that are attractive to regulatory proteins. In this regard, the interaction of H3K9 methylation and heterochromatin protein 1 (HP1) is particularly important^[44-46].

The opposing process of histone methylation is demethylation which is catalyzed by histone demethylases. The first identified histone demethylase was lysine-specific demethylase 1 (LSD1), described by Shi *et al.*^[47]. This enzyme demethylates histone H3K4 or H3K9 under specific conditions. Interestingly, when LSD1 attacks repressive histone mark H3K9 methylation by its demethylating activity, it leads to activation of androgen receptor target genes^[48]. Conversely, H3K4 demethylation seems to be fundamental for inactivation of enhancer function, which leads to specific gene silencing, according to differentiation demands, especially in ESCs. Whyte *et al.*^[49] showed that LSD1 controls the enhancers of transcriptionally active loci, which is essential for maintaining of ESC pluripotency. Interestingly, ESCs that lack LSD1 activity have no potential to differentiate. Moreover, at active enhancers LSD1 associates with the Nucleosome Remodeling and histone Deacetylase (NuRD) complex and this protein interaction appears to be fundamental for ESC differentiation^[49]. The importance of the NuRD complex in ESC integrity, and some other functional events in ESC nuclei, was also reported by Reynolds *et al.*^[47].

These authors observed that the NuRD complex, which is required for ESC lineage commitment, modulates both transcriptional heterogeneity and the dynamic properties of pluripotency-related genes^[50]. Moreover, HDAC1, HDAC2, and HDAC3, are present in four distinct multi-protein complexes Sin3, NuRD, CoREST, NCoR/SMRT (summarized by Hayakawa and Nakayama^[51]). Thus, NuRD protein complex, consisting of HDAC1/2, is characterized by both histone deacetylase and nucleosome remodeling activity. This complex plays a role in various cellular processes including cell cycle regulation^[52,53], maintenance of stem cell pluripotency^[54], self-renewal, and cellular differentiation (summarized by Hayakawa and Nakayama^[51]). These findings partially clarify our recent data on Oct4 recruitment to locally induced double strand breaks in living ES cells, which occurred simultaneously with HDAC1 recruitment; under specific histone acetylation conditions^[55]. Considering that Nanog and Oct4 interact with various core components in the NuRD complex^[54], these data collectively suggest that especially Oct4 can associate with specific regulatory complexes to control ESC fate.

DNA methylation

The concept of DNA methylation has been recognized since 1948 when the first modified base, 5-methylcytosine, was discovered by Hotchkiss using the method of paper chromatography^[56]. DNA methylation can be divided into two categories, maintenance methylation and *de novo* methylation. *De novo* methylation takes place on previously modified DNA whereas maintenance methylation occurs on a newly established strand of DNA that is methylated according to the pattern of the old strand^[33]. Covalent DNA methylation is a post-replication modification that mainly, but not exclusively, occurs on cytosine residues in CpG dinucleotides. Based on an earlier study, it is thought that 75%-85% of DNA methylation occurs at CpG islands. These CpG dinucleotides are found primarily at the promoters of genes^[57]. DNA methylation is mediated by DNA methyltransferases (Dnmts) that catalyze the transfer of a methyl group within a nucleic acid. During DNA methylation the methyl groups are accumulated in the major groove of DNA, preventing the binding of transcription factors to these genomic regions. An accompanying event related to gene silencing is the binding of the methylation-specific protein MeCP2 to methylated CpG sites resulting in chromatin conformational changes^[33]. The processes of DNA methylation are associated with the function of particular methyltransferases: Dnmt1 and Dnmt2 are among the enzymes responsible for maintenance methylation whereas the only *de novo* methyltransferases are Dnmt3a and Dnmt3b. These two enzymes operate in early embryonic development, between the stages of morula and blastocyst, when the methylation profile is determined. In later development methylation is maintained by Dnmt1^[58].

It is now well recognized that differentiation of ESCs is accompanied by Oct4 down-regulation as a consequence of

de novo DNA methylation of the Oct4 regulatory region. As Athanasiadou *et al.*^[59] showed *de novo* methylation mediated by Dnmt3a and Dnmt3b occurs at two discrete sites: the proximal enhancer and distal promoter of the *Oct4* gene. For efficient complexity, the functional inter-connection of Dnmts is essential for Oct4 transcriptional regulation. Interestingly, even H3K9 methyltransferase G9a can recruit Dnmts to the Oct4 locus and other loci upon ESC differentiation^[60], but generally, CpG methylation throughout most of the regulatory genomic region accumulates even in the absence of G9a^[59]. These data support the notion that histone modifications and DNA methylation are basic transcriptional regulators acting specifically and simultaneously in pluripotency-related genes.

Mapping of 5-hydroxymethylcytosine in embryonic stem cells

DNA methylation is considered as well explored epigenetic phenomenon in ESCs. However, function of 5-hydroxymethylcytosine (5-hmC) in maintaining ESC pluripotency and differentiation is right now a hot topic of ESC biology. 5-hmC is generated by the TET family of Fe(II) and 2-oxoglutarate-dependent enzymes through oxidation of 5-methylcytosine (5-mC). Recent studies of Tahiliani *et al.*^[61] or Kriaucionis and Heintz^[62] revealed a high level of 5-hmC in Purkinje neurons (described by Czech physiologist Jan Evangelista Purkyně) and ESCs. Interestingly, the level of 5-hmC can decrease during differentiation of ESCs, thus functional Tet1 enzyme is required for maintenance of ESC pluripotency^[63]. Genome-wide analyses showed enrichment of 5-hmC at enhancers abundant on H3K4me1 and H3K27ac. Moreover, binding sites of OCT4 and NANOG were also characterized by cytosine hydroxymethylation^[64]. High-throughput sequencing of 5-hmC containing DNA revealed 5-hmC within exons and near transcription start sites enriched in both H3K27me3 and H3K4me3^[65]. Interestingly, these authors suggested a model of how 5-hmC contributes to “poised” chromatin signature. They claimed that similarly as 5-mC, 5-hmC at promoters caused gene down-regulation. However, in ESC differentiation-specific genes, “poised” for transcription, 5-hmC could be fundamental for subsequent gene up-regulation responsible for induction of given differentiation pathway^[65]. Other report showed a novel phenomenon of how 5-hmC can further modulate epigenetic events responsible for ESC pluripotency^[66]. For example, selected enhancers with a high level of 5-hmC were in parallel enriched in H3K4me1, H3K18ac, H4K5ac, H3K27ac in human ESCs. Mentioned experimental approach, and especially genome-wide analyses seems to be a promising tool how to distinguish between epigenetic landscape in pluripotent ESCs and their differentiated counterpart.

HETEROCHROMATIN PROTEIN HP1

HP1 (or chromobox homolog) was first identified in *Drosophila melanogaster* by the American scientists James and

Elgin^[67]. HP1 is a highly conserved non-histone protein that plays an important role in epigenetic regulation^[37,68]. The main function of HP1 is the regulation of gene expression through the binding of protein complexes to heterochromatin, thus maintaining the integrity of heterochromatin. Complex studies have confirmed this role by demonstrating specific interaction of HP1 with other histone and non-histone proteins^[44-46].

Structure and function of HP1

HP1 homologues (HP1 α , HP1 β , HP1 γ) have been identified in almost all eukaryotic organisms from yeast to human. HP1 proteins are the basic units of heterochromatin, including centromeres and telomeres^[37,68,69]. HP1 protein subtypes are characterized by an N-terminal chromodomain (CD) and C-terminal chromoshadow domain (CSD), which are separated by the so-called "hinge region"^[70]. Three-dimensional structures of the CD and CSD were determined by nuclear magnetic resonance spectroscopy^[71] and X-ray crystallography^[72]. Both of these domains represent globules with a diameter of approximately 30 AA. Each domain is composed of anti-parallel three-fiber β -sheets wrapped around one (α_2) or two (α_1 , α_2) α -helices. Conserved non-polar residues represent the backbone of this characteristic fold and form a hydrophobic groove on the β -sheet. This groove is not very accessible in the CSD but is relatively open in the CD, where it provides potential sites for protein-protein interactions^[73].

As mentioned above, binding of HP1 protein to methylated H3K9 is important for the formation of heterochromatin^[69]. The CSD binds proteins that are responsible for chromatin remodeling. The CD interacts with the N-terminal end of H3, therefore HP1 therefore gradually binds to methylated histone H3 leading to an effective increase in the local concentration of HP1. Direction of HP1 to pericentric heterochromatin requires not only H3 methylation, but also histone deacetylation^[73]. Moreover, HP1 protein is not accumulated in facultative heterochromatin, such as the inactivated X chromosome, despite the fact that these chromosome territories contain methylated H3K9, and preferentially H3K27me3 appears in female inactive chromosome X. In addition, mutations in the HP1-associated genomic regions lead to re-location of HP1 from this heterochromatin^[74] despite the fact that this area is rich in H3K9 methylation^[75]. These observations indicate that, in addition to H3 methylation, the interaction of HP1 with other proteins is also important for directing HP1 to specific chromatin domains.

HP1 as a repressor and activator

HP1 was originally characterized as a fundamental component of heterochromatin^[76]. This view has been changed by the observation that HP1 γ exhibits features highly characteristic of euchromatin^[77]. Recent data suggest that the presence of HP1 outside of the constitutive heterochromatin has functional relevance because euchromatin also contains genes whose transcription is

repressed by the presence of HP1 sub-types^[78]. This is in accordance with the fact that HP1 interacts with Krüppel associated box (KRAB) and KRAB domain-associated protein (KAP-1), co-repressors of the KRAB domain and a zinc finger protein^[79]. Considering that KRAB domain proteins represent the largest family of transcription repressors, it is not surprising that HP1 proteins are attracted to areas where gene silencing is expected regardless of whether this region is in heterochromatin or euchromatin^[68].

One explanation for the activating and repressive effects of HP1 is that homo- and hetero-oligomers of each of the three HP1 variants play different roles. Although this depends on the chromatin context, HP1 γ homodimers seem to have an activating function. HP1 α -HP1 γ , and HP1 β -HP1 γ heterodimers act variously and HP1 α and HP1 β homodimers act exclusively as repressors. This hypothesis is supported by the fact that HP1 α and HP1 β suppress the activity of several human genes while HP1 γ supports their transcription^[80]. An example of HP1 acting as a positive regulator of transcription can be seen in the ribosomal genes (rDNA), in which HP1 β and HP1 γ proteins, in a complex with RNA polymerase I and additional above mentioned proteins, cause activation of transcription rather than silencing^[40,81]. *Drosophila melanogaster* provides another example of the HP1 protein acting as a positive regulator. Analysis of larval stages showed that gene activity of heat shock protein 70 (Hsp70) was increased in the presence of HP1 protein^[82].

CHROMATIN STRUCTURE AND PLASTICITY IN ESCS

Basic features of nuclear organization

The nucleus of every eukaryotic cell is characterized by its well-organized structure (summarized by Cremer *et al.*^[83]). Structural integrity of the nucleus ensures proper functioning of the whole cell. As a result of co-operation among a number of regulatory factors, the nucleus consists of DNA and histone proteins that are packed into higher order chromatin structures with specific functions. Chromatin is arranged into large compartments called chromosome territories (CTs) that are partially separated by interchromatin space. Intermingling of CTs is a matter of discussion and seems to depend on the method of CT visualization, but certain interactions of CTs must exist because of the presence of chromosome translocations in tumor cells^[83-85]. Taken together, current data indicate that the nucleus contains macromolecular complexes that function in basic nuclear processes, including replication, transcription, splicing, and/or DNA repair. Moreover, these processes are influenced by physiological condition of additional structures, such as the nuclear lamina, nucleolus, promyelocytic leukemia (PML) bodies or Cajal bodies that are characterized by well-defined functions^[86,87].

A very important compartment of the cell nucleus is

the nuclear lamina (NL), which predominantly consists of lamin proteins that form part of the nuclear membrane. The NL is not obvious at all stages of the cell cycle; it is disrupted at the beginning of mitosis by the activity of cyclin-dependent kinases and joins together again in late anaphase^[86]. Other clearly distinguished structures are Cajal bodies, described in the early twentieth century by the Spanish neurobiologist Santiago Ramón y Cajal. Cajal bodies are spherical structures with a typical size of 0.1 to 2 μm . They contain high concentrations of the protein p80 coilin, small nuclear ribonucleoprotein particles, and small nucleolar ribonucleoproteins. The majority of these proteins are involved in the proper progression of RNA processing^[88]. Moreover, it is generally accepted that formation of Cajal bodies is limited in hESCs, and only low level of coilin is homogeneously dispersed through hESC nucleoplasm (summarized by Morris *et al.*^[89]). Other structures with a regulatory function are PML bodies, the number of which varies from 5-30 per cell depending on the cell type and the current phase of the cell cycle^[90-92]. PML bodies reach a maximum size of 1 μm and are formed by the accumulation of PML protein isoforms, which are freely distributed in the cell nucleus. PML bodies are discussed in the literature under different names, for example PML oncogenic domains, Kramer (Kr) bodies, or nuclear domains 10. PML bodies are considered protein reservoirs, and their main function is indirect regulation of gene expression, DNA repair, proteolysis, apoptosis, antiviral response, tumor suppressor function, and anti-proliferative processes^[92,93]. In ES cells, PML bodies not only differ in their morphology, but also in protein composition.

Morphological differences in chromatin between ESCs and differentiated cells

Chromatin plasticity affects many nuclear processes including transcription, replication, cell cycle kinetics, and dynamics of nuclear proteins. Therefore, chromatin is a basic regulatory unit that controls the developmental and functional status of the cell and in this regard *in vitro* cultivated ESCs are no exception^[94].

In ESCs an open, more relaxed, chromatin configuration is observed. This chromatin is highly dynamic and is significantly different from the chromatin in terminally differentiated cells^[95]. In pluripotent ESCs, highly condensed state of chromatin appears rarely in comparison with differentiated counterparts, characterized by pronounced chromatin compaction. In terminally differentiated cells, especially the periphery of interphase nuclei occupies condensed heterochromatin, while more relaxed euchromatin appears in more central parts of the nucleus^[83]. As a consequence, transcriptionally inactive chromatin predominates in differentiated cells whereas in pluripotent ESCs there is a large amount of transcriptionally active chromosomal regions^[96]. Concerning morphological differences, chromatin in particular ESCs is spread out in relaxed regions, diffused, and amorphous, compared with smaller condensed chromatin domains

in differentiated cells. In addition, nuclear lamina, which is characterized by a reduced level of A-type lamins in ESCs, becomes densely stained by the appropriate lamin antibodies after induced differentiation^[97-99]. Specific distribution of euchromatin- and heterochromatin-related factors can be also observed within colonies of ESCs. Recently, we have found that Nanog-positive cells are present in the very interior regions of pluripotent ESC colonies. However, H3K27me3 shows high positivity in the ESC nuclei positioned on the periphery of the mESC colony^[100]. Here, we show that nuclei with a high positivity for the splicing factor SC-35 are located in the colony interior (Figure 2A-D), but HP1 β -positive cells are mainly at the periphery of the mESC colony, moreover, similar HP1 β pattern was found after induced differentiation (Figure 2E, F). PcG-related BMI1 protein showed no preferential positioning within the colonies (Figure 2G-J). These data unambiguously showed that in majority of cases, nuclear pattern of ESC cells must be analyzed in view of the whole colony, because analysis on single cell level could be unsatisfactory in some cases^[100].

Chromatin and chromatin-related proteins in pluripotent ESCs are additionally highly dynamic, as proved by living cell studies^[98]. For example, hyperdynamic binding of structural chromatin proteins is a functionally important feature of pluripotent ESCs and is probably responsible for the undifferentiated state of ESCs^[98]. In ESCs, HP1 protein and histone H1 are only loosely bound to chromatin unlike neural progenitor cells and primary mouse embryonic fibroblasts, in which HP1 and H1 binding is much stronger and results in a more “closed” chromatin configuration^[87]. This raises the idea that the dynamics of chromatin proteins is regulated during cellular processes and not just a consequence of the overall biophysical state of chromatin, such as simple diffusion that can be studied by the fluorescence recovery after photobleaching technique (FRAP). Moreover, the formation of an open chromatin structure and the hyperdynamic plasticity of chromatin correlate with biological properties of ESCs^[98]. This is again linked to specific transcriptional profiles, associated with fast localized movement of epigenetically important proteins^[41,43,94,96].

Arrangement of chromosome territories in ESCs

As outlined above, it is well known that chromatin is present in the nucleus in two forms: basically, heterochromatin, which is compact, highly conserved, and transcriptionally inactive, but euchromatin is relaxed and transcriptionally active. This is also the case in ESCs. In addition, heterochromatin may be facultative, with the ability to change from a transcriptionally active to a silent state during ontogenesis^[101]. The inactive chromosome X represents one of the best-known examples of facultative heterochromatin^[102]. Moreover, X chromosome inactivation occurs during differentiation of female ESCs and is accompanied by repositioning of the X chromosome closer to the nuclear periphery and increased level of H3K27me3 appears^[28].

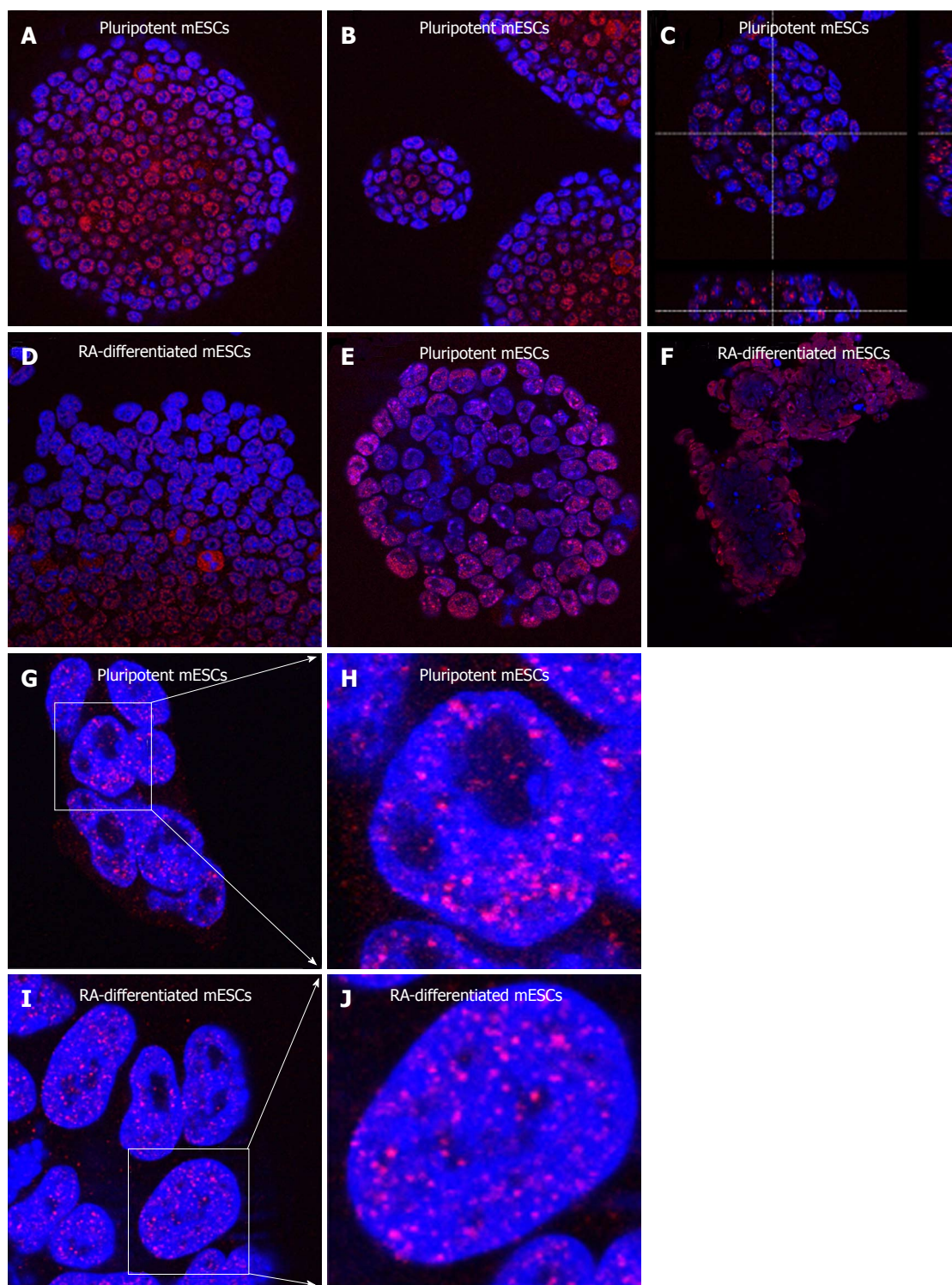


Figure 2 Morphology of colonies of mouse embryonic stem cells (line D3). A-D: The splicing factor stem cells (SC)-35 (red) was visualized in the cell nuclei (blue) within mouse embryonic stem cells (mESC) colonies in pluripotent cells and after retinoic acid (RA)-induced cell differentiation; E, F: Cell nuclei (blue) that were positive for heterochromatin protein 1 (HP1)β (red) were mapped within a pluripotent mESC colony and after RA-induced differentiation; G-J: Pattern of polycomb group protein BMI1 (red) in pluripotent and RA-differentiated ESCs (blue). Individual nuclei in frames G, I were magnified in panels H and J.

Territories of autosomes also follow general principles with respect to nuclear architecture^[83,103]. The general principle of nuclear organization is based on the polarized distribution of gene-rich and gene-poor chromosomes^[104]. Gene-rich chromosome domains, or even whole chromosome 19, are mostly localized inwards the

cell nucleus. Gene-poor chromosomes are more frequently oriented towards the nuclear periphery, for example chromosome 18^[105]. The radial arrangement of territories of chromosomes 6, 8, 10, 12, 15, 17, and 19 is similar in differentiated and pluripotent hESCs (Bártová *et al.*^[28]; radial distribution is the average distance from the nuclear fluo-

rescence center and is normalized to the average nuclear radius). For example, in hESCs the short arm of chromosome 12, which carries the *Nanog* gene, is oriented more towards the center of the nucleus as in lymphoblastoid cells^[106]. Although the distribution of autosomes is not significantly changed during ESC differentiation, as mentioned above. One exception is the transcriptionally inactive X chromosome in the female genome^[28]. Moreover, the majority of centromeric heterochromatin is re-located closer to the nuclear periphery during differentiation of hESCs^[107]. This suggests that chromosomal domains are relatively dynamic structures and individual chromosomal sub-regions can be moved during cellular processes, but always with respect to other chromosomal regions and related to general nuclear structures. Thus, the dynamics of chromosomal regions seems to be important for the regulation of expression of certain genes^[106,108,109].

Positioning of gene loci in ESCs

The spatial arrangement of specific genes in the nucleus also represents a very interesting phenomenon specific to ESCs. As mentioned above, in hESCs the short arm of chromosome 12 carrying the *Nanog* gene is oriented more towards the center of the nucleus compared with lymphoblastoid cells^[106]. Similarly, human chromosome 6, which carries the major pluripotency gene *Oct4*, did not change its nuclear radial position during differentiation of hESCs^[107]. Another aspect related to chromosome architecture is mapping of genes within a related chromosome territory. For example, the position of the *Nanog* and *c-myc* loci within their territories remained constant after differentiation. However, in pluripotent hESCs, the *Oct4* locus was located outside of its chromosome territory on large de-condensed chromatin loops, but differentiation caused *Oct4* repositioning in close proximity to the related chromosome territory^[106]. This robust structural change did not affect the nuclear radial distribution of the *Oct4* gene^[107]. Interestingly to this fact, the transcriptionally active *Oct4* locus is located more internally in mESCs than in hESCs. A possible explanation for this incongruity is the level of expression of neighboring genes or even whole chromosomes, which is significantly different in human and mouse cell nuclei and might influence the general nuclear architecture of mouse and human genomes^[110].

CONCLUSION

Embryonic stem cells represent a promising tool for future regenerative medicine. Recently it becomes more evident that epigenetic process and chromatin plasticity are responsible for self-renewal and pluripotency of ESCs. Thus, genome-wide studies on histone signature, DNA methylation and cytosine hydroxymethylation enable us to better understand principles of stem cells pluripotency. It is important especially from the view of complete reprogramming of iPS cells that represent an advanced methodological tool how to get pluripotent cells with high potential as

therapeutic tool. Also, we must not forget the study performed on individual cellular level, including living cell studies. For example, in such experimental systems it is possible to analyze distribution and function of pluripotency factors within colonies of ESCs^[100] or nuclear events, such as transcription regulation during specific ESC differentiation^[111] or during DNA repair^[55,112].

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Nutrient supplemented serum-free medium increases cardiomyogenesis efficiency of human pluripotent stem cells

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Abstract

AIM: To develop an improved p38 MAPK inhibitor-based serum-free medium for embryoid body cardiomyocyte differentiation of human pluripotent stem cells.

METHODS: Human embryonic stem cells (hESC) differentiated to cardiomyocytes (CM) using a p38 MAPK inhibitor (SB203580) based serum-free medium (SB media). Nutrient supplements known to increase cell viability were added to SB medium. The ability of these supplements to improve cardiomyogenesis was evaluated by measurements of cell viability, total cell count, and the expression of cardiac markers *via* flow cytometry. An improved medium containing Soy hydrolysate (HySoy) and bovine serum albumin (BSA) (SupSB me-

dia) was developed and tested on 2 additional cell lines (H1 and Siu-hiPSC). Characterization of the cardiomyocytes was done by immunohistochemistry, electrophysiology and quantitative real-time reverse transcription-polymerase chain reaction.

RESULTS: hESC cell line, HES-3, differentiating in SB medium for 16 d resulted in a cardiomyocyte yield of 0.07 ± 0.03 CM/hESC. A new medium (SupSB media) was developed with the addition of HySoy and BSA to SB medium. This medium resulted in 2.6 fold increase in cardiomyocyte yield (0.21 ± 0.08 CM/hESC). The robustness of SupSB medium was further demonstrated using two additional pluripotent cell lines (H1, hESC and Siu1, hiPSC), showing a 15 and 9 fold increase in cardiomyocyte yield respectively. The age (passage number) of the pluripotent cells did not affect the cardiomyocyte yields. Embryoid body (EB) cardiomyocytes formed in SupSB medium expressed canonical cardiac markers (sarcomeric α -actinin, myosin heavy chain and troponin-T) and demonstrated all three major phenotypes: nodal-, atrial- and ventricular-like. Electrophysiological characteristics (maximum diastolic potentials and action potential durations) of cardiomyocytes derived from SB and SupSB media were similar.

CONCLUSION: The nutrient supplementation (HySoy and BSA) leads to increase in cell viability, cell yield and cardiac marker expression during cardiomyocyte differentiation, translating to an overall increase in cardiomyocyte yield.

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Key words: Soy hydrolysate; Bovine serum albumin; Differentiation; Cardiomyocyte; Human embryonic stem cells; Human induced pluripotent stem cells

Core tip: Nutrient supplements were screened for improving cell survival during the cardiomyocyte differentiation process of human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC) in

a serum-free medium based on the inhibition of p38 MAPK (SB media). Soy hydrolysate and bovine serum albumin supplementation was found to improve cell viability and therefore increased the yield of HES-3 cardiomyocytes by 2.6-fold over non-supplemented SB medium. The enhancing effect of this medium was demonstrated in an additional hESC line (H1) and Siu1-hiPSC cell line (15 and 9 fold respectively). The cardiomyocytes formed expressed canonical cardiac markers (sarcomeric α -actinin, myosin heavy chain and tropomyosin-T) and demonstrated all three major phenotypes: nodal-, atrial- and ventricular-like.

Ting S, Lecina M, Chan YC, Tse HF, Reuveny S, Oh SKW. Nutrient supplemented serum-free medium increases cardiomyogenesis efficiency of human pluripotent stem cells. *World J Stem Cells* 2013; 5(3): 86-97 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v5/i3/86.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v5.i3.86>

INTRODUCTION

Heart disease is one of the most common causes of mortality in the world and accounts for more than 800000 deaths per year on average in the United States alone^[1]. After an episode of major cardiac insult (such as a myocardial infarction), the heart can lose up to 2 billion cardiomyocytes. Being an organ that cannot auto-regenerate, progressive heart failure develops. Cardiomyocyte cell therapy can thus be a potential cure for heart failure after myocardial infarction, and it is suggested that at least one billion cardiomyocytes will be required per patient for such treatment^[2].

Human embryonic stem cells (hESCs) present an attractive cell source for generating large amounts of cardiomyocytes, due to their pluripotency and ability to proliferate for multiple passages^[3]. Several studies have reported techniques to efficiently differentiate hESCs to cardiomyocytes *via* growth factors and small molecule inhibitors^[4-8]. Current cardiomyocyte differentiation protocols can be divided into two groups: differentiating hESC in 2D monolayers culture or in 3D suspended embryoid bodies (EBs) cultures^[9]. Although monolayer differentiation protocols have achieved high yields of cardiomyocytes^[6,10], the scalability of these methods is problematic and they have limited capability in generating the amounts of cardiomyocytes needed for cell therapy. On the other hand, methods that involve EBs formation, which have better potential for scale up, have lower yields of cardiomyocyte, and requires extensive use of expensive growth factors like BMP4 and activin A at multiple specific time points during differentiation^[7,11-13]. In addition, the growth factors have to be optimized for different cell lines, growth platforms or passage numbers^[14,15]. As such, there is a lack of protocols for cardiomyocyte differentiation in EB suspended cultures that are cost-effective, scalable and most importantly robust. Previously, we have developed a simple scalable methodology to dif-

ferentiate hESCs to cardiomyocytes using a serum-free differentiation medium^[16,17] containing a small molecule p38 MAP kinase inhibitor SB203580 (SB media)^[18,19]. The enhancing effect of SB203580 on cardiomyogenesis of hESC has been correlated to the expected inhibition of the p38 pathway as well as the activation of JNK^[20]. This suggests a regulatory interlink between the JNK and p38 pathways during cardiomyogenesis. Compared to protocols based on growth factors, small molecules are less costly and more amenable for good manufacturing practice (GMP) manufacturing of cells^[21]. However, the SB medium is essentially protein-free and lacks nutrients (*e.g.*, lipids) and growth factors. From the low cell viability and yield observed, we hypothesized that SB medium has nutritional deficiencies that limit cardiomyogenesis, especially in the initial stages of the differentiation process.

In this study, we sought to improve the survival of cells in SB medium and thereby enhance cardiomyogenesis using the embryoid body method of differentiation. For successful growth and maintenance of metabolic functions of differentiated human cells *in vitro*, appropriate culture conditions are required to mimic the physiological conditions *in vivo*^[22,23]. The culture medium is one of the most important factors in maintaining cell and tissue culture as it provides nutrients and salts, hormones and growth factors, buffering elements and oxygen supply^[22-24]. While media supplements have been developed for a variety of cell types, none have been performed for stem cell differentiation.

In this study, nutritional components were screened for an improvement in overall yields of cardiomyocytes of HES-3 cells using serum-free and insulin-free SB medium. Two supplements, Soy-hydrolysate (HySoy) and bovine serum albumin (BSA), resulted in improved cardiomyocyte differentiation efficiency. The concentrations of both supplements were optimized, resulting in an increase in cell growth and differentiation. The robustness of this new medium (SupSB media) was evaluated using the H1 and HES-3 hESC, as well as the Siu1-hiPSC cell lines (2.6 to 15.0 fold increase in cardiomyocyte yield). Similar cell yields and differentiation efficiency were obtained using 9 different batches of BSA and HySoy indicating that batch variability is not a major concern. Cardiomyocytes formed in SupSB media expressed canonical cardiac markers and electrophysiological studies demonstrated successful cardiac differentiation to give all the three major phenotypes of cardiomyocytes: nodal-, atrial- and ventricular-like. In summary, we have developed a cost-effective, scalable and robust protocol for cardiomyocyte differentiation by improving the p38 MAP kinase protocol with the addition of BSA and HySoy. SupSB media increases cell yield and cardiac expression markers during cardiomyocyte differentiation, translating to an overall increase in cardiomyocyte yield.

MATERIALS AND METHODS

Culture of hESCs and hiPSCs

HES-3 [(46, XX); ES Cell International], H1 [(46, XY);

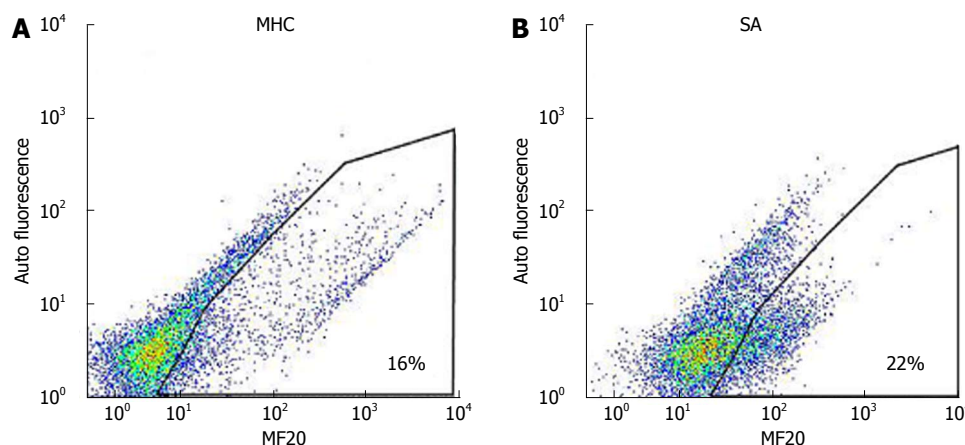


Figure 1 Dot plot of anti-myosin heavy chain and anti-sarcomeric α -actinin of HES-3 differentiated cardiomyocytes. A: Myosin heavy chain (MHC); B: Sarcomeric α -actinin (SA).

WiCell], and Siu1-hiPSC (Professor Tse HF, The University of Hong Kong) with normal karyotypes were cultured in KNOCKOUT medium on inactivated immortalized mouse feeders. The medium was refreshed daily and the cells were passaged weekly. Cultures were kept at 37 °C with 5% CO₂.

Differentiation to cardiomyocytes

hESC cultures were washed using phosphate buffered saline (PBS) (Invitrogen), cut into small clumps (EZ-passage tool; Invitrogen), and seeded at 1.33×10^6 cells/mL in ultra-low attachment 12-well plates (Nunc). The plates were agitated for 1 h and then cultured in static conditions at 37°C in a humidified atmosphere with 5% CO₂. The differentiation medium (SB media) comprised Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 2 mmol/L L-glutamine (Invitrogen), 0.182 mmol/L sodium pyruvate (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1 mmol/L 2-mercaptoethanol, 5.6 mg/L transferrin (Invitrogen), and 20 mg/L sodium selenite (Sigma). A solution of 5 mmol/L of p38-MAPK inhibitor (SB203580; Sigma), dissolved in dimethylsulfoxide (Sigma), was added to the medium at a final concentration of 5 μ mol/L. The medium was refreshed every 2 d following the previously described protocol^[16].

Supplements for cardiomyocyte differentiation

Various supplements (Table 1) were added to the SB medium and tested for cell yields and cardiomyogenesis efficiency using the above differentiation protocol.

Cardiomyocytes harvesting and quantification

Following the protocol established by Lecina *et al.*^[15] and Ting *et al.*^[16], beating aggregates collected from 1 well were washed with 5 mL of PBS without Ca²⁺/Mg²⁺ (PBS-) (Invitrogen), and incubated for 30 mins in 500 μ L PBS-solution containing 1.6 mg/mL collagenase (Sigma) and 20% fetal bovine serum (Hyclone). Thereafter, 500 μ L of 0.25% trypsin EDTA (Invitrogen) was added to generate single-cell suspensions. Cell suspensions were finally filtered through a 40- μ m cell strainer (Becton Dickinson), fixed, and permeabilized (Caltag Laboratories). For FACS analysis, cells were incubated with anti-myosin heavy

chain (anti-MHC; MF20, dilution 1:200; Developmental Studies Hybridoma Bank), anti-sarcomeric α -actinin (anti-SA; 1:100; Sigma) and anti-Troponin T (anti-cTnT; 1:200; Thermo Scientific). Fluorescein isothiocyanate-conjugated antimouse antibody (1:500; DAKO) was used as a secondary antibody. Dot plots for both anti-MHC and anti-SA are shown in Figure 1. All incubations were carried out at room temperature for 30 min. FACS measurements were done using Guava (Millipore).

Cell counting and calculations of cardiomyocyte/hESC yield and normalized yield

Cell concentration was determined by the nuclei count method using NucleoCounter (Chemometec). Apoptosis and cell proliferation were evaluated using Annexin V (Invitrogen) and Ki-67 (BD Biosciences) respectively via FACS. Cardiomyocyte yields were calculated following published protocols^[16,17], namely: CM hESC yield = (cell count_{Day 16}/cell count_{Day 0}: Initial hESC seeded) \times (% positive cells_{MHC, SA/100}); normalised yield = (CM/hESC yield_{Condition})/(CM/hESC yield_{Control}).

qRT PCR

Total RNA was isolated from the cells ($< 5 \times 10^6$) using RNeasy Mini Kit (Qiagen) following the supplier's protocol. Reverse transcription was carried out with 1 μ g total RNA using SuperScript III (Invitrogen). Real-time PCR was performed by applying a standard two-step amplification protocol on an ABI 7500 system (Applied Biosystem) to detect mRNA expression. Normalization of the results was done using a house-keeping gene, GAPDH. Primer sequences for Nanog, OCT 4, T-bra, Mesp 1, Nkx 2.5, MHC and GAPDH are provided in Table 2.

Immunocytochemistry

Cell aggregates were harvested, washed with PBS-, mechanically dissociated by pipetting, and plated in 24-well plates for 2 d in the SupSB medium at 37°C in a humidified atmosphere with 5% CO₂. The cells were then washed twice with PBS- and fixed with 4% paraformaldehyde (2 mL for 20 minutes at room temperature). After washing twice with PBS-, permeabilization and blocking was done using 0.1% Triton X-100 and 10% goat serum

Table 1 List of nutritional supplements

Supplements	Concentration/addition	Source
MEM vitamin solution ($\times 100$)	30 $\mu\text{L}/\text{mL}$	Gibco 11120
Yeastolate ultrafiltrate ($\times 50$)	20 $\mu\text{L}/\text{mL}$	Gibco 18200
Vitamin E	70 $\mu\text{g}/\text{mL}$	Sigma T3376
Syntheschol (cholesterol) ($\times 500$)	12 $\mu\text{L}/\text{mL}$	Sigma S5442
Yeast extract	50 $\mu\text{g}/\text{mL}$	Sigma Y1625
Bovine serum albumin	50 $\mu\text{g}/\text{mL}$	Gibco A10008-01
Soy hydrolysate	50 $\mu\text{g}/\text{mL}$	Kerry Bio-science 5X59022

respectively. The following antibodies were used: anti-MHC (MF20; Developmental Studies Hybridoma Bank), anti-SA (Sigma), and anti-troponin-T (Thermo Scientific). Nuclear staining was done using SlowFade Glow with DAPI (4'-6-diamidino-2-phenylindole) (Invitrogen). The fluorescence was observed using an Olympus IX71 fluorescence microscope (Olympus) coupled with Olympus imaging software Cell P.

Electrophysiology

Standard whole-cell patch-clamp recordings were performed at $37 \pm 0.5^\circ\text{C}$ to record the action potential phenotypes (HEKA Instruments Inc. Southboro, MA, United States) of beating cardiomyocyte aggregates as previously described^[25,26]. Patch pipettes were prepared from 1.5-mm thin-walled borosilicate glass tubes using a Sutter micropipette puller P-97 and had typical resistances of 3–4 $\text{M}\Omega$ when filled with an internal solution containing (mmol/L): 110 K^+ aspartate, 20 KCl, 1 MgCl_2 , 0.1 Na-GTP, 5 Mg-ATP , 5 Na_2 -phosphocreatine, 5 EGTA, 10 HEPES, and pH adjusted to 7.3 with KOH. The external Tyrode's bath solution consisted of (mmol/L): 140 NaCl, 5 KCl, 1 MgCl_2 , 0.4 KH_2PO_4 , 1.8 CaCl_2 , 10 Glucose, 5 HEPES, with pH adjusted to 7.4 with NaOH. Twenty consecutive action potentials from spontaneously firing HES3-derived cardiomyocytes were recorded per cell to ensure stable waveforms for analysis. For the electrically quiescent cardiomyocytes, a stimulation of 0.1–1 nA for 5 ms was given to elicit an action potential. The sampling frequency was 2.00 kHz and data were corrected for the liquid junction potentials of +15.9 mV. Maximal diastolic potential as well as action potential duration at 90% (APD90) and 50% repolarization level (APD50) were measured.

Statistical analysis

Experiments were done using three independent replicates. The significance of the results was calculated by Student's *t*-test or by one-way ANOVA ($P < 0.05$, $P < 0.01$).

RESULTS

Identifying nutritional supplements that can improve cardiomyocyte differentiation

In order to identify elements that can increase cardiomyocyte differentiation, we selected a panel of defined and

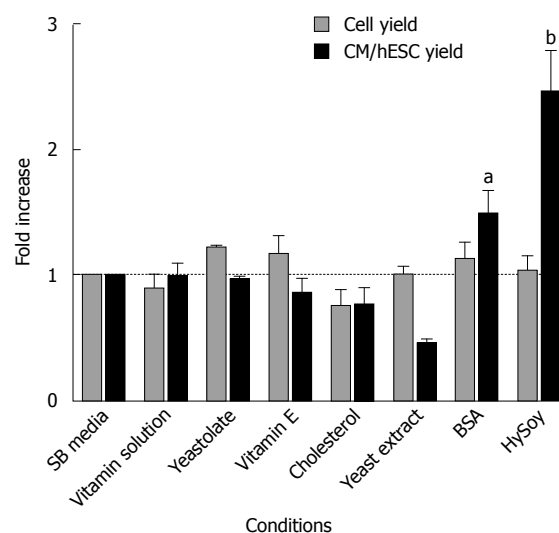


Figure 2 Effect of nutritional supplements added to SB medium on HES-3 differentiation to cardiomyocytes. Differentiation efficiency is based on two FACS markers: myosin heavy chain (MHC) and sarcomeric α -actinin (SA). Cell and cardiomyocyte (CM) yields were measured at day 16 of culture. Results were normalized against the yields obtained in SB medium (control). ($n = 3$, $^aP < 0.05$, $^bP < 0.01$ vs cell yield). BSA: Bovine serum albumin; HySoy: Soy-hydrolysate.

non-defined nutritional supplements (Table 1) which are known to support cell growth of a variety of cell lines^[27]. The supplements were used at concentrations typically reported for animal cell culture.

HES-3 cells were seeded at a concentration of 1.33×10^6 cells/mL and differentiated in SB medium supplemented with the various supplements described in Table 1. Control cultures were differentiated in non-supplemented SB medium. After 16 d, cells were harvested and measured for cell yield and expression of the cardiac markers sarcomeric α -actinin (SA) and myosin heavy chain (MHC). A third antibody, cTnT which detects cardiac troponin was also used to verify the results (data not shown). The differentiation efficiency of the supplement was evaluated by dividing the yields of cardiomyocytes produced per seeded hESC in the supplemented culture with the one in the control culture (normalized cardiomyocyte yield). This parameter considers both the differentiation efficiency (percentage of cardiomyocytes) and final number of total cells, making it meaningful for process analysis and evaluation^[16,17].

The nutritional supplements did not significantly affect cell counts. However, supplementing the media with BSA and HySoy improved the metabolic state of the cells, leading to an increase in normalized cardiomyocyte yield with improvements of 1.47 ± 0.20 and 2.45 ± 0.33 , fold on average respectively, compared to the control (Figure 2). Addition of vitamins had no effect on the differentiation process. Yeastolate, Yeast extract, and Vitamin E reduced cardiomyocyte yields showing a negative effect on cardiac marker expression (data not shown), while cholesterol had a negative effect on cell yields.

In order to further increase cardiomyocyte yields, the effect of HySoy and BSA concentration on cardiomyocyte yield was further investigated (Figure 3). At a BSA

Table 2 Quantitative real-time reverse transcription-polymerase chain reaction primers

Primers for qPCR	Forward (5'→3')	Reverse (5'→3')
Nanog	GAAAAACAACCTGGCCGAAGAAT	GGTGCTGAGGCCTTCTGC
4-Oct	AACGACCATCTGCCGCTTT	GGCCGCAGCTTACACATGTT
T-bra	AATTTGGTCCAGCCTTGGAAAT	CGTTGCTCACAGACCACAG
Mesp 1	GACGTGCTGGCTCTGTIG	TGTCAGTTGGGCTCCTCAG
Nkx 2.5	CAAGTGTGCGTCTGCCCTT	TGTCCGCCTCTGTCTTCTC
MHC	ATTGCTGAAACCGAGAATGG	CGCTCCTTGAGGTTGAAAAG
GAPDH	GTCGGAGTCAACGGATTGG	AAAAGCAGCCCTGGTGACC

qPCR: Quantitative polymerase chain reaction; MHC: Myosin heavy chain; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

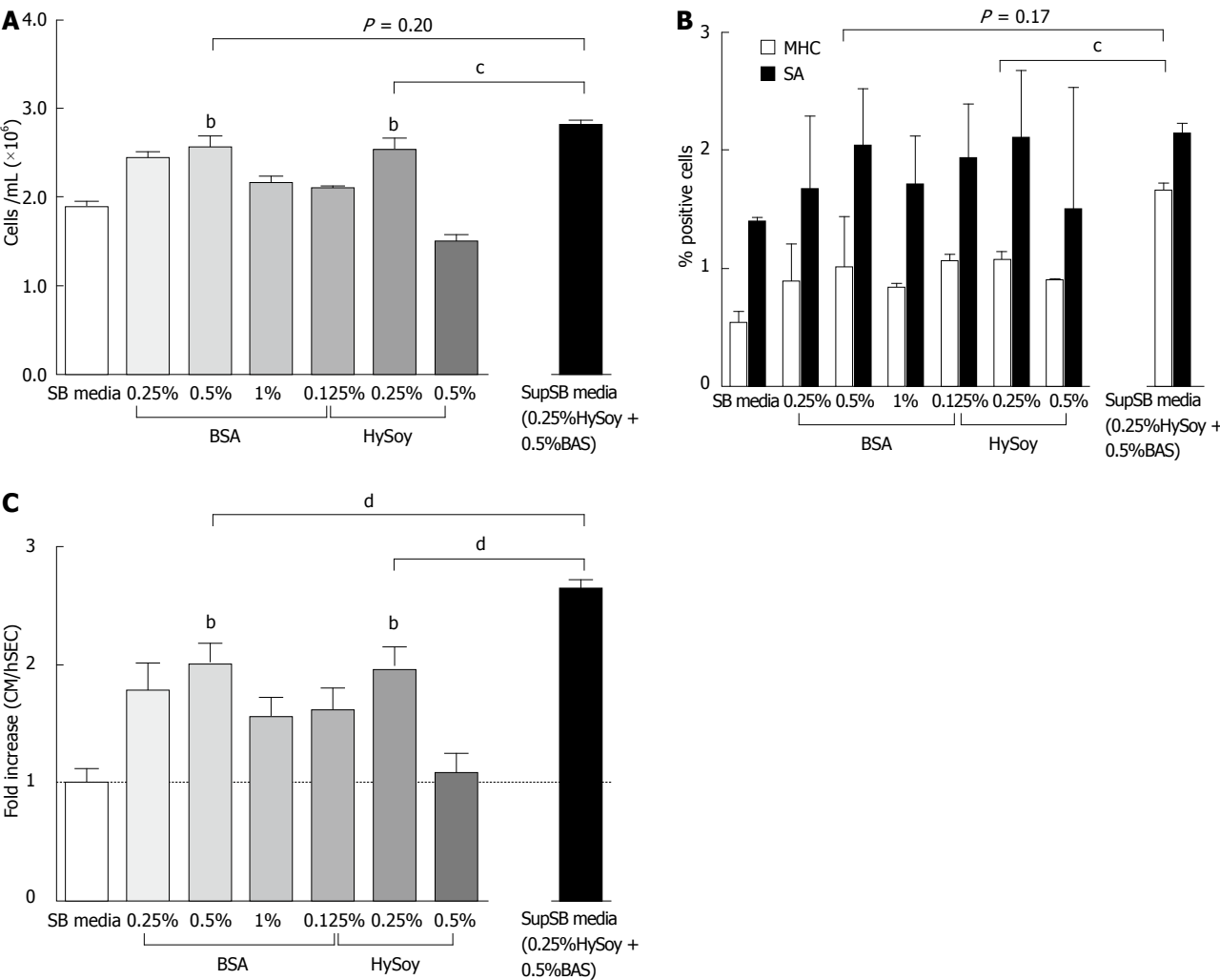


Figure 3 Additive and dose effect of Soy hydrolysate and bovine serum albumin supplementation on HES-3 differentiation to cardiomyocytes. HES-3 cells differentiated in SB medium containing varying concentrations of bovine serum albumin (BSA) and Soy hydrolysate (HySoy), as well as a combination of both supplements (SupSB media), were harvested on day 16 and evaluated for total cell count (A) and cardiac specific markers, myosin heavy chain (MHC) and sarcomeric α -actinin (SA) (B); C: The results were summarized by calculating the normalized yield which takes into account the ratio of cardiomyocyte at day 16 compared to the initial human embryonic stem cell seeded. Normalized yields are based on both MHC and SA markers. Dot plots of both MHC and SA are shown in Figure 1. ($n = 4$. $^aP < 0.01$ vs SB medium; $^bP < 0.05$; $^cP < 0.01$ vs SupSB media).

concentration of 0.5% v/v, an increase in cell growth compared to the control was observed, achieving an average of 2.54×10^6 cells/mL (Figure 3A). Similarly, cultures supplemented with 0.25% w/v HySoy achieved a maximum cell density of 2.51×10^6 cells/mL. The

supplements also improved cell viability, showing > 85% viability as compared to 65% in the controls (SB media). This increase in cell growth and viability resulted in a 2-fold increase in normalized cardiomyocyte yield for both BSA and HySoy (Figure 3C). Although there was no

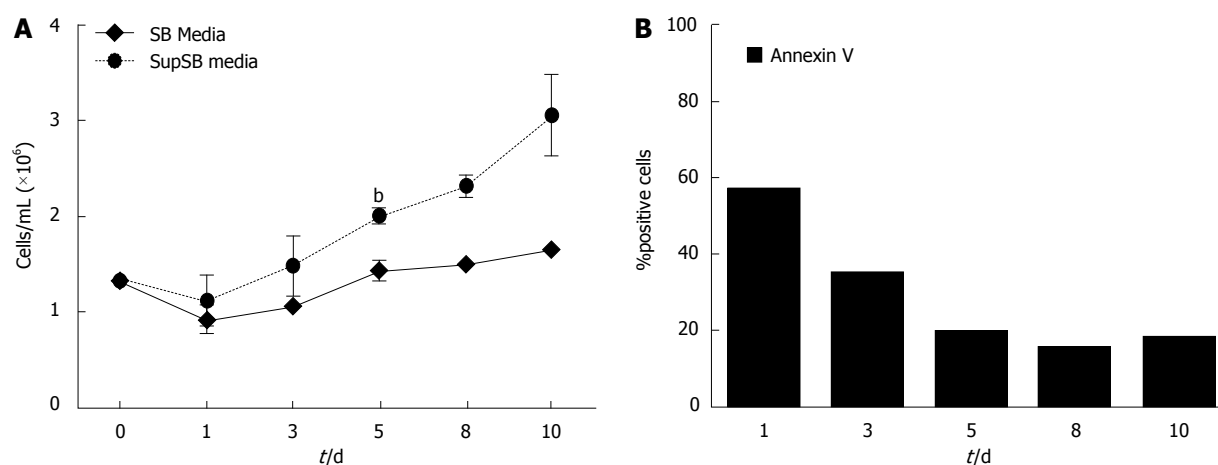


Figure 4 Cell growth and apoptosis in HES-3 cells during cardiomyocyte differentiation. A: Cell growth kinetics of SB and SupSB media cultures; B: Kinetics of apoptosis in SupSB media cultures (measured by annexin V antibody) ($n = 3$, ^b $P < 0.01$ vs in ten days).

statistical difference in the level of expression of cardiac markers (SA and MHC) between the cultures supplemented with different concentrations of BSA and HySoy as compared to the control, a general trend of increased level of expression was observed in cultures supplemented with 0.25% w/v HySoy and 0.5% v/v BSA (Figure 3B). Embryoid bodies of all cultures started beating at day 9, indicating that the supplements did not affect the process of cardiac differentiation temporally.

Additive effect of HySoy and BSA supplements further increases cardiomyocyte yields

Next, we explored the effects of combining both supplements on cell yield and cardiac marker expression (SA and MHC). HES-3 cells were seeded in medium containing both 0.25% w/v HySoy and 0.5% v/v BSA (SupSB media) and compared to control cultures supplemented by HySoy and BSA alone or not supplemented at all.

Results show a further increase in normalized cardiomyocyte yield (2.6-fold) when HySoy and BSA were combined, compared to only a 2-fold increase with the individual supplements alone ($P < 0.01$) (Figure 3C). This increase in cardiomyocyte yield can be attributed to a higher percentage of differentiated cardiomyocytes as indicated by a higher percentage of MHC-expressing cells (16.5% of cells) cultured in the SupSB medium vs. the individual supplements (10%-12%) (Figure 3B). In addition, there was also an increase in cell yield of cultures in SupSB media resulting in 2.79×10^6 cells/mL (Figures 3A and Figure 4), that was statistically significant ($P < 0.01$). The variability between different batches of BSA and HySoy were also tested. Differentiation experiments using 9 different batches of BSA and HySoy were conducted and little variability was observed. A high level of apoptotic cells (60% of total cells), measured by annexin V, was observed during the first day of differentiation, which correlates to cell death probably as a result of mechanical manipulation of the culture during seeding. Thereafter, gradual increase in cell yields and down regulation of

apoptotic markers were observed (Figure 4). In summary, the additive effect of HySoy and BSA increased cardiomyocyte yield further over individual supplements alone, through increasing cell yield and expression of cardiac markers.

Kinetics of cell growth and marker expression during HES-3 differentiation

Next, we investigated the underlying factors that resulted in the overall increase in cardiomyocyte yields using the optimal concentrations of both supplements. Differentiating HES-3 cultures supplemented with optimal concentrations of HySoy and BSA were monitored for 10-16 d. Every 2-3 d cells were harvested and analyzed for cell yield, expression of pluripotent markers (Nanog and OCT4), mesoderm markers (Mesp 1 and T-bra), the early cardiac marker Nkx 2.5 and the late cardiac marker MHC *via* qRT-PCR (Figure 5).

More than 30% of cell death was observed during the first day in both the supplemented as well as the control cultures. This phenomenon can be attributed to the mechanical cutting of the HES-3 cultures and the adaptation of the cells to the new differentiation media. Thereafter, BSA and HySoy supplemented cultures showed 34% and 39% increase in cell yields respectively by day 3 and achieved cell densities of 2.16×10^6 and 2.32×10^6 cells/mL on day 10, as compared to 1.65×10^6 and 1.44×10^6 cells/mL in the control cultures respectively (Figure 5A and B). At the end of the culture, addition of BSA and HySoy resulted in a rise in cell yield by 23% and 19% respectively as compared to the controls.

qRT-PCR analysis show that both supplemented cultures and SB control culture displayed similar trends of down regulation of pluripotent markers (Nanog and OCT4) as well as up-regulation of mesoderm markers (Mesp 1 and T-bra) (Figure 5C and D). However, cultures with supplements showed a significantly higher expression of cardiac progenitor gene *Nkx 2.5* as well as a higher general trend of late cardiomyocyte gene *MHC*

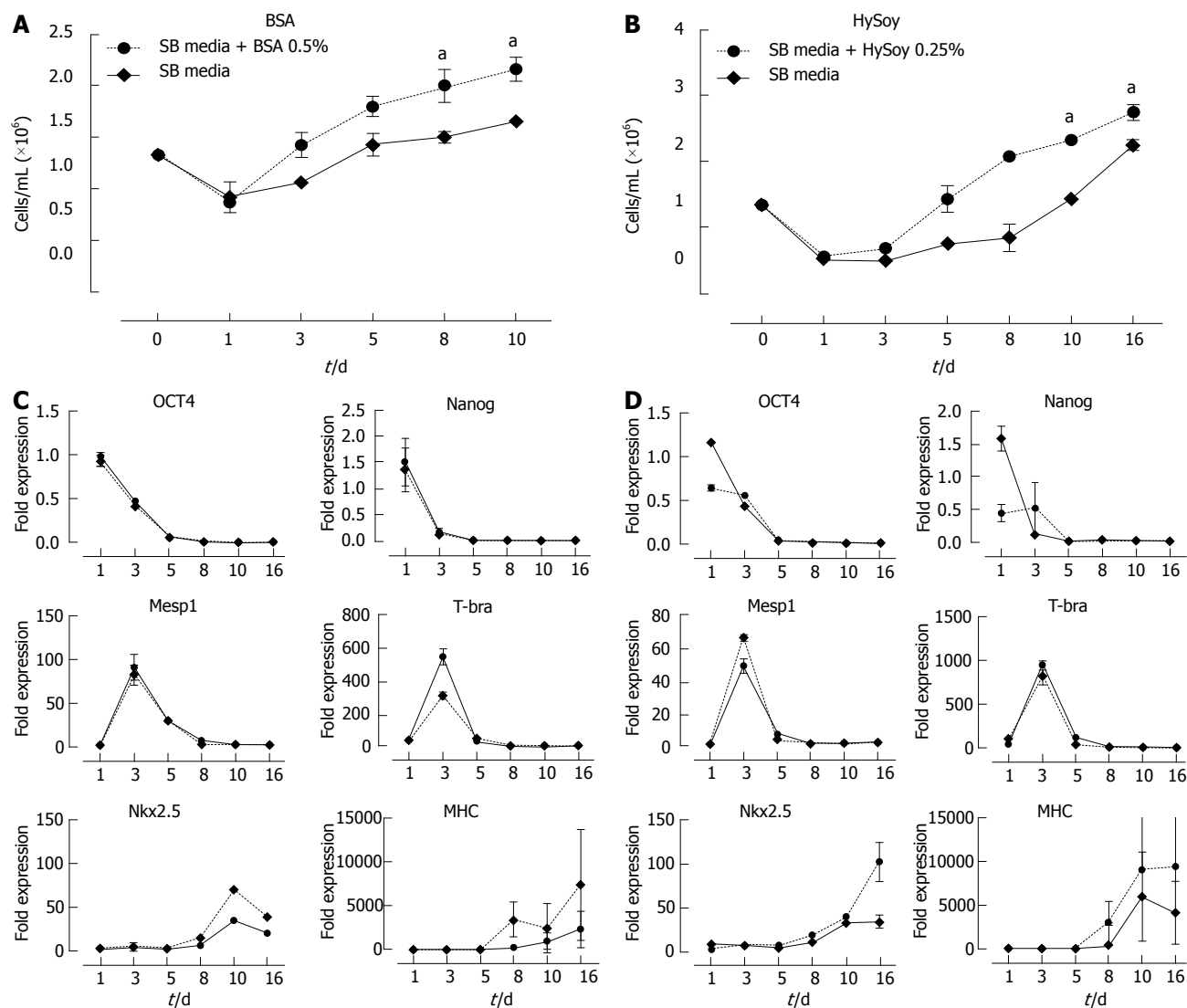


Figure 5 Cell growth and gene expression kinetics of HES-3 cells grown in cultures with and without supplements. Cell densities at multiple times points over a period of 10 to 16 d were recorded with cultures with and without supplements bovine serum albumin (BSA) (A); Soy hydrolysate (HySoy) (B). Gene expression profile recorded via quantitative real-time reverse transcription-polymerase chain reaction at multiple time points over 16 d during the course of cardiomyocytes (CM) differentiation. 6 genes: hESC pluripotency (OCT4, Nanog), Mesoderm (Mesp1, T-bra), cardiac progenitors (Nkx2.5), and cardiomyocytes (MHC) were monitored for BSA (C); Hysoy (D). $n = 3$, $^aP < 0.05$, $^bP < 0.01$ vs SB medium.

expression during the later stages as compared to SB control cultures (Figure 5C and D). In summary, the addition of supplements, BSA and HySoy, not only enhanced cardiomyogenesis *via* improvement in cell yield, but also increased the expressions of both Nkx 2.5 and MHC.

Robustness of SupSB medium: Increased cardiomyogenesis with other pluripotent cell lines

In order to demonstrate the universality and robustness of SupSB media for increased cardiomyogenesis induction efficiency, we cultured two additional cell lines, H1 hESC and Siu1-hiPSC. These cells were differentiated in SupSB medium for 16 d and tested for cell yields and cardiac marker expression, with cells differentiated in SB medium used as controls. Results show that culturing cells in SupSB medium led to increased cell density from 1.10×10^6 to 3.12×10^6 cells/mL for H1 and from

0.67×10^6 to 1.22×10^6 cells/mL for Siu1-hiPSC as compared to SB media (Table 3). Moreover, significantly higher expression of the cardiac specific marker MHC was observed in cultures with SupSB medium (Table 3). H1 cultures differentiated in SupSB medium showed a significant increase in normalized cardiomyocyte yield of 15-fold over the control, while Siu1-hiPSC showed a 9-fold increase (Table 3). Cardiomyocyte yield per hESC seeded was 0.59 cardiomyocyte/hESC for H1 cultures and 0.04 cardiomyocyte/hESC for Siu1-hiPSC cultures.

Another factor that can influence the yield of cardiomyocyte differentiation is the age or passage number of the cells. As such, HES-3 cells at early (P12 to P13) and late (P21 to P39) passages were differentiated using SupSB medium and cardiomyocyte yields were measured at day 16. Results in Figure 6 show that cells from early or late passage showed similar cardiomyocyte yields.

Table 3 Summary of results: Robustness of SupSB vs SB media for cell lines HES-3, H1 and Siu1-human induced pluripotent stem cells

Conditions		Cell expansion		Differentiation efficiency		CM yield
Cell type	Medium	Cell yield (10^6 cells/mL)	Cell expansion fold	Sarcomeric actinin	MHC	Cardiomyocyte/hESC
HES-3	SupSB	2.27 ± 0.58	1.65 ± 0.47	$14.70\% \pm 6.30\%$	$13.59\% \pm 3.40\%$	0.21 ± 0.08
	SB	1.66 ± 0.48	1.25 ± 0.36	$9.25\% \pm 6.19\%$	$6.17\% \pm 2.95\%$	0.07 ± 0.03
H1	SupSB	3.14 ± 0.24	2.35 ± 0.18	$26.70\% \pm 2.09\%$	$25.20\% \pm 5.41\%$	0.59 ± 0.01
	SB	1.10 ± 0.19	0.82 ± 0.14	$20.23\% \pm 1.87\%$	$4.76\% \pm 1.62\%$	0.04 ± 0.01
Siu1-hiPSC	SupSB	1.22 ± 0.15	0.92 ± 0.11	$20.76\% \pm 4.74\%$	$2.11\% \pm 0.73\%$	0.04 ± 0.04
	SB	0.67 ± 0.26	0.51 ± 0.20	$16.90\% \pm 1.95\%$	$1.01\% \pm 0.20\%$	0.004 ± 0.001

MHC: Myosin heavy chain. Cultures with SupSB medium were compared against SB medium for cell expansion and differentiation efficiency at the end of culture (day 16). The results were summarized by calculating the cardiomyocyte yield [cardiomyocytes (CM) yield] which takes into account the ratio of cardiomyocytes at day 16 compared to the initial human embryonic stem cells (hESC) seeded ($n = 3$ to 9).

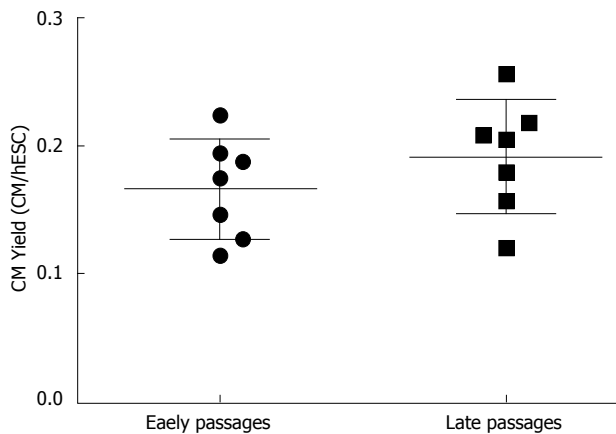


Figure 6 Robustness of SupSB medium over different cell ages (passage). HES-3 cells from different passages (P12 to P39) were differentiated using the supplements, Soy hydrolysate (HySoy) and bovine serum albumin (BSA). The passages were divided into two groups: Early passage (P12 to P13) and Late Passage (P21 to P39). There is no significant difference in cardiomyocyte yield at different passages. Student *t*-test and one-way ANOVA test were used to calculate the statistical significance of the data.

Characterization of cardiomyocytes obtained from SupSB differentiation medium

HES-3, H1 and Siu1-hiPSC cells differentiated in SupSB medium all showed increases in cell yield, expression of cardiac markers and cardiomyocyte yields (Table 3). The increase in cell yield can be attributed to better metabolic conditions manifested by an increase of 20%-30% in cell viability over the control and an increased expression of proliferation marker Ki-67^[28]. Specifically, at day 2 of differentiation, 12% increase in Ki-67 expression was observed in cultures differentiated in SupSB medium compared to SB medium. By day 4 onward, Ki-67 expression was similar in both cultures (data not shown). The percentage of beating aggregates in cultures with SupSB medium was also higher than that in SB medium (Figure 7).

Cardiomyocytes differentiated in SupSB medium were also characterized via immunostaining of cardiac proteins. 16 d old EBs were harvested, mechanically dissociated, and plated on 0.1% gelatin-coated 24-well plates before staining with a set of antibodies against cardiac-specific markers (Figure 8A-C). Cells stained positive for cytoskeleton structural proteins (SA) and contractile

functional proteins responsible for motility (MHC, and troponin-T)^[29]. Electrophysiological study *via* whole-cell patch-clamping was performed. Action potential durations at 90% and 50% repolarization levels as well as maximal diastolic potential were recorded using HES-3 cells differentiated for 23 d. Cells differentiated in both SB and SupSB medium gave similar results. All the three major phenotypes of cardiomyocytes: nodal-, atrial- and ventricular-like were obtained (Figure 8D). The APD and maximal diastolic potential of the cardiomyocytes were similar for cells cultured in both SB and SupSB media (Figure 8E-G) and are comparable to those of the control hESC cell line H7 (data not shown).

Furthermore, a normal karyotype was observed in 20 d old cardiomyocytes differentiated in SupSB medium (Figure 9).

DISCUSSION

In this paper, we have demonstrated that the media supplements, HySoy and BSA, can improve the differentiation efficiency of hESCs into cardiomyocytes when added to SB media containing the p38 MAP kinase inhibitor, SB203580. In particular, when combined at optimal concentrations (0.25%HySoy and 0.50%BSA), the cardiomyocyte yield is enhanced by 2.6-fold compared to the SB medium without supplements for the HES-3 cell line. This increase is due to the improvement in cell growth, as well as differentiation efficiency. Cell lines such as, H1 and Siu1-hiPSC, which initially did not differentiate efficiently to cardiomyocytes in the SB media, showed significant improvements in yields of cardiomyocyte/hESC or cardiomyocyte/hiPSC (15- and 9-fold respectively) when these supplements were added. The variability in cardiomyocyte differentiation yields between cell lines is not surprising and it likely reflects genetic and epigenetic differences between pluripotent stem cell lines^[30-32] that influences their cardiac differentiation efficiencies^[14,15,33]. Moreover, the level of differentiation obtained in SupSB medium was consistent and not affected by cell age (passages 12 to 39). We have observed varying ratios of expression levels for MHC and SA, ranging from 1:1 to 1:16 (MHC: SA) (Table 3). These differences can be attributed to the stages in the differentiation process which the cells

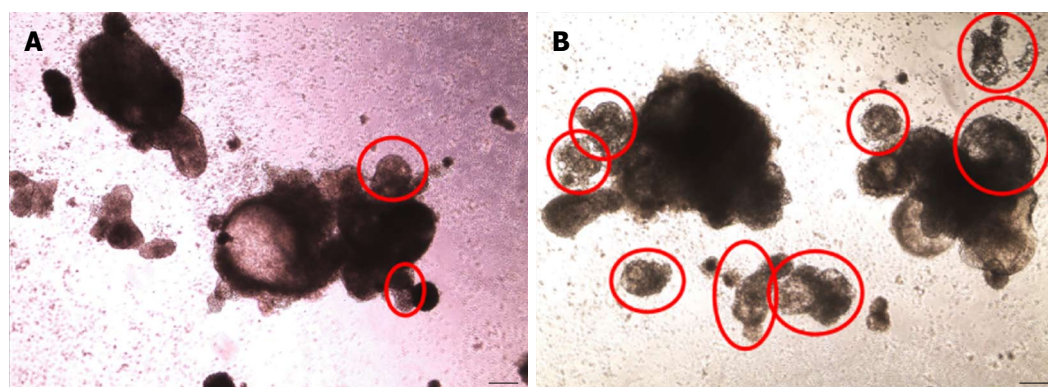


Figure 7 Phase contrast microscopy of cardiomyocytes obtained from differentiation of HES-3 cells. Pictures of aggregates formed from human embryonic stem cells differentiated in SB media (A) and SupSB media (B) were taken after 16 d. Beating areas are indicated by red circles. Total number of beating aggregates was higher in cultures differentiated in SupSB medium (about 80% of total aggregates) compared to in SB medium (about 60% of total aggregates). Scale bar = 200 μ m.

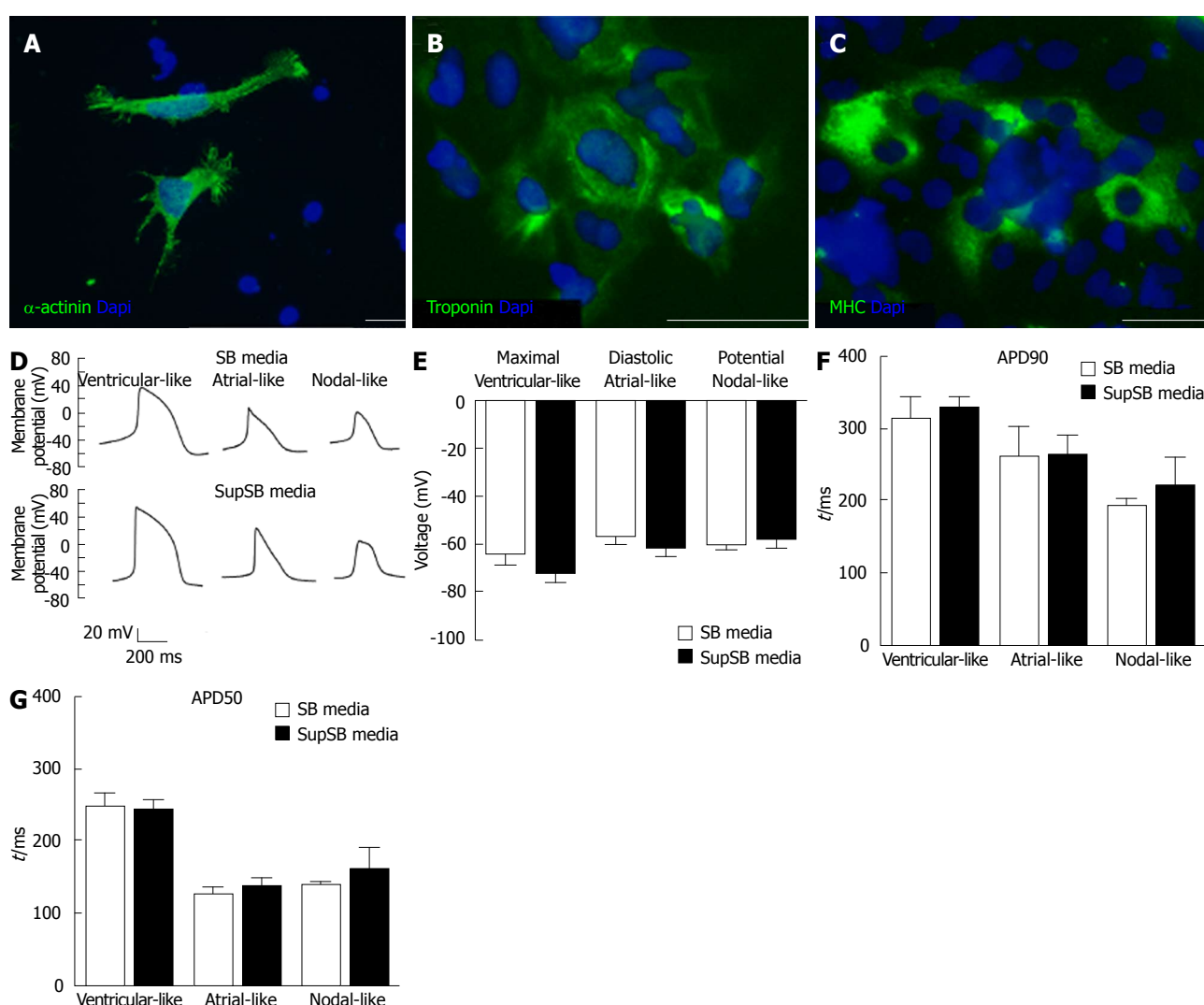


Figure 8 Characterization of cardiomyocytes: Immunocytochemistry and electrophysiology. HES-3 differentiated cardiomyocyte aggregates at the end of differentiation (day 16) were mechanically dissociated and plated on 24-well plates and stained for markers. A: Sarcomeric α -actinin (Structural protein); B: Troponin-T (contractile function protein); C: Myosin heavy chain (MHC) (contractile function protein). Nuclei stained with DAPI (blue). Scale bar = 20 μ m. MHC, myosin heavy chain; DAPI: 4'-diamidino-2-phenylindole. D: Whole-cell patch-clamp recording was performed on beating cardiomyocyte aggregates at day 23 of differentiation. The recording shows the successful derivation of all three cardiac phenotypes as well as no difference between cells grown in SB media (control) and SupSB media (supplements); E: Maximal diastolic potential; F: Action potential duration at 90% repolarization (APD90); G: Action potential duration at 50% repolarization (APD50).

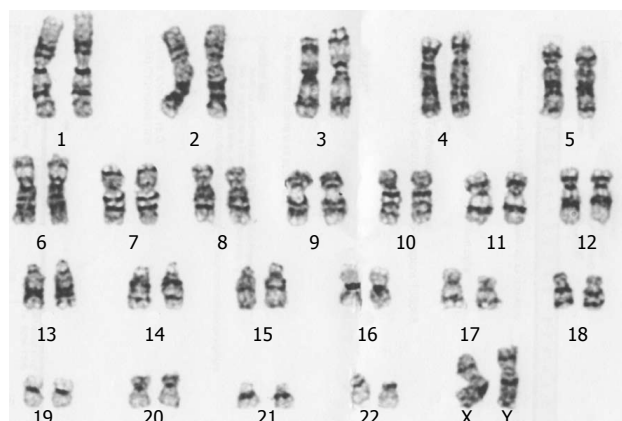


Figure 9 Karyotype of cardiomyocytes differentiated from HES-3 in SupSB media.

are at. SA is expressed earlier than MHC during cardiac differentiation^[34]. Cultures expressing a higher ratio of SA to MHC (16:1) indicate an earlier stage of differentiation in comparison to cultures with lower ratios.

The results from qRT-PCR analysis showed no acceleration in the differentiation process when cells cultured with supplements (BSA and HySoy) were compared to SB media, with expression of pluripotent and mesoderm markers showing similar trends. However, there was a significantly higher expression of *Nkx 2.5*, indicating a higher population of cardiac progenitor cells in the cultures with supplements. MHC expression showed a higher general trend, but not a significant increase, when comparing cells cultured with individual supplements to those cultured in SB media only. However, when the supplements were combined, their additive effect produced a significantly higher expression of MHC, indicating that SupSB media improves the differentiation efficiency of cardiomyocytes.

Albumin, as a major serum protein with a typical concentration of 50 mg/mL in blood, accounts for approximately 60% of the total protein in mammals^[35]. Although there is such an abundance of albumin, its physiological actions and molecular mechanisms are not fully understood^[36,37]. The main functions of albumin have been summarized to include (1) maintenance of blood oncotic pressure and pH; (2) binding and transport of physiologically important nutrients, including lipids, metal ions, amino acids and other factors; and (3) antioxidant functions but mainly from the perspective of its role in blood circulation^[35].

Protein hydrolysates are known as a potential source of metabolizable materials including amino acids, oligopeptides, iron salts, lipids and trace elements. Their beneficial effects to the growth of animal cell culture have been known for more than two decades^[38] and are generally thought to act as a concentrated balanced nutrient mixture that may partly or fully replace serum^[39,40]. In recent years suppliers have improved their production processes to generate more consistent products with less batch-to-batch variability for the biopharma industry^[41]. Multiple reports have shown the effect of protein hydro-

lysates on growth in a variety of cell lines including both animal and rodent cells^[40,42]. This study further validates HySoy as a suitable supplement for cardiomyocyte differentiation.

The increase in cardiomyocyte efficiency was attributed to two reasons: (1) increased cell growth; and (2) increased differentiation efficiency, probably due to the improved metabolic state of the cells. Both BSA and HySoy have been repeatedly reported to increase cell densities in various different cell lines, and thus, the increase in cell growth observed during cardiomyocyte differentiation is not surprising. BSA and HySoy are also more defined in comparison to Fetal Bovine Serum, which is widely used in current EB and monolayer differentiation protocols^[4,10]. We assume that the addition of albumin and HySoy to the differentiation medium improves cardiomyocyte cell yields and viability by their antioxidant activity, conferring needed metabolized nutrients and the ability to transport nutrients (*e.g.*, lipids) to the cells. Previously it has been shown that insulin inhibits the process of cardiomyogenesis, therefore it was removed from the SB media^[43]. BSA and HySoy supplements were able to restore the proliferative capacity of cardiomyocytes in this insulin-free SupSB media. Moreover, it was reported that BSA can also help in the transport of small molecules such as the p38 MAPK inhibitor to the cells^[35]. Maturation of cardiomyocytes into ventricular, atrial and nodal phenotypes occurred within 16 d of differentiation as shown by the electrophysiology characterization. Ventricular phenotype maturation was much faster in comparison to other works which indicated a time frame of 60 d^[8].

Currently, there are multiple companies offering complete medium for hESC cardiac differentiation, these media use expensive ingredients and thus they are considerably more expensive than SupSB media. This work is the first step in developing an inexpensive and efficient cardiomyocyte differentiation protocol that can be used by commercial companies to produce cardiomyocytes. To the best of our knowledge, this is the first report of the benefits of BSA and HySoy on improving cardiomyogenesis of pluripotent human stem cells. Future works into cardiomyocyte purification and increase cardiomyocyte yield is needed.

In conclusion, we have created a simple, robust and cost effective media that significantly improves cardiomyocyte differentiation over many passages for multiple pluripotent cell lines that will be useful for research and cell therapy applications.

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COMMENTS

Background

Heart disease is one of the most common causes of mortality in the world

and accounts for more than 800000 deaths per year on average in the United States alone. After an episode of major cardiac insult (such as a myocardial infarction), the heart can lose up to 2 billion cardiomyocytes. Being an organ that cannot auto-regenerate, progressive heart failure develops. Cardiomyocyte cell therapy can thus be a potential cure for heart failure after myocardial infarction, and it is suggested that at least one billion cardiomyocytes will be required per patient for such treatment

Research frontiers

Human embryonic stem cells (hESCs) present an attractive cell source for generating large amounts of cardiomyocytes, due to their pluripotency and ability to proliferate for multiple passages. As such, several studies have reported techniques to efficiently differentiate hESCs to cardiomyocytes via growth factors and small molecule inhibitors. Specifically, a protocol based on the inhibition of p38 MAPK with small molecules displayed the ability to be scaled up in a cost efficient manner. However, low cell yield and viability were inherent to this protocol, reducing the output of cardiomyocytes.

Innovations and breakthroughs

Soy hydrolysate (HySoy) and bovine serum albumin (BSA) were found to improve cell viability during cardiomyogenesis of human embryonic and induced pluripotent stem cells in serum free medium. The addition of both supplements leads to an increase in cell viability, cell yield and cardiac marker expression during cardiomyocyte differentiation, translating to an overall increase in cardiomyocyte yield (2.6 fold increase over non supplemented medium).

Applications

Authors have created a simple, robust and cost effective serum free medium (SupSB media) that significantly improves cardiomyocyte differentiation of human pluripotent stem cell in suspended embryoid bodies culture. The new medium can be used for research and cell therapy applications using human embryonic and induced pluripotent cultures.

Terminology

p38 MAPK are a class of protein kinases involved in pathways that deal with differentiation, apoptosis and autophagy. p38 MAPK was inhibited with small molecule, SB203580.

Peer review

The authors have carried out an interesting study in which they developed an inexpensive and efficient cardiomyocyte differentiation protocol that can be used to produce cardiomyocytes. They demonstrated the benefits of BSA and HySoy on improving cardiomyogenesis of pluripotent human stem cells.

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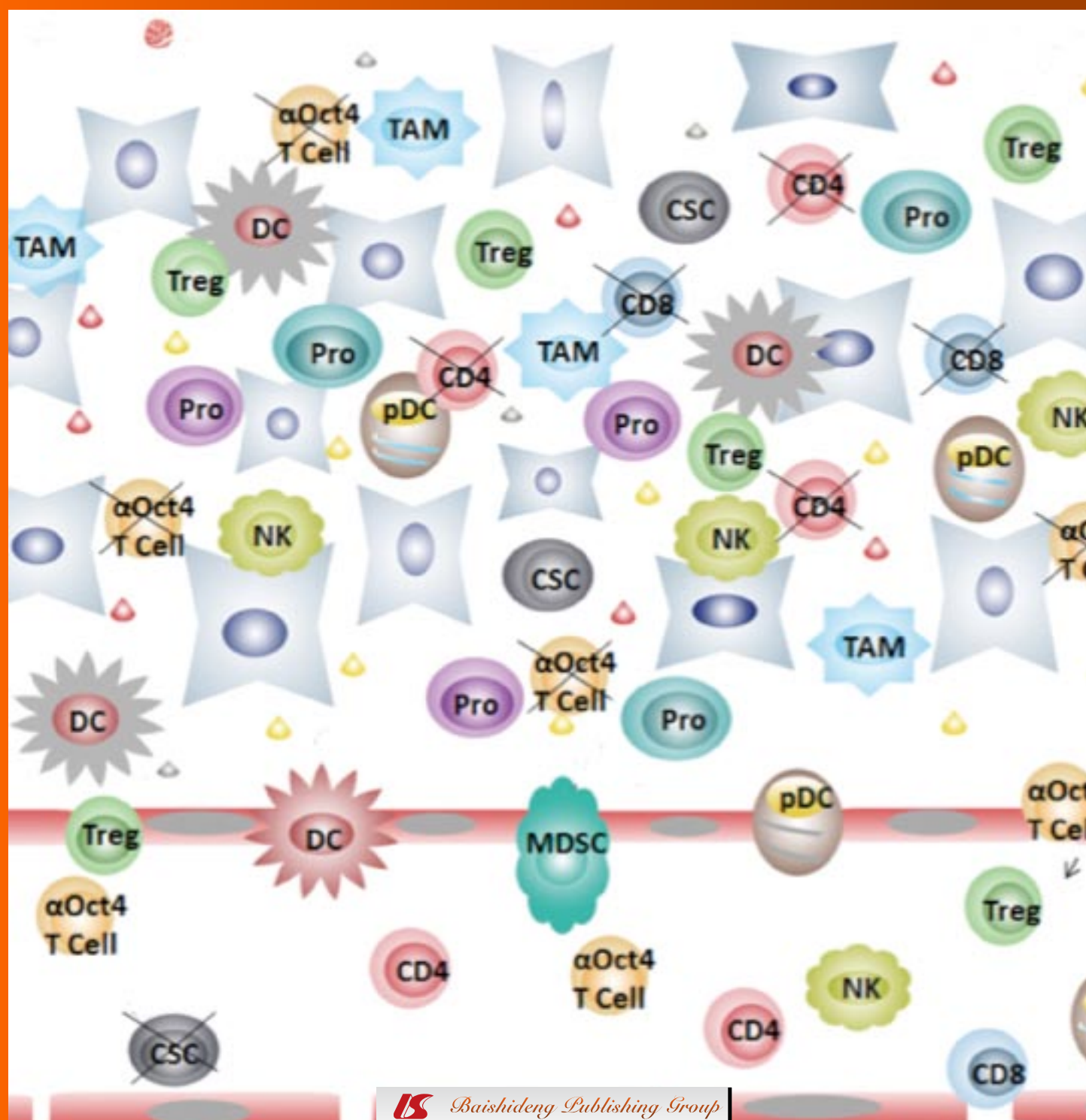
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Advances in homology directed genetic engineering of human pluripotent and adult stem cells

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Engineered viruses; Synthetic restriction endonucleases

Core tip: This manuscript focuses on the development of novel technologies to precisely alter the human stem cell genome and highlights their implications for both basic and applied stem cell research. Specifically, we discuss the development of two main technologies: molecular engineering of viral vectors and design of artificial endonucleases. We also discuss the merits of combining these complementary approaches and suggest other possible strategies that could be explored to further improve genetic engineering of human stem cells.

Abstract

The ability to introduce precise genomic modifications in human cells has profound implications for both basic and applied research in stem cells, ranging from identification of genes regulating stem cell self-renewal and multilineage differentiation to therapeutic gene correction and creation of *in vitro* models of human diseases. However, the overall efficiency of this process is challenged by several factors including inefficient gene delivery into stem cells and low rates of homology directed site-specific targeting. Recent studies report the development of novel techniques to improve gene targeting efficiencies in human stem cells; these methods include molecular engineering of viral vectors to efficiently deliver episomal genetic sequences that can participate in homology directed targeting, as well as the design of synthetic proteins that can introduce double-stranded breaks in DNA to initiate such recombination events. This review focuses on the potential of these new technologies to precisely alter the human stem cell genome and also highlights the possibilities offered by the combination of these complementary strategies.

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Key words: Human stem cells; Genetic engineering;

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INTRODUCTION

Gene targeting *via* homologous recombination (HR) presents a precise way to manipulate the mammalian genome at specific loci. Such legitimate site-directed gene targeting requires homology between the donor DNA and the endogenous chromosome, and results in the substitution of DNA between the homologous donor and the endogenous chromosomal sequence (Figure 1). Creating or correcting specific mutations in human stem cells provides great utility in stem cell research; for example, gene knock-in and knockout studies can be carried out to identify and study specific genes expressed and involved in stem cell fate decisions, as well as to create lineage-specific reporter cell lines to screen for signaling pathways *in vitro* and to monitor differentiation and proliferation of stem cells following transplantation *in vivo*^[1-5]. Targeted gene mutations also facilitate the generation of models to investigate human developmental diseases and screen

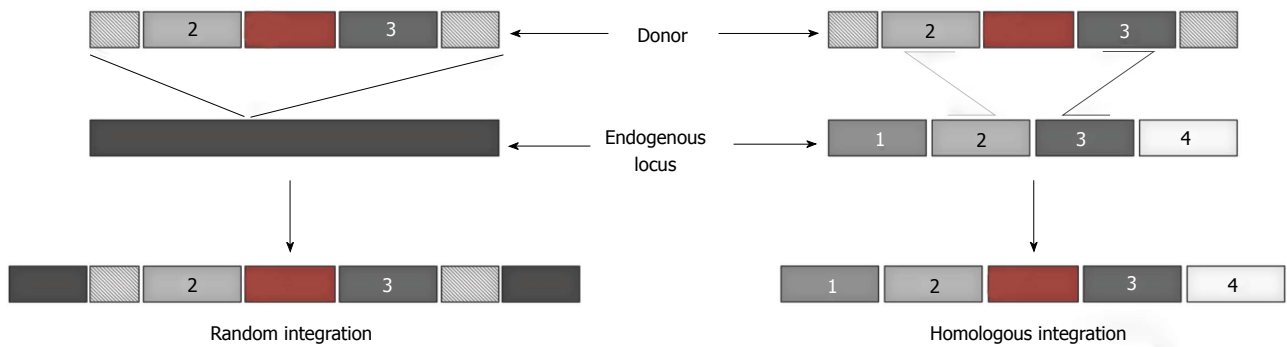


Figure 1 Random and homologous integration. Introduction of a foreign gene into mammalian cells can either result in its random integration into the endogenous chromosomal DNA, or site-specific integration at the desired location dictated by the homology between the donor DNA and the endogenous target locus.

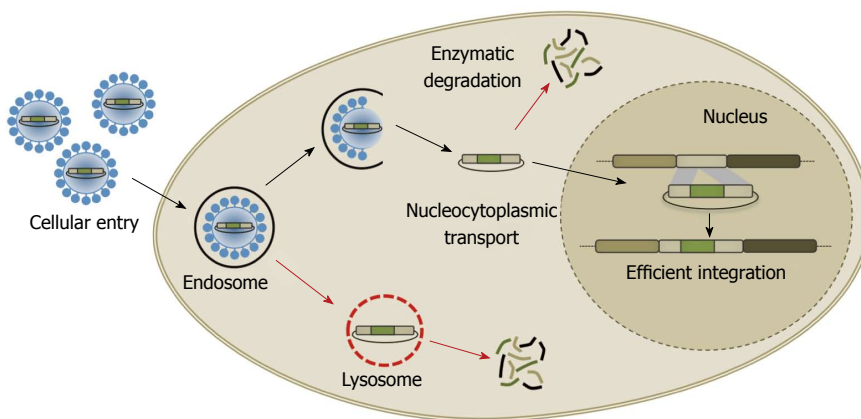


Figure 2 Barriers to gene targeting. The different barriers that gene delivery vectors must overcome for successful gene targeting include cell binding and internalization, intracellular trafficking and endosomal escape, translocation through the nuclear envelope, and efficient site-specific integration.

for potential therapies^[6]. Therefore, mediating efficient gene targeting in human stem cells impacts the future application of these cells in tissue engineering, regenerative medicine, and high-throughput drug discovery and toxicology. Current strategies to manipulate the mammalian cell genome are mostly viral-based; several viral gene delivery systems, including adenovirus, retrovirus, and lentivirus provide high transduction efficiencies, integration into the host cell genome, and high levels of gene expression^[7-9]. Lentiviral vectors in particular are highly efficient and result in stable, long-term transgene expression without gene silencing and have been extensively employed in studies of human stem cells^[9]. However, viral approaches are often complicated by random vector integration into the host genome that results in genomic instability leading to increased risk of oncogenesis, a major concern for downstream clinical applications^[10,11]. In contrast, non-viral methods, such as encapsulation of DNA by lipid or cationic polymer vesicles and naked DNA delivery by either physical (electroporation) or chemical methods, deliver DNA episomally and therefore can in principle avoid insertional mutagenesis due to random integration; however, their application for gene targeting is often restricted by inefficient delivery and by the inability to provide sustained gene expression^[12,13]. Thus, despite the enormous potential of genetically modified stem cells in basic and translational research, low rates of targeted gene modifications in human stem cells have limited the realization of these applications.

This review focuses on recent advances in gene targeting techniques, with particular emphasis on technologies that significantly enhance the rates of homology directed genetic engineering of human pluripotent and adult stem cells. These advances open up a range of experimental and therapeutic possibilities, including instruction of stem cell differentiation into therapeutically relevant cells and development of stem cell-based disease models for drug development.

BARRIERS TO GENE TARGETING

Low efficiencies of gene targeting can result from a combination of extracellular and intracellular barriers and increasing our understanding of the contributions of these barriers to gene targeting, as well as strategies to successfully overcome them will benefit the design of efficient vectors for enhanced gene transfer and targeting. The series of barriers that challenge the progress of the gene targeting constructs to the cell nucleus include inefficient transport across the cell membrane and the nuclear envelope, as well as diffusional and metabolic barriers of the cytoplasm that reduce the amount of intact donor DNA constructs that reach the nuclear envelope (Figure 2)^[13-16]. Inside the nucleus, exogenous DNA can integrate at predetermined sites *via* homologous recombination into the genome, although in general the rates of random integration are far greater than those of homologous integration; this low spontaneous frequency of site-specific

integration necessitates identification of the small fraction of cells undergoing homologous integration by a combination of positive and negative selection, which require considerable time and expertise^[17,18].

Recent years have witnessed the emergence of two main strategies to overcome these barriers: (1) molecular engineering of viral vectors to improve their safety and efficiency and (2) artificial endonucleases that introduce site-specific DNA double-stranded breaks to stimulate targeted integration. Here we will review the use of these novel technologies to carry out homology directed gene knock-outs, knock-ins, and corrections in human stem cells and their potential for advancing basic and applied stem cell research.

MOLECULAR ENGINEERING OF VIRAL VECTORS

Lentivirus-based gene delivery vectors have been widely examined for *in vivo* and *in vitro* applications owing to their inherent advantages - specifically, they can efficiently transduce both dividing and non-dividing cells, can be pseudotyped to optimize tropism to a specific cell or tissue of interest, can transduce both dividing and non-dividing cells, and do not induce significant immune responses^[19,20]. However, their broad applicability has been limited by the risk of mutagenesis associated with random vector integration into the target genome. Recently, it has been demonstrated that the random insertion event can be prevented by simply introducing mutations in the integrase protein of the virus that facilitates viral genome integration^[21]. Such integrase-deficient lentiviral vectors (IDLV) represent new avenues for targeted genetic engineering of human stem cells due to their ability to deliver episomal donor DNA for homologous recombination, while retaining their unique ability to efficiently infect both dividing and non-dividing cells. For example, IDLV have been shown to efficiently transduce both dividing (human embryonic kidney 293) and non-dividing cells (primary neurons and astrocytes) *in vitro*. And the transgene expression, monitored using GFP fluorescence, was transient in dividing cells and stable in non-dividing cells, consistent with transcription from episomal genetic sequences. Moreover, residual integration activity studies confirmed significantly reduced occurrence (10^2 - 10^3 fold lower) of integration events compared to wild-type lentiviral vectors. IDLVs have also been used to provide clinical benefits for patients with X-Linked adrenoleukodystrophy (ALD)^[22]. ALD is caused by a mutation in the *ABCD1* gene encoding for the ALD protein, which leads to accumulation of abnormally high levels of very long chain fatty acids (VLCFAs) causing the adrenal cortex and the myelin membranes that surround nerves to cease functioning. A lentiviral vector encoding the wild-type *ABCD1* was introduced into CD34⁺ cells obtained from ALD patients and the transformed cells were then re-infused into patients. Quantitative analysis using RT-PCR showed that the expression of *ABCD1* transgene was

four- to five-fold higher than the endogenous mutated *ABCD1* gene. Furthermore, after 20-24 mo, the VLCFA levels in patients decreased by up to 39%. Finally, IDLVs encoding for cytokines have been used to induce human monocytes to differentiate into dendritic cells and the resulting induced dendritic cells were functional and capable of stimulating multivalent immune responses *in vitro* and *in vivo*^[23]. In addition to developing non-integrative vectors, researchers are also pursuing approaches to engineer safer viral vectors by overriding the intrinsic preferences of viral genomic integration for transcriptional start sites (TSS); for example, Lim *et al*^[24] discovered that the addition of DNA binding domains at key sites within retroviral vectors shifted their integration patterns toward regions where TSS are relatively rare. Another approach is the incorporation of Cre recombinase into lentiviral vectors for directed gene targeting^[25]. Although this approach is limited by Cre's specificity for loxP sites that are not native to the mammalian genome, researchers have reported the presence of pseudo loxP sites in the human genome that can be targeted by either wild-type Cre or Cre variants^[26].

Recently, adeno-associated virus (AAV) based gene targeting vectors have attracted significant attention as an alternative to the more commonly used lentivirus- and adenovirus-based vectors, mainly because of their ability to deliver transgenes in episomal form and mediate long-term gene expression in dividing and non-dividing cells of numerous human tissues^[27]. Additionally, it has been demonstrated that AAV vectors can also be used to introduce site-specific genetic modifications in human cells with high efficiencies (up to 1%) that are 10^3 - 10^4 -fold higher than plasmid constructs delivered using electroporation^[28]. There is evidence supporting that AAV genome's inverted terminal repeats facilitate its stable integration into the host cell genome using the RAD51/RAD54 pathway of HR in eukaryotes^[29]. The ability of AAV to introduce targeted genetic modifications in human cells has been exploited in a variety of applications including the creation of isogenic (knock-in) cells that could be used to study cancer genes in a high-throughput manner. Di Nicolantonio *et al*^[30] recently used this approach to evaluate the effect of drugs on isogenic breast epithelial cells expressing wild-type and mutant alleles of *BRAF*, *KRAS*, *PIK3CA*, and *EGFR* genes previously implicated in oncogenic pathways. Erlotinib and gefitinib (*EGFR* tyrosine kinase inhibitors) were shown to prevent cell proliferation and induce apoptosis selectively in cells carrying the *EGFR* delE746-A750 deletion, while the cells with *KRAS* or *BRAF* mutations were more resistant to these drugs.

AAV-based vectors have also been evaluated for their ability to perform HR mediated gene targeting in human stem cells, and these studies revealed that the gene-targeting frequency for AAV-based vectors was about 1×10^{-5} ; the low frequencies observed in this study could be attributed to low efficiency of naturally occurring AAV variants in mediating gene delivery to human stem

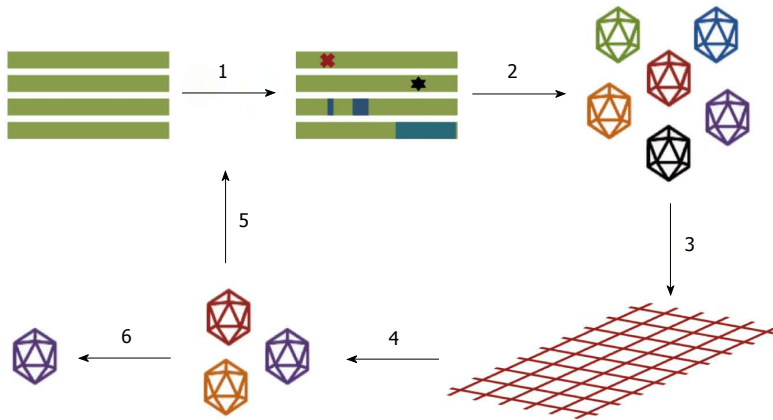


Figure 3 Schematic of steps in the directed evolution of adeno-associated virus. The wild-type adeno-associated virus (AAV) cap genes are mutated to create large genetic libraries (1) and the mutant cap genes are packaged to generate libraries of AAV particles (2), with each AAV composed of a variant capsid surrounding a viral genome encoding that capsid. The resulting AAV libraries can be subjected to appropriate selective pressures (3) to isolate vectors with modified capsids that facilitate the AAV variants to efficiently surmount these pressures (4). Examples include isolation of AAV variants with the ability to evade neutralizing antibodies, altered receptor binding and cell tropism, and enhanced gene delivery. Successful AAV variants are amplified and recovered (6), or can be subjected to additional rounds of mutagenesis and selection (5). Adapted from Maheshri *et al.*^[33] and Bartel *et al.*^[35].

cells^[31,32]. Directed evolution approaches, therefore, have been applied to rapidly engineer AAV mutants with the capacity for high efficiency gene delivery to human adult and pluripotent stem cells. Unlike the more deliberate rational design approaches where the virus is modified based on an understanding of the mechanistic consequences of a particular set of changes, directed evolution is a rapid, high-throughput selection approach to create and isolate novel virus mutants with specific properties of interest through appropriate selection pressures from millions of genetic variants. Central to the approach is the highly evolvable AAV capsid that determines the viral infectivity and tropism. This approach employs iterative rounds of genetic diversification, either *via* error-prone PCR or DNA shuffling and artificial selection pressures to incrementally alter the desired phenotypes. For example, it has been demonstrated that directed evolution of AAV capsids can be used to select variants with the ability to evade antibody neutralization and deliver genes more efficiently than wild-type AAV^[33] (Figure 3). Other successful examples include the isolation of AAV variants with altered receptor binding and cell tropism *in vitro* and *in vivo*, and enhanced gene delivery^[34,35]. Using this approach, researchers have recently created a novel AAV variant - AAV *r3.45* that mediates efficient gene delivery to both murine (infection efficiency > 40%) and human (infection efficiency > 30%) neural stem cells (NSCs) when compared to naturally occurring AAV variants (infection efficiency < 1%)^[36]. More importantly, this increase in gene delivery efficiency was accompanied by up to 10-fold enhancement in the ability to repair single-base pair mutations in rat NSCs, relative to naturally occurring AAV. In a related study, researchers have also demonstrated the ability of directed evolution approaches to isolate a new AAV variant, AAV *1.9* that exhibited enhanced gene delivery to both human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (infection efficiencies: 40%-50%) that was accompanied by a corresponding increase in AAV *1.9*'s capacity for gene targeting, with efficiencies as high as 0.12%, in human ESCs and iPSCs^[37].

Another strategy that has been used to improve the ability of AAV vectors to deliver genes to human cells is to facilitate AAV's intracellular trafficking (from the

cytoplasm to the nucleus) following cellular uptake. Previous studies have shown that inhibition of the EGFR-PTK mediated phosphorylation of AAV capsid proteins at tyrosine residues results in decreased ubiquitination of AAV capsids and their proteasome-mediated degradation, thereby enabling nuclear transport of AAV vectors and improved gene delivery to the cell nucleus^[38]. Based on these results, Zhong *et al.*^[39] hypothesized that substitution of surface-exposed tyrosine residues on AAV capsids may lead to the design of AAV vectors that facilitate efficient delivery of genes to the nucleus of target cells at lower doses. Consistent with this hypothesis, they observed that site-directed mutagenesis of surface-exposed tyrosine residues significantly improve the transduction efficiency of AAV vectors (about 10-fold increase) in human epithelial cells *in vitro*. It has also been demonstrated that tyrosine mutant AAV vectors display enhanced gene delivery efficiencies *in vivo* when compared to wild-type AAV vectors^[40]. Although further investigation is warranted, such enhanced AAV vectors engineered through rational design to avoid cytoplasmic degradation could also potentially enhance the efficiencies of AAV-mediated gene targeting in human stem cells.

INTRODUCTION OF SITE-SPECIFIC DNA DOUBLE-STRANDED BREAKS

It has been previously demonstrated that the rate of homologous recombination with donor DNA can be enhanced by the introduction of double-stranded breaks (DSBs) in the mammalian genome using nucleases such as I-SceI^[41]. Building upon this important prior work, scientists have recently developed highly specific nucleases such as zinc finger nucleases that can be in principle engineered to target and introduce DNA DSBs at any site in the genome and thus facilitate targeted modifications of endogenous genes. Zinc finger nucleases (ZFNs) are synthetic proteins composed of a DNA-binding domain that can be engineered to target desired DNA sequences fused to a non-specific endonuclease domain usually derived from the FokI enzyme^[42]. The zinc finger binding domain contains several amino acids stabilized by a zinc ion; such zinc finger domains used in tandem can guide the non-specific DNA cleaving domain to create a double-strand

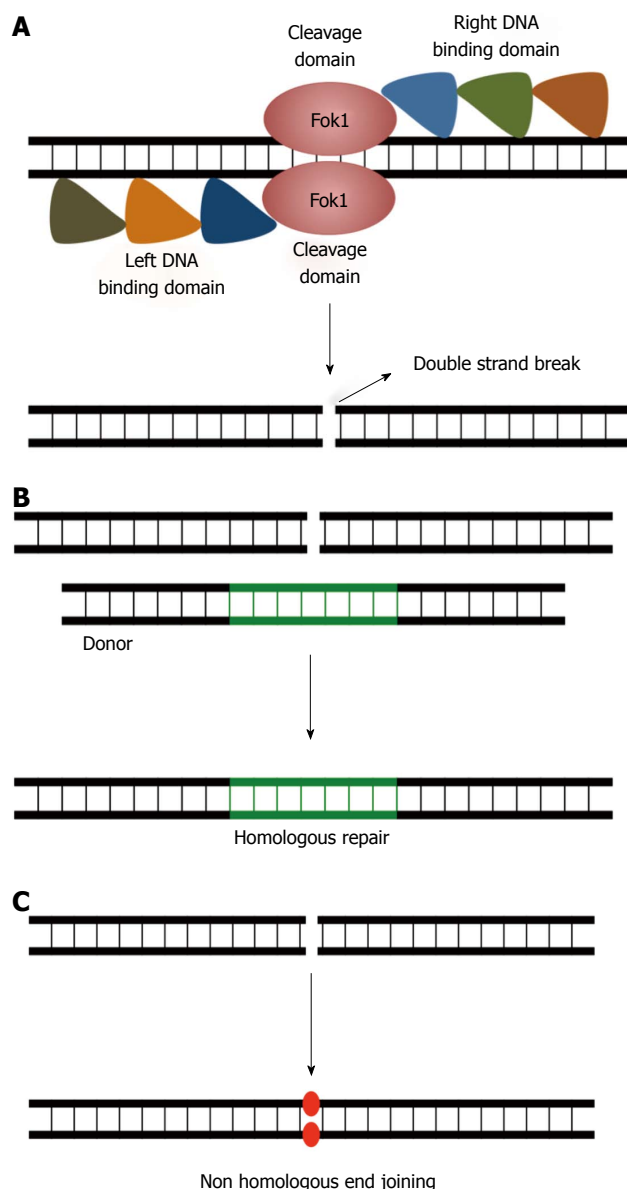


Figure 4 Gene editing using Zinc finger nucleases. **A:** Schematic showing a zinc finger nuclease (ZFN) dimer bound to its target locus to introduce a site-specific double-stranded break (DSB) in the chromosome, each ZFN monomer consists of three to six zinc finger domains fused to the DNA-cleavage domain of the FokI endonuclease. **B:** The DNA DSB can be repaired via either the homologous recombination (HR), or **C:** the non-homologous end-joining pathway. HR requires a homologous DNA template to accurately repair DSBs in the chromosome, and ensures high fidelity DNA repair. In contrast, non-homologous end joining does not rely on sequence homology between the DNA ends for ligation and can be error-prone.

break at a specific target site within a genome. The cleavage domain must dimerize in order to become active and cleave DNA, thus two ZFN subunits are assembled as heterodimers at the target cleavage site (Figure 4A). Such double-strand breaks are typically repaired by two cellular repair pathways: non-homologous end joining (NHEJ) and homologous recombination (Figure 4B and C).

NHEJ is the major DNA DSB repair pathway in eukaryotic cells and it repairs DNA breaks by direct ligation without the need for a homologous template. Typically, this repair pathway does not create mutations if the two

ends are compatible; however, when the two ends are not compatible then NHEJ is prone to cause mutations (insertion or deletion), thereby knocking out the gene (Figure 4C). The ability of ZFNs to facilitate targeted gene knockouts has been recently exploited to disrupt the *CCR5* gene in human CD4⁺ T-cells^[43]. *CCR5* is a co-receptor used by the human immunodeficiency virus (HIV) to infect cells of the immune system; the disrupted gene produces malformed *CCR5* proteins and confers resistance to HIV infection. The safety and efficacy of T-cells modified by ZFNs targeting the *CCR5* gene are currently being evaluated in a clinical trial (ClinicalTrials.gov identifier NCT00842634). Continuing to build upon this work, researchers have also used ZFNs to disrupt the *CCR5* gene in human hematopoietic stem/progenitor cells^[44], and this approach may lead to providing individuals with a self-renewable and potentially lifelong source of HIV-resistant immune cells.

In contrast, DNA repair using the HR pathway relies on a homologous DNA sequence that promotes accurate repair of DSBs (Figure 4B). In addition to gene knockout, this approach also allows precise correction of mutated or dysfunctional human genes to restore normal function^[45,46] and targeted gene addition into a specified location in the human cellular genome^[47]. Correction of endogenous genes in human cells was first demonstrated in a study using ZFNs that recognize an X-linked severe combined immune deficiency mutation in the *IL2R γ* gene, with targeting frequencies as high as 5.3% in human CD4⁺ T-cells^[45]. Recently, it has also been demonstrated that ZFNs can enable targeted gene correction in human embryonic and induced pluripotent stem cells; in this study, the authors demonstrate gene targeting efficiencies of up to 0.24% when the cells were cotransfected with donor DNA and ZFNs specific to the target site, a > 2400-fold increase in the gene correction efficiencies when compared to without ZFNs (< 10⁻⁶). This ability of ZFNs to facilitate site-specific gene corrections has immense potential to facilitate the generation of genetically corrected, patient-derived cells for autologous transplantation therapies. Recently, Joung and coworkers demonstrated *in situ* correction of the disease causing mutation in iPSCs derived from sickle cell anemia patients^[48]. Finally, homology directed gene addition has also been demonstrated in human pluripotent stem cells; ZFNs can be used to target both expressed (*OCT4*) and non-expressed (*PITX3*) genes in human pluripotent stem cells and knock-in reporter genes (*eGFP*) to create reporter cell lines^[47]. ZFNs can also be used to knock-in mutations in the mammalian genome to generate *in vitro* models of human disease. For example, Soldner *et al.*^[49] reported the use of ZFNs to develop genetically defined human *in vitro* models of Parkinson's disease (PD) by introducing PD-causing α -synuclein mutations into hESCs.

Despite the huge therapeutic potential for ZFNs there are complications with toxicity and specificity. Extensive testing of several variants of ZFNs is required to find the right ZFN pair to minimize off-target binding, therefore making the process both time-consuming and expensive.

Recent discoveries of proteins in bacterial pathogens in plants have revealed new DNA binding domains: TAL effectors, which have tandem repeats of amino acids that bind to a single base pair of DNA. These TAL effectors in conjunction with the FokI nuclease (TALENs) have been shown to function similar to ZFNs^[50,51]. The TALEN technology provides scientists with an increase in modularity allowing for the creation of many variants at little time and cost. Although in its infancy, this new technology has shown great promise; TALEN-mediated double-strand breaks have initiated HR at similar frequencies and precision as ZFNs in human stem cells. For example, Hockemeyer *et al.*^[52] demonstrated the use of TALENs to target three endogenous loci (PPP1R12C, OCT4, and PITX3) in hESCs and iPSCs at efficiencies comparable to those reported for ZFNs for the same loci. TALENs have also been recently used to generate human stem cell-based disease models. Cowan and co-workers reported the generation and use of TALEN pairs to efficiently generate mutant alleles of 15 genes in human somatic and pluripotent stem cells and observed minimal off-target events. Further, they demonstrated the utility of TALEN-mediated gene editing to develop detailed genotype-phenotype relationships for four genes known to be directly linked to various human disease conditions^[53]. Despite these advances, further research is required to fully evaluate the potential of TALENs before they replace ZFNs for facilitating targeted gene modifications in human stem cells.

CONCLUSION AND FUTURE PERSPECTIVES

This review highlights the development of two complementary approaches to efficiently modify the human stem cell genome. The first approach involves molecular engineering of viruses, either *via* rational design or directed evolution, to yield novel vectors that can mediate safe and efficient gene targeting in human stem cells. The second approach is the design of custom endonucleases to target unique predetermined sites in the human genome and facilitate efficient homologous recombination with donor DNA. Each approach individually has yielded remarkable enhancements in gene targeting efficiencies and it is possible that a combination of these complementary approaches can further enhance gene targeting. For example, it has recently been demonstrated that an evolved AAV variant can function effectively in conjunction with ZFNs to mediate highly efficient gene targeting (> 1%) in human pluripotent stem cells. In this study, it was observed that novel AAV vectors can be created to mediate efficient gene targeting in human pluripotent stem cells and in the presence of targeted DSBs generated by the evolved AAV mediated co-delivery of ZFNs, these efficiencies can be further enhanced^[57]. The observed enhancements in gene targeting efficiencies of engineered viral vectors in the presence of local DSBs are not limited to AAV; researchers have also demonstrated

that baculoviral and IDLV vectors can be used to co-deliver both ZFNs and DNA donor templates to induce efficient gene targeting in human stem cells^[54-56]. In these cases, researchers observed high rates of homology directed gene targeting accompanied by low frequencies of random chromosomal integration.

Other strategies may explore the possibility of combining rational vector design with directed evolution - for example, can gene delivery efficiencies of engineered AAV vectors evolved to infect human stem cells be further improved by site-directed mutagenesis of surface-exposed tyrosine residues?

In summary, the considerable progress in enhancing the capacity for homology directed gene targeting bear profound implications on basic and applied human stem cell research. Combining complementary approaches and as-of-yet unexplored possibilities offered by such strategies will yield the next set of developments in this exciting and challenging field.

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New insights for pelvic radiation disease treatment: Multipotent stromal cell is a promise mainstay treatment for the restoration of abdominopelvic severe chronic damages induced by radiotherapy

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Abstract

Radiotherapy may induce irreversible damage on healthy tissues surrounding the tumor. It has been reported that the majority of patients receiving pelvic radiation therapy show early or late tissue reactions of graded severity as radiotherapy affects not only the targeted tumor cells but also the surrounding healthy tissues. The late adverse effects of pelvic radiotherapy concern 5% to 10% of them, which could be life threatening. However, a clear medical consensus concerning the clinical management of such healthy tissue sequelae does not exist. Although no pharmacologic interventions have yet been proven to efficiently mitigate

radiotherapy severe side effects, few preclinical researches show the potential of combined and sequential pharmacological treatments to prevent the onset of tissue damage. Our group has demonstrated in preclinical animal models that systemic mesenchymal stromal cell (MSC) injection is a promising approach for the medical management of gastrointestinal disorder after irradiation. We have shown that MSCs migrate to damaged tissues and restore gut functions after irradiation. We carefully studied side effects of stem cell injection for further application in patients. We have shown that clinical status of four patients suffering from severe pelvic side effects resulting from an over-dosage was improved following MSC injection in a compassionate situation.

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Key words: Cell therapy; Radiotherapy; Irradiation; Stem cells; Multipotent stromal cells; Mesenchymal stem cells; Clinical trial

Core tip: Multipotent stromal cells (MSCs) provide a long-term effect in inhibition of chronic inflammation and a fistulisation, arrest of hemorrhagic syndromes for the hemorrhagic cystitis. MSCs are successfully used to treat the late effects of radiotherapies for breast cancer and radiodermatitis. Their efficiency was also demonstrated on pain reduction. Concerning clinical trials to cure abdominal late severe damages of radiotherapy, one compassionate trial has demonstrated the feasibility of cell therapy treatment for patients overdosed.

Chapel A, Francois S, Douay L, Benderitter M, Voswinkel J. New insights for pelvic radiation disease treatment: Multipotent stromal cell is a promise mainstay treatment for the restoration of abdominopelvic severe chronic damages induced by radio-

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INTRODUCTION

The number of people with cancer is expected to increase from 12.7 million in 2008 (latest available figure) to 20.3 million in 2030^[1]. Sixty percent of these patients receive radiation therapy with a chance of recovery of fifty percent. The number of radiotherapy centers in 2011 is about 7500, linear accelerators in clinical use are approximately 10000 (IAEA 2011). Up to 500000 patients per year undergo abdominal or pelvic radiotherapy worldwide. Eight out of ten will develop acute gastrointestinal symptoms and 5% to 10% will develop severe intestinal complications within 10 years after treatment. The efficacy of abdominal or pelvic radiotherapy requires an optimal compromise between normal tissue damage and tumor control that is the risk/benefit ratio. The lack of curative treatment at present and the potential severity for the disorder highlight the importance of novel and effective therapeutic strategies for gastrointestinal complications after radiation exposure^[2]. Proton therapy is an attractive method to attenuate toxicity of radiotherapy because of the decrease of integral radiation dose to normal tissues, which should lead to fewer late side effects. Unlike other types of radiation therapy that use X-rays to destroy cancer cells, proton therapy uses a beam of special particles called protons, inducing less damage to the surrounding healthy tissue. There will be a lower risk of normal tissue toxicity associated with proton therapy because of a lower delivered dose outside of the target tissue^[3].

POST-RADIATION DAMAGES

Radiation lesions' etiology focused on epithelial ulceration, microvascular destruction, mucosal and submucosal inflammation for the acute radiation enteropathy. The severity of acute radiation enteritis may be predictive for more severe chronic gastrointestinal symptoms. Acute or chronic side effects can also be aggravated after a radiotherapy accident such as an overdose. The risk factors for complications are age, irradiated volume, histories of abdominal surgery, androgenic hardship, diabetes, hemorrhoids and inflammatory intestinal diseases^[4].

Treatments to manage post-radiotherapy pelvic damages

Treatments usually applied are only symptomatic. The systematic study of therapy complications shows: (1) coagulation with the plasma argon is insufficient in the long-term; (2) a moderated effect of the short chains fatty acids and of hyperbaric oxygen therapy; and (3) an insufficiency of proof for the efficiency of formaline,

5-amino-salicylic acid, sulfanazine, vitamin A and pentoxifylline. Corticoids remain scarcely effective, because the cause is more ischemic than inflammatory. Hence, more effective approaches are of primary clinical importance^[5].

Targeted organ of pelvic radiation and chronic effects

The use of radiation therapy for the treatment of pelvic malignancy, including that of the prostate, cervix, uterus and ovaries, has increased in recent years. The frequency of complications is of 12% for uterus cancers and 7% for prostate cancers. The symptoms are proctopathies and radic cystitis. Intra-abdominal organs located close to cancerous tumors can be affected during radiotherapy. The most radiosensitive organ of the intra-abdominal area is the small intestine. Acute radiation responses affect patient quality of life, causing abdominal pain, diarrhea/constipation sequences, and malabsorption that may interrupt or delay the radiotherapy protocol. Radiotherapy is associated with a high incidence of undesirable acute and/or chronic gastrointestinal complications that can affect the patient's quality of life and may even be life threatening. Exposure of the small intestinal tissue to ionizing radiation may lead to loss of its integrity by a dose-dependent stem cell depletion initiating gastrointestinal disorders. Radiation proctitis or proctopathy occur frequently and can be debilitating side effects of radiation therapy. There are 2 forms of radiation proctopathy, acute and chronic. The acute form occurs in nearly all patients. The incidence of the chronic form ranges from 2% to 20%. However, the true incidence may be underestimated. Chronic radiation proctopathy includes symptoms that occur as a continuation of early symptoms 3 mo after the completion of radiation therapy. The median onset is 8 to 12 mo, but the onset can occur as late as 30 years post therapy completion. Common symptoms include diarrhea, tenesmus, mucus/blood loss via the rectum, urgency, incontinence and pain. The most common symptom is rectal bleeding. Endoscopic finding include mucosal pallor, friability, spontaneous oozing, angiectasia and infrequently ulceration. These combinations of symptoms significantly affect patients' quality of life^[5].

Acute radiation tissue injury to the bladder is caused primarily by damage to the bladder mucosa, which contains cells that divide rapidly. This usually occurs during the treatment period. The underlying pathology of late adverse effects is different from that seen in acute reaction. Late responding tissues such as vascular and connective tissues, have a slow turnover rate, therefore, while they sustain radiation damages at the time of treatment, the effect are not expressed until repeated cell divisions are attempted. For this reason, late radiation tissue injury can take several months to many years to develop, and is largely function of the total radiation dose and fraction size. The pathological hallmark of late radiation tissue injury is obliterative endarteritis resulting in atrophy and fibrosis. Late radiation cystitis following radiation therapy for cancer in the pelvic region has an incidence of 5% to 10%. Late radiation cystitis can develop from 6 mo to

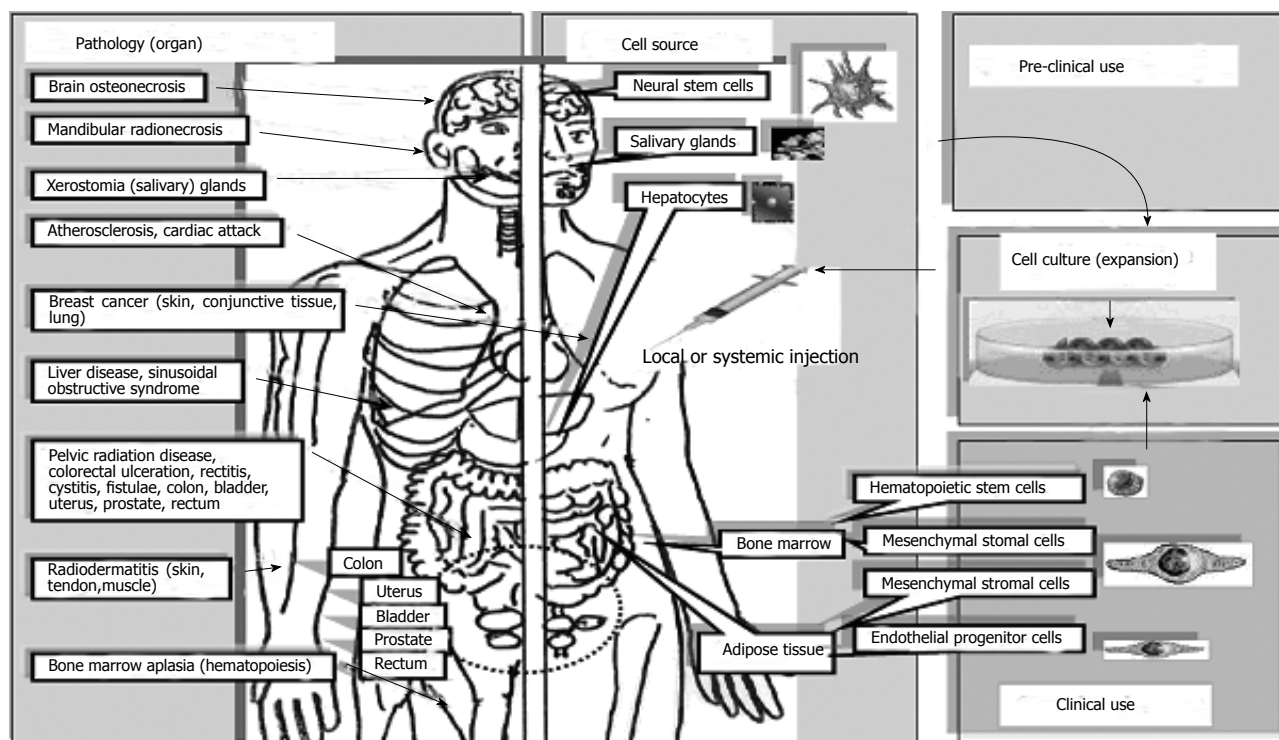


Figure 1 Clinical trials to treat consequences of radiotherapy. Clinical and pre-clinical use of stem cells to treat toxic side effects induced by radiotherapy on healthy tissue of radio-sensitive patients. Pathologies treated are (left side) osteonecrosis, xerostomia, atherosclerosis, cardiac attack, breast cancer, liver disease, pelvic radiation disease, radiodermatitis and bone marrow aplasia. Targeted organs (brain, salivary glands, mandibles, skin, liver, heart and rectum/bladder, bone marrow) are mentioned on the left side. On the right side, stem cells are isolated from several tissue sources, expanded *in vitro*, then injected locally (i loc) or intravenously (iv). Stem cells from bone marrow and adipose tissues are used in clinic. Other sources of stem cells (neural stem cell, salivary gland stem cell, hepatocyte and embryonic stem cell) are only used in animal models (pre-clinical use).

as long as 20 years after radiation treatment, with a mean latent period of 35 mo. One chronic manifestation is recurrent haematuria or haemorrhagic cystitis, defined as diffuse vesical bleeding. Haemorrhagic cystitis present a significant clinical problem once established. A minority of patients will develop severe bleeding that may become life threatening; in such patients conservative treatment strategies are often inadequate and radical surgery may be the only curative option^[6-8].

STEM CELL THERAPY

Multipotent (mesenchymal) stromal (stem) cell (MSC) therapy is currently among the most advanced cell therapy tools, with the availability of three Food and Drug Administration-approved products: Prochymal, provacel, and chondrogen. Stem cell-based approaches using MSCs are promising for the development of future therapy in therapeutics^[9] to correct radiodermatitis^[10], to improve haematopoiesis^[11,12], and to prevent graft *vs* host disease (GVHD) post-haematopoietic stem cell transplantation^[13]. Clinical studies have reported effects of MSCs on gastrointestinal healing such as the reversion of colon peritonitis in patients with GVHD or the treatment of rectovaginal and perianal fistulas in patients with Crohn's disease^[14].

Stem cell therapy of radiation damages

Stem cells can be used to treat toxic side effects induced

by radiotherapy on healthy tissue of radio-sensitive patients. Figure 1 illustrates where stem cells could be used and where MSCs are already used. The organs impacted by irradiation side effects that are mostly studied are: brain salivary glands, mandibless skin, liver, heart, rectum/bladder and bone marrow. Stem cells isolated from bone marrow; adipose tissue are easily expanded *in vitro*. Expanded or un-expanded stem cells are injected locally or intravenously. Stem cells from bone marrow and adipose tissues are already in clinical use. Other sources of stem cells (neural stem cell, salivary gland stem cell, hepatocyte and embryonic stem cell) are only used in animal models (pre-clinical use).

Preclinical treatment of pelvic radio-induced damages

As demonstrated in a preclinical model, MSCs may offer a novel strategy to treat pelvic radiation disease. After abdominal irradiation, MSCs have the capacity to engraft into the enteric mucosa^[14-18]. MSCs are able to repair radiation-induced intestinal damage by inhibiting ulceration^[19-21]. Mitigation of radiation-induced lethal intestinal injury can similarly be achieved by transplantation of bone marrow-derived adipose stromal cells (BMASC). BMASC increase blood levels of intestinal growth factors and induce regeneration of intestinal villi, thereby, accelerating functional recovery of the intestine^[22]. Furthermore MSC injection improved muscle regeneration and increased contractile function of anal sphincters

after injury^[23]. The effects of MSCs are a consequence of their ability to improve the renewal capability of the small intestine epithelium. MSC treatment favors the re-establishment of cellular homeostasis by both increasing endogenous proliferation processes and inhibiting radiation-induced apoptosis of the small intestine epithelial cells^[24]. MSCs release cytokines and growth factors such as, interleukin (IL)-11, human hepatocyte growth factor, fibroblast growth factor-2 and insulin-like growth factors. Each of these factor may be involved; they have been described earlier as facilitating intestinal mucosa repair, either through enhancement of cell proliferation or inhibition of epithelial cell apoptosis. Repeated infusion of MSC-derived bioactive components after abdominal irradiation increased the survival rate, decreased diarrhea occurrence, and improved the small intestine structural integrity of irradiated mice. By reducing the levels of pro-inflammatory cytokines, while inducing anti-inflammatory cytokines, MSCs may also dampen the systemic inflammatory response syndrome in radiation-induced gastrointestinal syndrome^[25]. François *et al.*^[24] evidenced the potential of MSC to limit radiation effects on the small intestine in an IL-6 dependent manner. MSC actions involve cellular homeostasis stabilization^[24]. The rescue of epithelial cell integrity by MSC after total body irradiation or abdominal radiotherapy might favor renewal of healthy intestinal tissue. Furthermore MSC treatment of a target organ may have an effect on distant tissues. MSC regenerated the small intestine epithelium, which in turn restored the enterohepatic recirculation pathway initially damaged by irradiation. Another mechanism that should be considered is the role of cytokines and growth factors produced by MSCs that are homing to other organs, such as the observed distant hepatic protection without engraftment of MSC in the liver^[26]. This might reduce acute and/or chronic side effects arising from ionizing radiation and may be of therapeutic interest^[24,27].

Clinical treatment

In 2008, three Epinal patients presenting serious intestinal radiation induced lesions, after over dosage of radiotherapy, compassionately received MSC treatment. For all three patients, the systematic administration of MSCs was well tolerated; efficient analgesic and anti-inflammatory effects as well as hemorrhage reduction were observed. A fourth patient was successfully treated in 2012^[14]. Compassionate trial demonstrated the feasibility of cell therapy treatment for patients overdosed during radiation therapy of prostate cancer as in Epinal Medical Center. A new protocol will be under taken in 2013 for the treatment of late severe damages of abdominal radiotherapy.

Untoward effects

There is interest in using these cells in critical illness, however, the safety profile of these cells is not well known. The resistance to transformation of MSCs produced in 4 cell therapy facilities was investigated during clinical trials. This study demonstrated that MSCs with

or without chromosomal alterations showed progressive growth arrest and entered senescence without evidence of transformation either *in vitro* or *in vivo*^[28]. Authors conclude that genomic stability of cultured adult stem cells is robust and not a significant source of concern^[29]. Another related concern is the capacity of MSCs to potentially contribute to tumor growth and metastasis especially for cell therapy after cancer radiotherapy treatment. The question currently remains; do somatic adult stem cells support or suppress tumor growth? A variety of tumor models in which MSCs are added exogenously have been used^[30]. Many studies have reported that MSCs either promoted or suppressed tumor growth. Mechanisms implied chemokine signaling, modulation of apoptosis, vascular support, and immune modulation and interaction with cells in the tumor microenvironment.

While it is true that MSC therapy has shown utility in the reversal of tissue injury in nearly every model examined, there is more to consider with radiation damage that is not as important with other types of tissue injury. Radiations induce DNA damage and long-term inhibition of growth of exposed cells. This period of quiescence is mediated by P53 and other pathways. The biological effect of P53 activation is to stop cell growth long enough for DNA repair enzymes to attempt to repair the DNA damage. MSC therapy does nothing to improve DNA repair, so that MSC therapy will allow cells to continue to grow and repair the tissue over the short term, however the long-term consequences of this “repair” are not known. It might well be that allowing the cells to progress through cell cycle with a damaged DNA template will result in severe long-term consequences including cancer induction. However, we believe that the short-term effects far outweigh the possible long-term effects.

Understanding mechanisms by which adult somatic stem cells modulate tumor growth and long-term effects of MSCs after irradiation is essential to safely develop cell therapy as a therapeutic tool to treat radiation damages.

Lessons from clinical trials must be taken into account; since the first reported trial in 1995, cultured MSCs have been used in 125 registered clinical trials (registered at <http://www.clinicaltrial.gov/>) without any reported side effect of the cell therapy treatment. Clinical data support the long-term safety of MSCs. Furthermore the follow up of patients after cell therapy treatments post-radiotherapy for breast^[31], bladder or prostate cancers^[32] have never revealed side effects after a long period. A methodical review of clinical trials that examined the safety of MSCs was conducted using MEDLINE, EMBASE and the Cochrane Central Register of Controlled Trials (to June 2011). Systematic examination for adverse effects related to the use of MSCs did not identify any significant events other than transient fever. This systematic review provides some insurance to investigators and health regulators that this innovative therapy appears safe^[33]. The safety of cell therapy to treat the consequence of radiation on healthy tissue must involve a network of laboratories from the production to the

clinical units.

CONCLUSION

The number of individual with cancer is expected to increase from 12.7 million in 2008 (latest available figure) to 20.3 million in 2030. Sixty percent of these patients receive radiation therapy with 50% chance of recovery. Radiotherapy is associated with a high incidence of undesirable acute and/or chronic gastrointestinal complications that can affect the patient's quality of life and may even be life threatening. Currently, five percents will develop pelvic radiation disease. Some argue that modern therapy techniques will improve outcomes. However, chemo-radiation enhances survival but also increases the risk of pelvic radiation disease. Results will be an increasing cost for the society (repeated hospitalization for palliative care) and an ethical problem to help these patients with irreversibly degraded quality of life. The lack of curative treatment at present and the potential severity for the disorder highlight the need for novel and effective therapeutic strategies for gastrointestinal complications after radiation exposure. Stem cells can be used to treat toxic side effects induced by radiotherapy on healthy tissue of radio-sensitive patients. The expected results for patients will be potential/improved healing of chronic refractory diseases and an increase in their quality of life, leading to lower health expenses by reducing patient treatment and hospitalization. The interest of cellular therapy by injection of MSC in the treatment of pelvic pathologies and chronic severe damages of radiotherapy has already been established. Six clinical trials for the treatment of pelvic complications are currently in progress, three of which are in phases III.

MSCs provide a long-term effect in inhibition of chronic inflammation and a fistulisation, arrest of hemorrhagic syndromes for the hemorrhagic cystitis. MSCs are successfully used to treat the late effects of radiotherapy for breast cancer and radiodermatitis. Their efficiency was also demonstrated on pain reduction. Concerning clinical trials to cure abdominal late severe damages of radiotherapy, one compassionate trial has demonstrated the feasibility of cell therapy treatment for patients overdosed. A new protocol will be under taken in 2013 for the treatment of late severe damages of abdominal radiotherapy.

BIOGRAPHY

Alain Chapel, PhD, scientific investigator at Institut de Radioprotection et de Sûreté Nucléaire, is team leader in the Laboratory of radiopathology and experimental therapies. Over 20 years, he developed gene and cell therapy for non-human primate, immune-tolerant mice (NOD/SCID) and rats to protect against side effects of radiation. He has developed representative experimental models to investigate the effect of radiation on the radiosensitive hematopoietic cells and their bone marrow microenvironment, the skin and gut. He collaborates

with clinicians to develop new strategies for treatment of patients after radiation accidents or radiotherapy over-exposures. In compassionate trials, he has participated in the first establishment of a proof of concept for the therapeutic efficacy of MSCs for the treatment of hematopoietic deficit, radiodermatitis and the over dosages of radiotherapy. In collaboration with Saint-Antoine Hospital (Paris, France), he has contributed to the first reported correction of deficient hematopoiesis in patients (graft failure and aplastic anemia) thanks to the intravenous injection of MSC that restored the bone marrow micro-environment, mandatory to sustain hematopoiesis after total body irradiation. Currently, his work focuses on the development of radio-induced bone marrow aplasia using human hematopoietic stem cell derived from human induced pluripotent stem. He is a member of various national and international societies: European Bone Marrow Transplantation Group (EBMT), American Society for Hematology, International Society of Stem Cell Research, and Société Française de Greffe de moelle et de thérapie cellulaire. He is an associate editor of 5 international review journals: *World Journal of Stem Cells*, *World Journal of Gastrointestinal Surgery*, *World Journal of Radiology*, *The Open Gene Therapy Journal*, and the *Journal of Clinical Rehabilitative Tissue Engineering Research*. He participated in the scientific organization of the international conference EBMT Paris 2011.

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Regenerative medicine based applications to combat stress urinary incontinence

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Abstract

Stress urinary incontinence (SUI), as an isolated symptom, is not a life threatening condition. However, the fear of unexpected urine leakage contributes to a significant decline in quality of life parameters for afflicted patients. Compared to other forms of incontinence, SUI cannot be easily treated with pharmacotherapy since it is inherently an anatomic problem. Treatment options include the use of bio-injectable materials to enhance closing pressures, and the placement of slings to bolster fascial support to the urethra. However, histologic findings of degeneration in the incontinent urethral sphincter invite the use of tissues engineering strategies to regenerate structures that aid in promoting continence. In this review, we will assess the role of stem cells in restoring multiple anatomic and physiological aspects of the sphincter. In particular, mesenchymal stem cells and CD34⁺ cells have shown great promise to differentiate into muscular and vascular components, respectively. Evidence supporting the use of cytokines and growth factors such as hypoxia-inducible factor 1-alpha, vascular endothelial growth factor, basic fi-

broblast growth factor, hepatocyte growth factor and insulin-like growth factor further enhance the viability and direction of differentiation. Bridging the benefits of stem cells and growth factors involves the use of synthetic scaffolds like poly (1,8-octanediol-co-citrate) (POC) thin films. POC scaffolds are synthetic, elastomeric polymers that serve as substrates for cell growth, and upon degradation, release growth factors to the microenvironment in a controlled, predictable fashion. The combination of cellular, cytokine and scaffold elements aims to address the pathologic deficits to urinary incontinence, with a goal to improve patient symptoms and overall quality of life.

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Key words: Stress urinary incontinence; Smooth muscle; Tissue engineering; Regeneration; Stem cells; Biomaterials; Angiogenesis; Sphincter

Core tip: Stress urinary incontinence is a condition which affects millions of women on a world-wide basis. Current surgical strategies to alleviate the symptoms involved with this condition are temporary stop-gap measures. With the advent of tissue engineering strategies in combination with stem cells, the reality of creating a functional replacement for anatomic structures involved in stress urinary incontinence can be a reality.

Thaker H, Sharma AK. Regenerative medicine based applications to combat stress urinary incontinence. *World J Stem Cells* 2013; 5(4): 112-123 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v5/i4/112.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v5.i4.112>

INTRODUCTION

Symptoms of the lower urinary tract in women, such as urinary incontinence, account for a significant number of

outpatient consultations to urogynecologists^[1]. Several risk factors have been identified to explain the onset and persistence of this condition, particularly since the prevalence of urinary incontinence among adult women is approximately 16%-30%^[2,3]. The occurrence of urinary incontinence is closely correlated with rising age^[4], along with obesity through increased pressure on the pelvic floor, diabetes mellitus causing microvascular and neuropathic changes, and prior pelvic surgeries for the fascial, muscular and nerve stress it imparts. Among younger women, pregnancy and subsequent vaginal delivery leads to postpartum incontinence in up to 28% of the population. Bladder outlet compression, pelvic floor strain that elongates the pudendal nerve, and a prolonged second stage of labor all contribute towards urinary leakage^[5-7]. Less often, patients with a chronic cough or those with fascial weakness secondary to a defect in collagen metabolism may have incontinence as well^[8]. In adult men, direct injury to the neurovascular bundle and fascial planes during radical prostatectomy is a leading cause of incontinence^[9].

A Scandinavian study reports that as many as 64% of women with urinary incontinence issues do not consult their primary care physician for a diagnostic work up of their symptoms^[10]. A similar study in the United States (US) observed a rate of about 45%^[11]. The prevalence of urinary incontinence, therefore, could be much higher than documented. Though not life-threatening, patients endure a dramatic decline to their quality of life. A survey across Europe and the US documented this subjective aspect, showing that confidence, self-perception, levels of physical activity and social engagement were negatively impacted by incontinence^[12-14]. Not surprisingly, reports show a higher prevalence of anxiety and depression among these patients as well^[15,16]. For the elderly in nursing homes, uncontrollable passage of urine irritates the perineal skin, causing dermatitis and discomfort that is difficult to alleviate^[17].

Urinary incontinence is a condition in which there is involuntary leakage of urine that can be attributed to a number of differing factors. Depending on the presentation and history, incontinence is divided into several groups. Stress urinary incontinence (SUI) is an involuntary loss of urine that occurs with increased abdominal exertion, exemplified during coughing, sneezing, laughing or lifting. If the bladder is retaining urine in excessive amounts, small increases to abdominal pressure will produce an unwanted leak. Yet this symptom can still occur within the normal range of bladder fullness, such as in defects to the urethra or the intrinsic sphincter in retaining urine. A laxity of supportive structures to the pelvic floor and bladder means that increased pressures cannot be counteracted. Urge urinary incontinence presents with frequency causing low volume output, urgency and nocturia. The leakage of urine is typically accompanied by or preceded by a sense of urgency. A combination of these two types is described as mixed urinary incontinence, where increased abdominal pressures cause urinary leak-

age and concomitant urgency. Overactive bladder is characterized by urgency, with or without incontinence. Less common voiding abnormalities include conditions such as nocturnal enuresis and continuous urinary incontinence. An acute onset of urinary incontinence may suggest an underlying neurologic degenerative disease or malignancy, and should therefore be considered in the differential diagnosis^[18-20].

Though the development of urinary incontinence is multifactorial in nature^[21], all variations of disease in SUI ultimately manifest as either urethral hypermobility or urethral sphincter dysfunction. The effects of aging are particularly well described to demonstrate this. Increasing age leads to urethral musculature degeneration and neurologic injury^[22]. The number of striated muscle fibers diminishes with histologic thinning noted at the proximal vesicle neck (supported by the U-shaped detrusor) and at the dorsal wall of the urethra (encircled by striated sphincter muscles). In addition, an age-related weakening of the endopelvic fascia reduces the support to the urethra, causing an inability to maintain the physiologic 90-120 degree vesicourethral angle required to maintain continence^[23]. What results is termed urethral hypermobility. The endopelvic fascial layer is a dense, fibrous connective tissue layer surrounding the vagina, with attachments to the arcus tendinous fascia, the pubic bone ventrally, and the ischial spine dorsally^[23]. Degenerative changes diminish the ability of the urethra to generate pressure against stress. As an example, a staccato cough creates a 150 cmH₂O increase in abdominal pressure, which the urethra must counteract during non-micturition times. A more compliant fascial layer threatens to lose continence during such transient pressures changes.

Initiating therapy for SUI requires the identification and understanding of the functional components comprising the urinary sphincter. The nerve supply to the lower urinary tract consists of three important innervations (Figure 1). First, pelvic nerves (S2-S4) provide parasympathetic innervations to the detrusor muscle and urethral smooth muscle sphincter. As an excitatory motor efferent nerve, the pelvic nerves initiate and coordinate micturition by contracting the detrusor and relaxing the sphincter. Second, hypogastric nerves (T2-L3) are sympathetic innervations to the trigone, bladder neck internal sphincter and detrusor muscle. These nerves allow for urinary retention and inhibit detrusor activity. Lastly, the pudendal nerves (S2-S4) of the sacral plexus innervate the external urethral sphincter (EUS) and striated muscles of the pelvic floor. The EUS consists of circular striated "slow twitch" fibers that sustain long periods of contraction, and pelvic basin muscles like the pubococcygeus contains slow and fast twitch fibers for reflex contraction during a cough or sneeze. The pudendal nerve arises from Onuf's nucleus, traverses Alcock's canal and enters the ischiorectal fossa to innervate the EUS^[6]. This trajectory places the nerve between the sacrospinous and sacrotuberous ligaments, which makes it vulnerable to compression and injury.

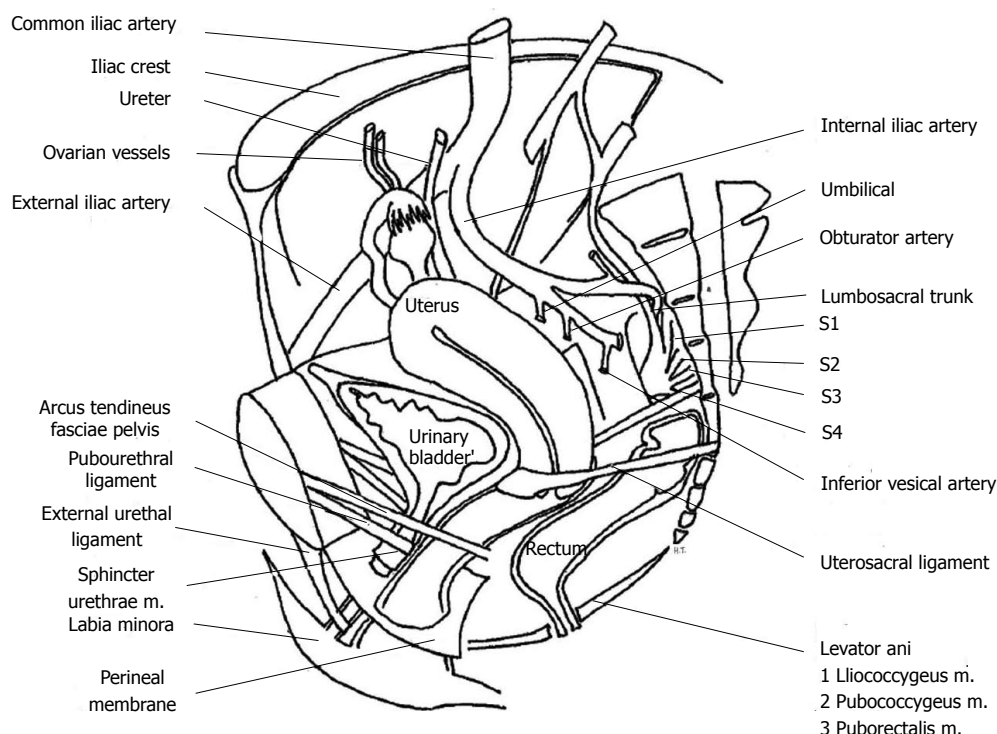


Figure 1 Sagittal section of female pelvis illustrating the position of the sphincter urethrae muscle in relation to adjacent structures and associated neuro-vascular components. Adapted from reference [21].

MEDICAL AND SURGICAL MANAGEMENT OF SUI

Conservative management of SUI involves lifestyle and behavioral changes, weight loss^[24], bladder training^[25], Kegel exercises to strengthen the pelvic floor and pharmacotherapy to improve urethral muscle tone^[26-28]. Unlike other forms of urinary incontinence, there is a dearth of pharmacologic options that are effective for SUI. Alpha-adrenergic receptors at the bladder neck and urethra can be targeted with pseudoephedrine and ephedrine to stimulate smooth muscle contraction^[27,29]. Though this use may theoretically be suited to improve symptoms, the cardiovascular side effect profile precludes many patients from this therapy^[30]. Imipramine, a tricyclic antidepressant with strong anti-cholinergic effects, also promotes urethral sphincter closure and urinary retention. This drug, however, poses a significant risk of orthostatic hypotension, which is often contraindicated in the elderly population. Lately, the use of duloxetine outside of the US has shown some promise, though the mechanism of action hardly addresses the underlying cause^[31,32]. Patients with SUI and concomitant urge symptoms have more therapeutic options, including anti-muscarinic agents and oxybutynin^[33].

Despite these initial efforts to gain continence, most of these patients are refractory to treatment and are invariably referred for surgical intervention. The goals of surgery are to reduce the number of episodes of incontinence each day or to reduce the volume of leaked urine, with the ultimate aim of achieving complete continence.

Surgery can either support the compression of the urethra, or augment coaptation to create a better seal. Pre-operative assessment of surgical candidates begins with a detailed history with bladder diary recordings, and a physical, including a bimanual exam. A urinalysis to rule out infectious causes is indicated, followed by a cough stress test. In this test, the clinician visualizes the leakage of urine from a full bladder while the patient coughs. The volume of urine leak, collected on chux padding, may signify the degree of stress incontinence. Once the diagnosis of SUI is confirmed, most patients move on to urodynamic testing.

Urodynamic testing is expensive and subject to operator variability^[34,35], but it provides two important measurements for the management of SUI. The first is leak point pressure (LPP). LPP measures the intravesical pressure at which urine leakage occurs when the patient is asked to strain or subjected to the Valsalva maneuver. The patient must be careful to not induce a detrusor contraction. This is a measurement of intrinsic sphincter deficiency, and a LPP less than 60 cmH₂O is suggestive of SUI. The second measurement is the maximum urethral pressure generated, which is part of the urethral pressure profile. Profilometry also documents maximum urethral closure pressure, functional urethral length and the pressure transmission ratio. Maximum urethral pressures less than 20-30 mmHg are suggestive of SUI, and warrants the use of sling placement or periurethral bulking agent injections^[36].

The surgical approach to SUI involves either placement of slings to support of the urethra, or injection of

biomaterials to increase urethral coaptation. Minimally invasive sling placement is currently the most common intervention for SUI^[37]. Through a vaginal incision, a thin mesh of polypropylene thread material is positioned at the midurethral or at the bladder neck^[38]. The sling behaves like an immobile floor, onto which the urethra can contract^[39]. This strategy assumes the incontinent sphincter is indeed caused by urethral hypermobility, so supporting the plane of contraction would limit the angulation. Midurethral slings may be placed with either a retropubic or transobturator approach. A multicenter, randomized trial shows equivalent outcomes regardless of approach^[40], though some studies still report better outcomes with a tension-free vaginal tape retropubic sling^[41]. Newer interventions using a single incision sling or an adjustable sling have also shown promise^[42].

Midurethral sling procedures have largely replaced the older Marshall-Marchetti-Krantz and Burch techniques of colposuspension^[38,43]. Though these procedures have success rates of up to 88%^[44], complications of urinary retention, de novo urgency, posterior vaginal wall prolapse and osteitis pubis have been documented^[45]. In contrast, a Cochrane review of sling surgeries showed that the minimally invasive approach lessens operative times and decreases post-operative voiding dysfunction^[38].

Sling materials have traditionally been sourced from autologous rectus fascia, fascia lata or small intestinal submucosa^[46-48]. Cadaveric allografts from the dura, dermis or fascia lata have been shown to be inferior to autologous grafts in the long term based on the frequency of recurrent incontinence^[49]. However, harvesting autologous fascial layers requires longer operative times, longer recovery times and more pain for the patient^[50], despite evidence suggesting they are equivalent to synthetic slings^[51]. For this reason, synthetic materials have become the mainstay in slings.

Sling placement, albeit a minimally invasive procedure, is not without risks and complications. Establishing an appropriate tension across the urethra is challenging, and can result in urinary retention or failure to alleviate symptoms. Both outcomes require release and adjustment of the sling, which is another surgical procedure. Other risks include bladder or urethral laceration, perforation and urinary tract infections (UTI)^[52]. Occasionally, these issues are secondary to erosion of the mesh through soft tissue. These patients suffer from vaginal discharge, post-coital spotting, dyspareunia, and frequent UTIs. Despite these potential complications, the majority of patients do achieve freedom from incontinence, and self-reported improvements to sexual activity^[53,54], and reductions in coital incontinence^[55].

Injecting biomaterials to augment the urethral mucosa has become an alternative to urethral slings. Bulking agents have the greatest benefit for intrinsic sphincter defects with a LPP of less than 60 cmH₂O. Regardless of where the injection is placed, studies have shown equivalent success with biomaterials in the periurethral, transurethral, midurethral and proximal neck areas^[56,57]. The differences then lie in the biomaterial composition.

APPLICATIONS OF TISSUE ENGINEERING TO SUI

Tissue engineering the urethral sling

Current surgical standards use non-antigenic synthetic materials for slings. Over the past few years, studies have investigated the potential role of stem cells in SUI treatment. In a study by Zou *et al.*^[58], acellular silk slings were tested against slings seeded with autologous bone marrow derived mesenchymal stem cells (MSCs) on rats with bilateral sciatic nerve transection. MSCs were isolated from bone marrow aspirates using flow cytometry against CD34, CD44 and CD105 cell surface epitopes. Sciatic nerve transection, among other methods, is a means to produce genuine SUI pathology^[59]. At 12 wk post-implantation, MSC/silk slings had double the collagen fiber formation of silk slings alone, evidenced by a higher Young's modulus (4.468 ± 0.510 MPa) and higher failure force (2.436 ± 0.192 N) as compared to silk slings alone. The mean Young's modulus of silk slings alone was 3.045 ± 0.388 MPa, with a failure force of 1.521 ± 0.087 N. The collagen formation improved sling integration with the native urethral tissue. However, both MSCs/silk and silk alone constructs performed equally in increasing the LPP (MSCs/silk at 36.3 ± 3.1 cmH₂O *vs* silk alone at 38.0 ± 3.3 cmH₂O). Nonetheless, this study demonstrates that the introduction of MSCs into the urethral environment does not cause any significant inflammation, scarring or adverse effects. Other scaffolds may be better suited in lieu of silk. It is important to note that even though a stem cell seeded construct could improve integration of slings into the urethra, the operative risks remain the same as that for current sling placements. Additionally, there is no evidence demonstrating that cellular slings have a decreased risk of mesh erosion over commercially available slings.

Tissue engineering the urethral sphincter

Attribution of SUI to intrinsic sphincter degeneration poses a challenging problem from a therapeutic standpoint. Sphincter degeneration involves the loss of multiple functional tissue types. Efforts to recreate the function of urethral sphincters are best demonstrated through artificial fluid-filled cuffs encircling the urethra. Artificial urinary sphincters have three components: a cuff of 4.5 cm in size, a reservoir with 61-70 cmH₂O to mimic urethral pressures, and a pump to permit inflation and deflation controlled by the patient. Sphincters are most commonly placed at the bulbar urethra in men who suffer from post-prostatectomy SUI^[9]. Though theoretically purposeful, artificial sphincters are associated with a multitude of complications. Acutely, urethral edema produces pain and discomfort for the patient. Chronically, patients experience atrophy and erosion of the sphincter resulting in irritative voiding symptoms, perineal pain and hematuria^[60]. There are presently no controlled trials showing an improvement to symptoms using an artificial device over conventional therapy^[61]. As such, we consider the employment of stem cells and tissue engineering

Table 1 Periurethral stem/progenitor cell injections improving leak point and urethral closure pressures in various studies

Study	Stem/progenitor cell source	SUI model	LPP findings in cmH ₂ O at 4 wk status post injection	Absolute difference in LPP in cmH ₂ O and percent improvement
Lee <i>et al</i> ^[117]	Allogenic gastrocnemius MDSC	Rat, sciatic nerve transection	MDSC = 44.1 ± 6.6 Saline = 18.6 ± 5.2 Control (-) = 25.8 ± 2.5	MDSC = 18.3 ± 9.1, 70.93%
Kwon <i>et al</i> ^[67]	Muscle derived cells and fibroblasts	Rats, sciatic nerve transection	MDSC = 38.2 ± 4.3 Fibroblast = 38.8 ± 1.2 MDSC/fibroblast = 34.5 ± 3.3 Control (-) = 25.8 ± 1.4 Control (+) = 43.3 ± 2.5	MDSC = 12.4 ± 5.7, 48.06% Fibroblast = 13.0 ± 2.6, 50.38% MDSC/Fibroblast = 8.7 ± 4.7, 33.72%
Fu <i>et al</i> ^[118]	Adipose derived stem cells induced into myoblasts with 5-azacytidine	Rats, vaginal balloon dilation	Myoblast = 32.43 ± 2.05 ADSCs = 30.75 ± 3.17 Control (-) = 36.19 ± 2.25 Control (+) = 45.42 ± 1.71	Myoblast = -3.76 ± 4.3, -10.38% ADSC = -5.44 ± 5.42, -15.03%
Kinebuchi <i>et al</i> ^[69]	Bone marrow derived mesenchymal stem cells	Rats, urethrolisis and cardiotoxic injection	BMSC = 25.66 ± 4.38 Control (-) = 18.19 ± 1.55 Control (+) = 35.98 ± 5.14	BMSC = 7.47 ± 5.93, 41.06%
Lim <i>et al</i> ^[119]	Human umbilical cord blood mononuclear cells	Rats, electrocauterization of periurethral soft tissue	HUCS = 91.75 ± 18.99 Control (-) = 65.02 ± 22.09	HUCS = 26.76 ± 41.08, 41.16%
Kim <i>et al</i> ^[120]	Allogenic mesenchymal stem cells	Rats, pudendal nerve transection	MSC = 43.1 ± 3.2 Control (-) = 22.0 ± 2.2 Control (+) = 29.1 ± 2.1	MSC: 21.1 ± 5.4, 95.90%
Zhao <i>et al</i> ^[93]	Autologous adipose derived stem cells + NGF	Rats, pudendal nerve transection	ADSC/PLGA = 17.8 ± 3.1 ADSC/NGF = 18.3 ± 2.4 ADSC/PLGA/NGF = 22.5 ± 6.1 Control (-) = 11.6 ± 2.7	ADSC/PLGA = 6.2 ± 5.8, 53.44% ADSC/NGF = 6.7 ± 5.1, 57.75% ADSC/PLGA/NGF = 10.9 ± 8.8, 93.96%
Corcos <i>et al</i> ^[72]	Bone marrow derived mesenchymal stem cells	Rats, pudendal nerve transection	MSC = 24.28 ± 1.47 Control (-) = 16.21 ± 1.26	MSC = 8.07 ± 2.73, 49.78%
Watanabe <i>et al</i> ^[121]	Adipose derived mesenchymal stem cells	Rats, pelvic nerve transection	Subtracted LPPs: Collagen = 9.39 ± 2.08 ASC = 8.86 ± 3.13 Control (-) = 10.94 ± 3.55	
Kim <i>et al</i> ^[122]	Human amniotic fluid stem cells	Rats, pudendal nerve transection	hAFCs = 20.2 ± 3.3 Control (-) = 15.2 ± 3.1 Control (+) = 27.6 ± 3.6	hAFCs = 5.0 ± 6.4, 32.89%
Li <i>et al</i> ^[123]	Adipose derived stem cells	Rats, vaginal balloon dilation	ADSC = 46.34 ± 2.63 Control (-) = 36.82 ± 1.68 Control (+) = 48.92 ± 2.71	ADSC = 9.52 ± 4.31, 25.85%
Chun <i>et al</i> ^[66]	Human amniotic fluid stem cells	Rats, pudendal nerve transection	hAFCs = 23.9 ± 1.85 Muscle progenitors = 38.43 ± 0.51 Control (-) = 15.24 ± 1.87 Control (+) = 36.54 ± 1.67	hAFCs = 8.66 ± 3.72, 56.82% Muscle progenitors = 23.19 ± 2.38, 152.16%
Lecoeur <i>et al</i> ^[124]	Myofiber core of satellite cells	Pigs, endoscopic destruction of striated urethral sphincter	Myofiber = 71.5 ± 17.8 Curarization sphincter = 33.5 ± 14.8	
Mitterberger <i>et al</i> ^[125]	Autologous myoblasts and fibroblasts	Humans (n = 123)	UCP = 28.8 ± 12.3 Post-operative UCP = 40.5 ± 15.8 after 1 year	11.7 ± 28.1, 40.62%
Mitterberger <i>et al</i> ^[126]	Autologous myoblasts and fibroblasts	Humans (n = 20)	Pre-operative UCP = 27.0 ± 13.3 Post-operative UCP (1 yr) = 39.4 ± 14.8 Post-operative UCP (2 yr) = 42.2 ± 12.1	66.4 ± 28.1, 45.92% (1 yr) 15.2 ± 25.4, 56.29%

SUI: Stress urinary incontinence; LPP: Leak point pressure; MDSC: Muscle derived stem cells; NGF: Nerve growth factor; ADSCs: Adipose derived stem cells; BMSC: Bone marrow stem cells; HUCS: Human umbilical cord serum; PLGA: Poly (lactic-co-glycolic acid); hAFCs: Human amniotic fluid cells; UCP: Pre-operative urethral closure pressures; MSC: Mesenchymal stem cell; ASC: Adipose stem cells.

techniques to reconstruct the urethral sphincter.

Several studies have established a foundation of infusing stem cells directly into the urethral sphincter. Preparations of MSCs, autologous progenitor muscle cells^[62], adipose cells^[63], processed lipoaspirate^[64,65], human amniotic stem cells^[66] and fibroblasts^[67] have all been used with variable results^[62,68,69] to bolster smooth muscle regeneration and to improve LPPs and urethral closure

pressures (Table 1). Few studies have assessed the role of stem cells for the subset of male patients with SUI from prostate-related surgery. In one study, transurethral injections of autologous muscle derived fibroblasts and myoblasts produced complete continence in 65% of the 63 participants, quantified by a pre-operative LPP of 46.3 ± 17.1 cmH₂O, and a post-operative LPP of 68.2 ± 24.3 cmH₂O^[70]. Another study using a similar approach

reported improvements to merely 12% of 222 male patients, with no improvements in 46%^[71]. While both studies showed that stem cell implantation is a safe procedure in eligible patients, the results do not show a clear benefit as seen in trials with women and SUI.

Using MSCs seems to show the greatest promise, as MSCs have displayed the potential to regenerate both muscle and ganglion components in the sphincter. Corcos *et al.*^[72] demonstrated in an animal model that injecting BMSCs into denervated urethral sphincters improved LPPs to almost normal, non-SUI levels. This result is argued to be due to the differentiation of MSCs into striated muscle within the urethral microenvironment^[72]. Though this evidence is merely histologic, and not in an improvement to symptoms for patients, the concept of creating a functional contractile tissue in the sphincter is worthy of further development.

MSC use in tissue engineering has become a prominent strategy in a multitude of fields, including urogynecology^[73-79]. MSCs express cell surface markers CD29, CD44, CD105, CD166, and are negative for hematopoietic markers such as CD14, CD34, CD40 and CD45^[80]. MSCs are also negative for leukocyte common antigen CD45, suggesting that these stem cells escape lymphocyte detection, and thus avoid immune rejection^[81,82]. Sourced from the bone marrow, MSCs can be easily isolated from other hematopoietic cells through flow cytometry. MSCs have the capacity to divide 24-40 times *in vitro*, allowing for multiple passages of expansion without losing their multipotent properties or differentiating spontaneously^[83]. This advantage, described by Pittenger *et al.*^[84], permits the differentiation of MSCs by external forces, such as the microenvironment of target tissue itself. Coupled to this environment, MSCs display an immunomodulatory effect^[85] that includes the secretion of cytokines to initiate and support tissue regeneration^[86]. For these reasons, MSCs must, by minimal criteria, differentiate into osteoblasts, chondrocytes and adipocytes. Yet, the plasticity inherent in MSCs has pushed researchers to generate neural, cardiac, muscular and other soft tissue lineages. In the realm of urinary tract healing, MSCs are considered a prime candidate since their presence has great therapeutic potential with minimal complications^[87,88].

Some centers are already offering stem cell injections into the urethra for patients^[89-91]. However, it is unclear whether these cells serve a functional purpose in regenerating damaged sphincters, or whether the cells are merely a bulking agent not unlike injectable biomaterials. In addition, it is recognized that inflammation at the implant site diminishes the ability of injected cells to survive long enough to participate in regeneration^[92]. If growth were not sustained over a 7-10 d period, the applications of MSCs would be significantly stunted. Providing a means to enhance cell viability *in vivo* could be achieved by introducing synthetic scaffolds and growth factors.

The harmony of using scaffolds, stem cells and growth factors together has shown promise in a number of tissue engineering projects. Zhao *et al.*^[93] harvested

adipose derived stem cells (ADSCs) and seeded them onto poly (lactic-co-glycolic acid) (PLGA) microparticles containing nerve growth factor (NGF). As a synthetic scaffold, PLGA has been shown to be safe in the urinary tract^[94,95]. With periurethral injection into mice, this combination improved the LPP to 22.5 ± 6.1 cmH₂O over treatments lacking either the PLGA or NGF. This result was explained by NGF prolonging the survival of ADSCs, enhancing the urethral muscle area on histology, and increased the density of neurofilaments supporting the sphincter lamina propria. This is the first iconic study where tissue engineering directly addresses the pathology underlying intrinsic sphincter defects. We propose a similar approach, where scaffolds and MSCs are injected not into the periurethral space, but directly into the urethral sphincter.

Poly(1,8 octanediol-co-citrate) scaffolds to support urethral sphincter regeneration

The number and variety of polymers synthesized for tissue engineering is rapidly expanding. A popular selection for research is PLGA^[96], possessing elastic properties that adapt well to dynamic soft tissue structures. A similar material, poly (1,8-octanediol-co-citrate) (POC), is used by our group for urologic tissue engineering efforts. First established by the Ameer Research Lab^[97], POC thin film (POCf) scaffolds are a highly reproducible elastomeric material^[98]. The POCf allows for cell growth, cell infiltration, and for unimpaired exchange of oxygen and nutrient delivery. During polymerization of the scaffold, several aspects of construction can be customized to mimic the compliance, elasticity, and tensile strength measured through Young's modulus. Equimolar amounts of citric acid and 1,8-octanediol are combined, melted and cooled to make a pre-polymer, and parameters such as temperature and time can be adjusted. Higher temperatures with short polymerization times produce dense films, while low temperatures and long polymerization times yield scaffolds that are less cross-linked. With these modifiable ester-bonding schemes, highly adaptable, labile and reproducible scaffolds can be created specifically for urinary tract tissue targets. Tailoring these parameters also reflects the degradation scheme of POCf, which degrades to nontoxic byproducts of CO₂ and H₂O *via* non-enzymatic hydrolysis.

Another feature of POC scaffolds, besides providing a highly conducive substrate for cell growth and proliferation, is the ability to deliver growth factors through a controlled release upon scaffold degradation^[99]. During the polymerization of POC, small peptides including growth factors and cytokines may be chemically coupled to the scaffold and released upon surface erosion. POCf scaffolds modified with heparan sulfate to hold vascular endothelial growth factor (VEGF), fibroblast growth factor 2 and insulin-like growth factor were studied by Sharma *et al.*^[99] in a rat model. Heparan sulfate, a highly sulfated glycosaminoglycan, protects bound growth factors to prevent enzymatic degradation. Delivery of the

pro-angiogenic growth factors upon break down of the scaffold led to increased vascular growth *in vivo* as compared to controls. The difference in results demonstrates that using POCf for a protracted but focused delivery of growth factors improves tissue healing.

The versatility of POCfs used in consonance with MSCs and growth factors offers the basis to potentially correct for sphincter deficiencies. For pregnancy related SUI, one pathway has been identified to be upregulated in response to vaginal distention and subsequent tissue damage^[100]. The pathway involves hypoxia inducible factor-1 α , a transcription factor stabilized in hypoxic conditions to induce expression of VEGF. This marker of tissue injury, and the resulting drive for angiogenesis, could potentially home stem cells to the site of injury. Studies by Cruz *et al.*^[101] have shown that pelvic injury *via* vaginal distension is a sufficient nidus for MSC homing to the urethra and levator ani. This phenomenon is speculated to be through chemokine ligand-7. Adding these chemokines to the POC delivery system could therefore boost the response of MSCs in tissue regeneration, and recruit circulating progenitors as well^[102,103]. Progenitors stationed in the tissue, such as intrinsic satellite cells, may also be recruited to striated muscle reconstruction^[104]. Direct and strong evidence exists that VEGF promotes the growth of myoblasts and increases capillary growth to the regenerating tissue. Interestingly, VEGF was capable of advancing the growth of myoblasts sourced even from older mice, where cells have less capacity to proliferate into functional tissue^[105]. Growth factors like basic fibroblast growth factor (bFGF), hepatocyte growth factor and insulin-like growth factor have also contributed to muscle regeneration^[106]. Characterization studies have also outlined a multitude of paracrine factors secreted by MSCs that are anti-apoptotic, immunomodulatory, anti-fibrotic and pro-angiogenic^[107]. As previously confirmed, VEGF from MSCs, along with IL-6, MCP-1 and extracellular matrix components, assist in supporting angiogenesis, laying down extracellular matrix, and preventing apoptosis secondary to hypoxia^[108].

The significance of bioactive compounds in regeneration was further strengthened by Choi *et al.*^[109]. In this study, plasmid DNA encoding bFGF was injected into rat periurethral submucosa *via* a PLGA synthetic delivery system. Results indicated that the levels of SM α -actin were elevated due to the bFGF, corresponding to a proliferation of tightly packed smooth muscle. Furthermore, contraction studies, conducted through electrical stimulation, showed a marked elevation in contractile properties for pDNA transfected urethras. Normal, continent urethras generated a contraction force of 36.4 ± 2.5 tension/mg of tissue, which is not dramatically different from the measured 32.3 ± 1.5 tension/mg tissue generated in pDNA/PLGA treated incontinent mice.

Three important concepts come out of this study: (1) that a sustained release of pDNA expressing bFGF through PLGA proved beneficial to regeneration; (2) the use of pDNA ensures that regeneration continues

beyond the half-life and denaturation of biogenic compounds; and (3) since neither MSCs nor any other cell lines were not utilized in the injection, the improvement to symptoms can be specifically attributed to bFGF.

Integrating POCfs with MSCs and cytokines addresses the muscular aspect of sphincter regeneration. But a more comprehensive approach pays attention to the vascular and neural components as well. Seeding POC with progenitor cells from the bone marrow alongside MSCs could complete these components. Recent insights into CD34⁺ hematopoietic stem cells (HSCs), harvested from the same bone marrow origin as MSCs, points to a promising adjunct to MSCs. HSCs express von Willebrand Factor, vascular endothelial-cadherin and Flk-1^[110-112]. These markers, in addition to CD133 and CD34^[113], allow HSCs to be distinguished from MSCs and other primitive cells^[114]. Placement of CD34⁺ HSCs onto compatible POCfs improved neovascularization and reduced fibrosis when injected into the site of injury^[115].

Angiogenesis in the diseased urethral sphincter is beneficial for two reasons. First, it will nourish the proliferation and regeneration of MSCs into muscular components. Second, the blood supply will contribute to the vascular plexus that surrounds the urethral smooth muscle lumen. This plexus, when perfused, helps forms a tight seal of the mucosal surfaces, just as muscle contraction would.

Addressing the need for neural components in tissue engineering has been challenging. One study from our own group demonstrated the ability of MSCs combined with CD34⁺ cells to form muscular, vascular and even neural tissue in a rat bladder augmentation model^[115]. Stem/progenitor cells were seeded onto POC scaffolds prior to implantation, which yielded well-organized fascicles of smooth muscle supported by collagen. CD34⁺ cells contributed greatly to the levels and distribution of blood vessels in MSCs/CD34⁺/POC constructs. Novel to this study was the detection of peripheral nerve regeneration from the surrounding healthy tissue. Stained with neuronal specific antibodies β III tubulin and synaptophysin, rat nerve bundles innervated the regenerated tissue significantly more in MSC/CD34⁺/POC grafts than in controls. The authors suggest that renewal of a blood supply to the area improves delivery of growth factors and cytokines promoting neuronal growth.

CONCLUSION

In the evolving field of tissue engineering, there has been an overwhelming trend towards therapy against the exact mechanism of disease causing SUI. Individual studies have lent credence to the importance of MSCs, CD34⁺ HSCs, scaffolds and growth factors in efforts to regenerate the urethral sphincter. A combination of these four components would create a plausible scenario in which to restore function in a structure as complex as the sphincter. Even with the advances in surgical slings, there still remains an inherent need to establish normal physiologi-

cal function. Paired with POC scaffolds, we exploit the vast potential MSCs to differentiate into muscle, and hematopoietic precursors to proliferate into blood vessels in the presence of cytokines and growth factors. The indices of LPP readings from pre-operative urodynamic studies can be correlated to different levels of POCf elasticities, suited for a specific patient. Immunohistochemical and calcium release assays would support the MSC contractile properties as muscle regenerates, and nicotinic receptors targeted by α -bungarotoxin would illustrate the presence of neuronal fibers^[116].

At least one study has observed the restoration of skeletal muscle and ganglionic elements from MSC injection into the rat urethral sphincter. Conducted by Kinebuchi *et al.*^[69], this study is a step in the right direction. However, follow up results did not confirm any improvement to LPP when compared to a control of cell free medium injection. The authors attribute the finding to inflammatory changes and to an insufficient bone marrow stem cells volume. In spite of this, the fallbacks can perhaps be accounted for by the absence of scaffolds and growth factors. Applications of this system expand beyond the treatment of SUI in adult women. Foremost, children born with neurogenic bladders secondary to myelomeningocele often have coexistent sphincter dysfunction. Likewise, post-prostatectomy men occasionally complain of incontinence as well. Patients with multiple sclerosis may have S2-S4 damage, leading to neuromuscular degeneration from the loss of incoming sensory nerve impulses and outgoing motor signals. The concept of urinary incontinence is similar to that of vesicoureteral reflux, so tissue engineering strategies provide an additional avenue to explore alongside ureteral reimplantation. To improve the symptoms of lower urinary tract symptoms in these patients, MSCs, HSCs, POC, and growth factors may one day supplement current surgical tactics.

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Engineering stem cell niches in bioreactors

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Abstract

Stem cells, including embryonic stem cells, induced pluripotent stem cells, mesenchymal stem cells and amniotic fluid stem cells have the potential to be expanded and differentiated into various cell types in the body. Efficient differentiation of stem cells with the desired tissue-specific function is critical for stem cell-based cell therapy, tissue engineering, drug discovery and disease modeling. Bioreactors provide a great platform to regulate the stem cell microenvironment, known as "niches", to impact stem cell fate decision. The niche factors include the regulatory factors such as oxygen, extracellular matrix (synthetic and decellularized), paracrine/autocrine signaling and physical forces (*i.e.*, mechanical force, electrical force and flow shear). The use of novel bioreactors with precise control and recapitulation of niche factors through modulating reactor operation parameters can enable efficient stem cell expansion and differentiation. Recently, the development of microfluidic devices and microbioreactors also provides powerful tools to manipulate the stem cell microenvironment by adjusting flow rate and cytokine gradients. In general, bioreactor engineering can be used to better modulate

stem cell niches critical for stem cell expansion, differentiation and applications as novel cell-based biomedicines. This paper reviews important factors that can be more precisely controlled in bioreactors and their effects on stem cell engineering.

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Key words: Stem cell engineering; Bioreactor; Differentiation; Microenvironment; Microfluidics

Core tip: Stem cells are promising cell sources for cell therapy, tissue engineering, drug discovery and disease modeling due to their ability of self-renewal and immense capability of lineage-specific differentiation. Bioreactor systems with engineered stem cell microenvironments, called "niches", play an important role in deriving functional cell populations from stem cells. Some important factors and their effects on stem cell engineering in bioreactors are reviewed in this paper. The understanding of bioreactor regulation of stem cell niches is of great interest in developing novel biomedicines.

Liu M, Liu N, Zang R, Li Y, Yang ST. Engineering stem cell niches in bioreactors. *World J Stem Cells* 2013; 5(4): 124-135 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v5/i4/124.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v5.i4.124>

INTRODUCTION

Stem cells are promising cell sources for cell therapy, tissue engineering, drug discovery and disease modeling due to their ability of self-renewal and immense capability of lineage-specific differentiation^[1-3]. Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have the potential to be expanded indefinitely and differentiated into all cell types in the body^[4,5]. In addition, adult stem cells, such as mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs) and neural stem cells (NSCs), also have limited

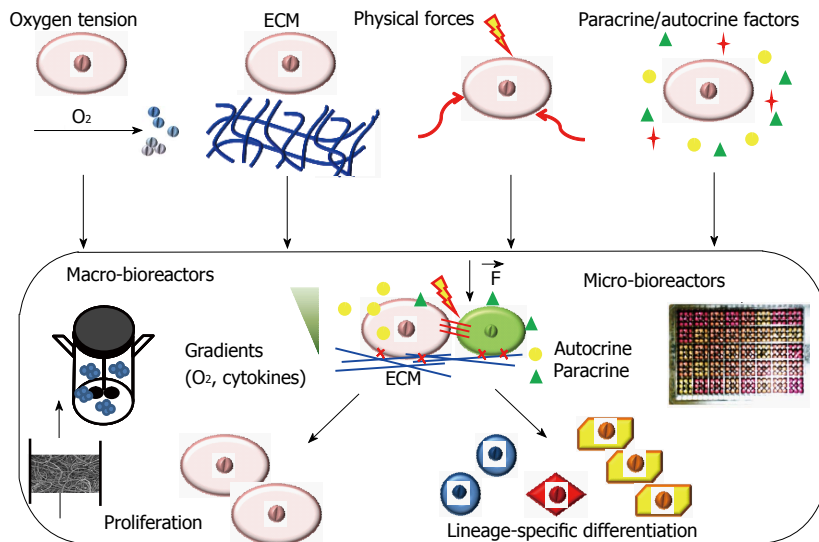


Figure 1 Important stem cell niche factors regulating stem cell proliferation and differentiation in bioreactors. The niche factors include regulatory factors (such as oxygen), extracellular matrix (synthetic and native), physical forces and paracrine/autocrine factors. ECM: Extracellular matrix.

lifespan and the restricted lineage differentiation, which may be extended by fetal stem cells such as amniotic fluid stem cells (AFSC)^[6]. Although stem cells open a new era for tissue regeneration, tissue modeling, and drug discovery, efficient differentiation of stem cells with the desired function is still the major challenge in stem cell engineering to fulfill their potential in biomedical applications.

Stem cells have been differentiated into a variety of cell types; however, the function of stem cell-derived tissue specific cells have not been well understood^[2]. For example, evidences indicated that the human PSC-derived cardiomyocytes more resemble embryonic cardiomyocytes rather than mature cardiomyocytes^[7]. Human ESC-derived erythroid progenitors expressed mostly fetal haemoglobin but have limited ability to activate mature haemoglobin^[8]. In the example of MSCs, trophic factor secretion by the cells, the most important characteristics of MSC therapy, is highly dependent on the culture systems^[9]. In addition, the efficiency of the lineage specific differentiations remain as the major challenge in stem cell bioprocessing because most current protocols resulted in low purity of the target lineages^[10]. To date, all *in vitro* studies have indicated that the recapitulation of stem cell microenvironment is critical to regulate stem cell fate decision and the functionality of the differentiated tissue-specific cells.

Bioreactor systems with engineered stem cell microenvironments, called “niches”, play an important role in deriving functional cell populations from stem cells (Figure 1)^[11]. These bioreactor systems with tightly controlled and highly reproducible environmental factors can be used to regulate stem cell differentiation and tissue formation^[12,13]. The critical culture parameters that have been widely studied in a bioreactor system for engineering stem cell niches include oxygen tension, 3-D scaffolds (synthetic or decellularized), physical forces such as hydrodynamic forces, mechanical strain, flow shear and electrical stimulation (Figure 1). Recently, microfluidic devices and microbioreactors have been designed and used for high throughput regulation of microenvironmental factors to better understand stem cell niches and for drug

screening^[14,15]. Paracrine/autocrine factors can also be revealed using microfluidic devices and microbioreactors to better control stem cell differentiation. While the design of novel bioreactor affects all types of stem cells, the key factors and the optimal values differ for different process objectives and different tissue-specific cell types. Some important factors and their effects on stem cell engineering in bioreactors are summarized in Table 1 and reviewed in this paper. The understanding of bioreactor regulation of stem cell niches is of great interests in developing novel biomedicines.

REGULATING OXYGEN TENSION IN BIOREACTORS

Oxygen is an important microenvironmental factor for stem cell differentiation via its role in regulating cell metabolism^[16,17]. Traditionally, *in vitro* cell cultures are performed in incubators supplied with air and 5% CO₂, in which the oxygen concentration is about 20%. However, the mean oxygen concentration *in vivo* is around 3% and varies with different tissue regions (*i.e.*, 12% for arterial blood while 1.5% for the brain)^[18,19]. In embryos where stem cells are abundant relative to adult tissues, the oxygen tension is at a lower level than those in mature somatic tissues. Bioreactors allow *in vitro* cultures at various scales to more exquisitely replicate oxygen levels in normal physiological conditions that cannot be easily done with traditional static cultures.

Role of oxygen tension

Reduced oxygen tension can maintain stem cells at primitive stage as well as regulating lineage-specific differentiation. Extensive studies have shown that hypoxia (0.9%-1% oxygen) maintains the primitive property of HSCs and their self-renewal ability^[20,21]. Human MSCs grown in hypoxia (2% O₂) had higher colony-forming unit activity and expressed higher levels of stem cell genes than those cultured at 20% O₂^[22]. For human ESCs, 5% O₂ reduced

Table 1 Examples of regulatory factors in bioreactors and their effects on stem cell engineering

Factors	Effects on stem cell niches	Ref.
Oxygen tension	Hypoxia promotes the proliferation of NSC, HSC, and MSC, inhibits spontaneous differentiation of human PSC, and promotes iPSC reprogramming and growth	[21-24,33]
	Hypoxia promotes lineage specific differentiation from NSC, MSC and human PSC	[27,29,32]
Scaffold/ substrate cues	Higher cell proliferation rates under higher mechanical stresses; Substrate stiffness directs stem cell differentiation; Control of cell shape via substrate size directs human MSC differentiation	[38,41,44]
	3-D fibrous matrix promoted neural differentiation of ESC, silk scaffold promoted bone tissue formation from MSCs, honeycombs for cardiac tissue formation	[42,43,47,50]
	Enhanced MSC proliferation in collagen scaffolds in a radial-flow bioreactor	[51]
Decellularized ECMs	Decellularized bone matrix in a perfusion bioreactor promoted human PSC differentiation into bone tissue; Decellularized cardiac matrix promoted human PSC differentiation into cardiac lineage.	[55,58,59]
	Human PSC-derived ECM supported PSC proliferation	[56]
Mechanical forces	Mechanical stimulation significantly improved the function of engineered ligaments	[64]
	Mechanical compression enhanced MSC differentiation	[66]
	Dynamic compression with deformational loading and hydrostatic pressure improved cartilage tissue engineering;	[61]
	Hydrodynamic shear, cyclic flexure, and cyclic stretch accelerated heart valve tissue formation	[68]
	Pulsatile flow and circumferential stretch improved the engineered blood vessels	[119]
Electrical stimulation	Induced cellular tension and promoted cellular and functional properties of engineered cardiac tissue	[71,72]
	Electrical stimulation enhanced neural differentiation	[69,70]
Flow shear force	Lower flow (shear) rates enhanced MSC proliferation and higher flow (shear) rate increased osteogenic differentiation; Parallel flow and transverse flow affected osteogenic differentiation of human MSCs	[80,81]
	Perfusion improved tissue architecture of engineered cardiac muscle and increased matrix synthesis in engineered chondrocytes	[73,74]
	Agitation preserved Oct-4 expressing cells during PSC differentiation	[82,83]

NSC: Neural stem cells; HSC: Hematopoietic stem cells; ESCs: Embryonic stem cells; MSCs: Mesenchymal stem cells; PSC: Pluripotent stem cells; iPSC: Induced pluripotent stem cells; ECM: Extracellular matrix.

the frequency of spontaneous differentiation through the up-regulation of hypoxia inducing factors (HIF)^[23]. Hypoxia (5%-6% O₂) also enhanced the efficiency of reprogramming to generate iPSCs, although the optimal O₂ concentration and duration of hypoxic culture in the reprogramming process still require further investigations^[24]. In the example of NSC differentiation, mild hypoxia (2.5%-5% O₂) enhanced human NSC differentiation into neuronal and oligodendrocyte cells, while under normoxia (20% O₂) human NSC showed the preferential commitment to astrocyte lineage^[25]. The beneficial effects of hypoxia may be due to the active mitochondria activity and better cell survival during differentiation. Similar to NSCs, oligodendrocyte progenitor cells were sensitive to oxygen^[26], because low oxygen was found to suppress bone morphogenetic proteins (BMP) signaling^[27]. For human ESC-derived neurospheres, hypoxic preconditioning promoted neuronal differentiation and up-regulated HIF-1 α , HIF-2 α and the genes of erythropoietin, vascular endothelial growth factor (VEGF), and Bcl-2 family members^[28]. All these studies demonstrated the important roles of oxygen in stem cell proliferation and differentiation.

Control of oxygen tension in bioreactors

Bioreactors allow the precise control of oxygen tension through the sensors and the feedback system. Lovett *et al.*^[29] demonstrated enhanced chondrogenic differentiation of human MSCs at 5% O₂ and adipogenic differentiation at 20% O₂ in a modular bioreactor with well characterized oxygen gradients^[29]. Neural progenitors were also prepared in bioreactors with controlled oxygen for clinical

trials^[30]. Agarose-encapsulated murine ESC aggregates cultured in a perfused stirred tank bioreactor were directed to cardiomyocyte differentiation at 4% O₂^[31]. Similarly, a study with size-controlled embryoid bodies (EBs) derived from human ESCs showed that 4% O₂ resulted in the up-regulation of cardiac genes compared to normoxia in a spinner bioreactor^[32]. Continuous production of iPSCs was also assessed in an acoustic perfused bioreactor at different oxygen tensions, which showed that a significant increase in iPSC growth at the low oxygen tension of 5% due to the preferential glycolytic metabolism over mitochondria oxidation^[33]. It has to be pointed out that the oxygen gradient could exist around the local cell organizations. Even that the medium was exposed to normoxia, different oxygen transfer and consumption rates in the culture system might expose the stem cells to the local hypoxia environment. Therefore, using a biosensor to measure the *in situ* oxygen level, as shown in Figure 2, would be useful to fully understand the effect of oxygen in stem cell fate decision.

BIOPHYSICAL CUES OF EXTRACELLULAR MATRIX

Exogenous extracellular matrix and scaffolds

Biophysical cues provided by a tissue scaffold as synthetic extracellular matrix (ECM) play an important role in engineering stem cells in the bioreactor microenvironment. A wide variety of natural and synthetic materials, including Matrigel, collagen, alginate, hyaluronic acid, poly (ethylene terephthalate) (PET) matrix, poly(ethylene glycol) hydrogels and self-assembling peptide gels, have been utilized

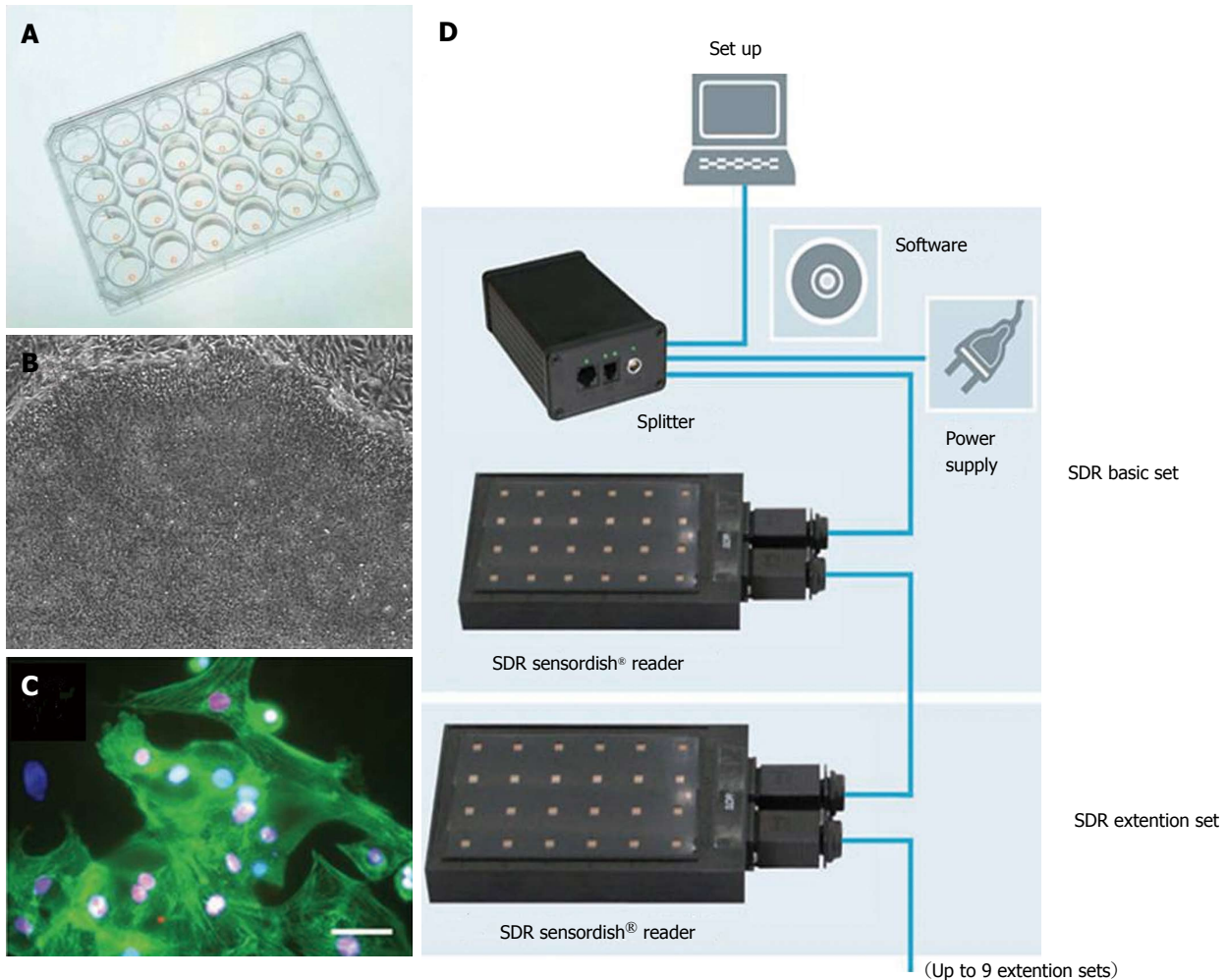


Figure 2 *In situ* measurement of oxygen concentration during stem cell differentiation. A: Multi-well plate with an oxygen sensor in each well; B: The starting undifferentiated human embryonic stem cells (ESCs); C: The human ESC derived cardiomyocytes expressing cardiac marker NKX2.5 and cardiac troponin I (adapted from reference Xu *et al.*^[120]); D: The bioreactor system with a sensor platform for reading the oxygen tension during *in situ* differentiation.

for culturing and engineering stem cells^[12,34-37]. The material properties including surface topology and stiffness or tensile strength have profound effects on cell adhesion, proliferation, and the differentiated cellular function^[38]. For example, substrates with high stiffness directed stem cells into osteoblasts, medium stiffness promoted myogenesis and low stiffness promoted neurogenesis^[39]. The scaffolds also provide 3-D biophysical cues that regulate cell organization and morphogenesis^[40,41]. Neural differentiation from ESCs was promoted in 3-D fibrous matrices compared to 2-D culture^[42,43]. It was believed that the biomechanical forces from the scaffold caused cytoskeletal tension that affected cell shape and signal transduction, which in turn regulated stem cell lineage commitment^[44]. The structures of the 3-D scaffolds, including pore size and porosity, affect mass transport and must be compatible with perfusion bioreactors for the development of thick and compact tissue grafts.

The optimal scaffold design for perfused bioreactor cultures should be guided by the structural and mechanical properties of native tissues. Hydrogels with tunable molecular, mechanical and degradation properties have

been applied in human ESC cultures^[45] and the engineering of soft tissues such as cartilage^[35]. For engineering bone and cardiac muscle, a scaffold must provide good balance in pore curvature for cell attachment, pore size for cell migration, and hierarchical structures including orientation, anisotropy and channels for vascular conduits^[46]. Mechanically strong, highly porous and mineralized silk scaffold can promote the bone tissue formation from MSCs^[47]. The pore size of the scaffold has been shown to affect the type of the engineered bone tissue with the formation of flat bone in small pore, trabecular bone in large pore and transient bone in a gradient of pore structures^[48]. In contrast, soft, highly porous and channeled elastomer scaffold has been preferentially used for the engineering of vascularized cardiac tissue^[49]. For example, an accordion-like elastomer scaffold with structural and mechanical anisotropy has been designed and used for cardiac tissue engineering^[50], which induced the alignment and coupling of neonatal heart myocytes and generated direction-dependent contractions close to native heart tissues. Bioreactors can also enhance the mass transfer in the scaffolds through perfusion and other

flow pattern to promote the stem cell proliferation or differentiation. For example, a radial-flow bioreactor was used to support 3-D expansion of human MSCs on a large collagen scaffold, which enhanced colony-forming unit activity of MSCs^[51]. ESCs differentiated on 3-D porous tantalum-based scaffolds inside spinner bioreactors showed significant enhancement in hematopoiesis compared to cells cultured under static conditions^[52]. Thus, the use of biomimetic scaffolds in the bioreactors is critical to recapitulate *in vivo*-like stem cell microenvironment to promote the desired lineage and the tissue-specific function.

Decellularized ECMs

Compared to synthetic ECMs, decellularized ECMs contain a mixture of adhesion ECM proteins, such as fibronectin and collagen, and the sequestered growth factors, such as fibroblast growth factor (FGF)-2, which can modulate the activities of receptor-ligand binding^[53,54]. These interactions constitute a dynamic and reciprocal relationship among the cells, growth factors and ECMs which cannot be readily reproduced by synthetic ECMs^[55,56]. For example, human PSC-derived ECM supported PSC propagation with the deposited growth factors acting in transforming growth factor (TGF)- β /Nodal pathways^[56]. Decellularized ECMs from the heart was repopulated in a perfusion bioreactor and the cells displayed pumping function^[57]. Decellularized cardiac matrices also preserved their signaling capacity and induced human ESC differentiation toward the cardiac lineage^[58]. Decellularized bone matrix in a perfusion bioreactor supported human ESC differentiation into stable bone-like tissue^[59]. Studies using the decellularized scaffolds supplied by nature will lead to the design of synthetic ECMs which mimic the native ECMs to be engineered in bioreactors.

PHYSICAL FORCES

Mechanical stimulation

Mechanical forces affect cellular differentiation pathways and are important to the control of tissue morphogenesis from stem cells^[60]. Mechanical stimulations affect cells in various ways: compression and shear can cause cellular deformation, pressure and shear force result in mechanical stress, and tension causes cell elongation and the deformation of cell nucleus^[46]. Therefore, mechanical signals play significant roles in the development and function of various tissues, including bone, cartilage, skeletal muscle and cardiovascular tissues. It is well-recognized that the mechanical forces and the magnitudes that govern tissue development and remodeling *in vivo* would also enhance tissue development and function *in vitro*.

Specialized bioreactors have been developed to study the effects of mechanical stimulation on stem cell differentiation. Deformational loading and hydrostatic pressure are the primary factors of cell microenvironment for

articular cartilage, and have been employed in cartilage tissue engineering^[12,61]. Mechanical stimulation such as longitudinal strain accelerated the bone morphogenesis from MSCs^[62]. Zhang *et al.*^[63] developed a biaxial rotating bioreactor (BXR) to generate tissue engineered bone grafts from human fetal MSC and showed that higher cellularity, confluence, and more robust osteogenic differentiation were achieved in the BXR as compared to spinner flasks, perfusion and rotating wall bioreactors. Human ligaments were engineered from human MSCs in a bioreactor by combining dynamic tension and torsion mimicking forces *in vivo*, which significantly improved the ligament function^[64]. Using MSCs and fibroblasts seeded in tendon constructs in a bioreactor with cyclic mechanical load, tendons with comparable ultimate tensile stress and elastic modulus to fresh tendons were obtained^[65]. Different cell types, such as MSCs and human EB-derived mesenchymal progenitors, may respond differently to the same mechanical force in a mechanical compression bioreactor^[66]. MSCs in hydrogels responded to mechanical stimulation in the absence of TGF- β 1 by upregulating chondrogenic genes while human EB-derived cells required the presence of TGF- β 1. Biomimetic perfusion systems have also been developed for engineering small-diameter blood vessel grafts. For example, Niklason *et al.*^[67] obtained the engineered vessels from smooth muscle cells cultured in fibrous scaffolds under pulsatile flow in a perfusion bioreactor^[67]. Cyclic flexure and laminar flow were also applied in the bioreactor, which synergistically accelerated the tissue formation of heart valve with significantly increased collagen contents and tissue stiffness^[68]. Thus, novel design of bioreactors can be and has been successfully used to recreate physiological loading environment and control stem cell differentiation.

Electrical stimulation

Besides mechanical forces, electrical stimulation is also an important microenvironmental factor for mediating stem cell differentiation. Electrical stimulation regulates the action potentials of excitable cells and is especially useful for differentiation into neural and cardiac lineages. Mild electrical stimulation strongly influenced ESCs to assume a neuronal fate by the induction of calcium ion influx^[69]. The application of a lateral current through the single-walled carbon nanotube/laminin composites stimulated the generation of neuronal action potentials during NSC differentiation^[70]. The application of direct current electrical fields to human ESC-derived EBs promoted cardiomyocyte differentiation by regulating the generation of reactive oxygen species^[71]. Cardiac-like electrical stimulation was also applied to generate excitation-contraction to induce cellular tension on cells cultured on scaffolds^[72]. Similar to the application of mechanical stretch, the electrically stimulated cells underwent electromechanical coupling and conducted electrical pacing signals over macroscopic distances with synchronously beating at the frequency of stimulation.

Table 2 Microfluidics devices and microbioreactors in engineering stem cell niches

Microfluidic devices and microbioreactors	Stem cell type	Applications	Ref.
Gradient-generating microfluidic device	NSC	Proliferation and astrocyte differentiation	[91]
MEMS automated microfluidic device	AFSC	Adipogenic and osteogenic differentiation	[92]
3-D hydrogel incorporated microfluidics	NSC	3-D differentiation into neuronal and oligodendrocyte differentiation	[93]
Micro-grooved PDMS sheets with cyclic strain	MSC	MSC proliferation and differentiation	[94]
Microfluidic device with logarithmical flow rate	Mouse ESC	ESC adhesion and proliferation	[95]
A microfluidic chip which creates arbitrary culture media formulations	MSC	Proliferation, osteogenic differentiation and motility	[96]
A microscaffold cell chip with precisely controlled microenvironment	Retinal stem cells	Decrease apoptosis during the retinal differentiation	[98,99]
Microbioreactor array for 2-D and 3-D hydrogel cultures	Human ESC	Adjust flow rate and evaluate vascular differentiation	[100]
Microbioreactor arrays for drug screening	ESC, MSC	Incorporate 3D culture, biomaterials, etc. to screen drugs in a high-throughput manner	[103]
Compartmentalizing microfluidic devices	Cancer stem cells	Understanding of cell migration and cancer invasion	[104]
Microbioreactor array with 3-D fibrous matrix	Mouse ESC	High-through cell-based assay for drug screening	[14,87,102,106]
Microbioreactor array with full factorial design of growth factor combinations	Human ESC	Screening exogenous and paracrine factors in human ESC differentiation into mesoderm cells	[15]
Microfluidics with patterning and temporal analysis	PSC	Reveal paracrine/autocrine signaling for PSC self-renewal	[110]
Microbioreactor array with 3-D cell culture setting	EBs derived from human ESC or iPSC	PSC mesoderm differentiation, with controlled cytokine gradients.	[101]
Microfluidics with varying flow rates	PSC	Reveal paracrine/autocrine signaling during PSC self-renewal	[108,115]

NSC: Neural stem cells; AFSC: Amniotic fluid stem cells; ESCs: Embryonic stem cells; MSCs: Mesenchymal stem cells; PSC: Pluripotent stem cells; iPSC: Induced pluripotent stem cells; EBs: Embryoid bodies.

Flow shear force

Flow shear force generated by perfusion or agitation can improve mass transfer in the scaffolds and thus provides better control on nutrient delivery. For example, perfusion improved tissue architecture of engineered cardiac muscle^[73], increased cellularity and matrix synthesis in chondrocyte cultures^[74], and enhanced chondrogenesis of human ESC-derived MSCs^[75]. Perfusion also improved cellularity and bone matrix components of engineered constructs using human adipose-derived MSCs^[76]. Grayson *et al.*^[77] engineered anatomically shaped human bone grafts using human MSCs in a bioreactor with continuous perfusion and found that the bone matrix architecture and density correlated with the interstitial flow pattern and intensity. In addition, perfusion was found to give better pO₂ control and more uniform cell distribution within 3-D scaffolds^[78], and facilitated long-term ESC culture to reach a high cell density^[79]. MSC proliferation was enhanced in highly porous matrices at different flow rates (0.1-1.5 mL/min), and the higher flow rate of 1.5 mL/min upregulated osteogenic differentiation^[80]. Different perfusion flow configurations like parallel flow and transverse flow were found to affect osteogenic differentiation of human MSCs due to the regulation of ECM and FGF-2 secretion by different flow patterns^[81]. Flow shear induced by agitation preserved Oct-4 expressing cells during PSC differentiation^[82,83]. It was suggested

that shear stress modulated gene expression through mechano-transduction to induce autocrine or paracrine signaling to suppress spontaneous differentiation^[84,85]. These findings underscore the importance of reciprocal interactions of flow shear force and cell signaling in 3-D cellular organizations.

MICROFLUIDIC DEVICES AND MICROBIOREACTORS

Microfabrication techniques, especially soft lithography, can be used to control features at a micrometer scale between 1 and 1000 µm in microdevices^[86], which provide controllable microenvironments for engineering stem cell differentiation as well as for cytotoxicity screening in high-throughput assays (Table 2)^[14,87]. Via laminar flow within microchannels, microfluidic systems can be used to generate biochemical gradients and deliver cytokines at well-defined concentrations^[88,89]. Microfluidic devices also can be used to regulate molecular and biomechanical signals, and to study their effects on cell morphogenesis, migration, proliferation and cell-cell interaction. Microbioreactors, or microfluidic-based cell culture arrays, allow the spatial and temporal control of stem cell microenvironment compared to large scale bioreactors. With microfluidic devices and microbioreactors, stem cell differentiation can be regulated by controlling substrate

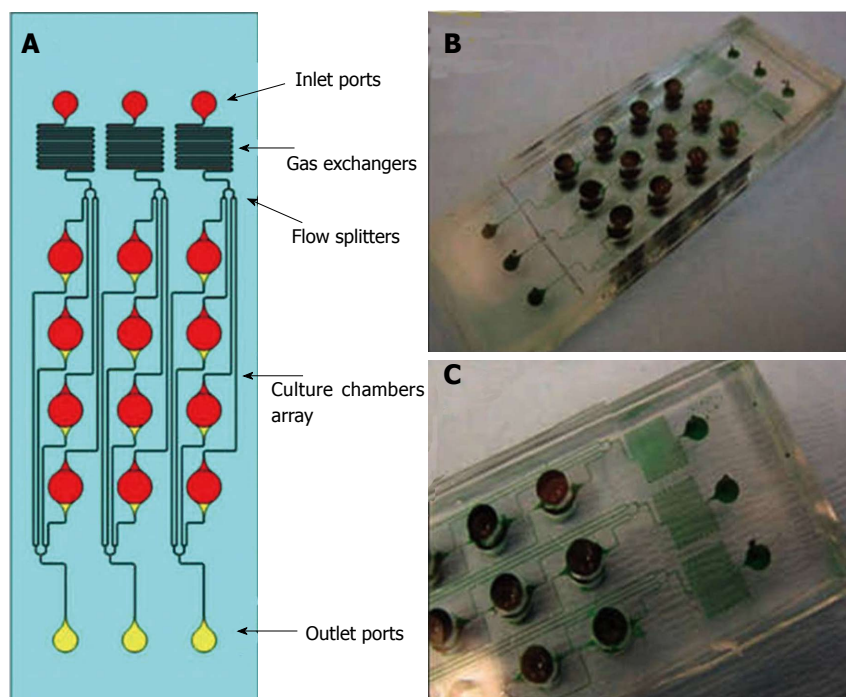


Figure 3 Microbioreactor array for human pluripotent stem cells differentiation. A: Top view of the microbioreactor array assembly; B, C: Photographs of the assembled microfluidic platforms. A contrast dye was used in images (B) and (C) to show the fluid paths. Adapted from Cimetta *et al.*^[100].

size and stiffness, cytokine gradients, flow rate and auto-crine/paracrine signaling^[90].

Applications in stem cell differentiation and drug screening

Microfluidics and microbioreactors have been studied recently for the specific lineage differentiation from various stem cell types. Spatial control of the proliferation and differentiation of NSCs was achieved by controlling gradients of growth factors in microfluidic devices^[91]. An automatic microfluidic system for adipogenic and osteogenic differentiation of human AFSCs has been developed using micro-electro-mechanical-systems (MEMS) technology^[92]. Neuronal and oligodendrocytic differentiation of NSC was significantly enhanced by 3-D microenvironments in microfluidic channels, suggesting that mimicking *in vivo* microenvironment should promote NSC differentiation^[93]. Microdevices have also been incorporated with cyclic strain; for example, micro-grooved polydimethylsiloxane (PDMS) sheets have been used to study the mechanosensing properties of MSCs^[94]. Kim *et al.*^[95] designed microfluidic arrays for perfused ESC culture over a logarithmic range of flow rates and observed the changes of colony sizes and shapes in response to flow rate^[95]. A microfluidic chip was used to automatically analyze proliferation, motility and osteogenic differentiation of MSC in a range of cell culture regimes including medium formulation, seeding density and feeding schedules^[96]. Besides controlling soluble and chemical niches in both 2D and 3D stem cell cultures, microdevices have been applied to study the synergistic effect of soluble/chemical factors and biomaterials in a miniaturized high-throughput manner^[12]. Synthetic biomaterial arrays were incorporated with microfluidics to test the interactions of stem cells with a variety of ex-

tracellular signals^[97]. A microscaffold cell chip, made of poly-methyl-methacrylate bonded to a perforated polycarbonate membrane, has been used to study the single spheroid behavior of retinal stem cells with the precisely controlled microenvironment^[98,99]. Using a microarray, thousands of polymeric materials and their effects on the differentiation of human ESCs and MSCs could be evaluated. A microfluidic platform coupling with an array of microbioreactors (Figure 3) has been applied for high throughput studies of human ESC differentiation into mesoderm cells^[100]. The microfluidic platform allows quantitative assessments of human ESC characteristics in both 2-D and 3-D microenvironments and can be used to determine the effects of cytokine gradients (*i.e.*, BMP-4, Activin A and Wnt3a) on cell differentiation^[101]. These microbioreactors can serve as the high-throughput platform for screening cytokines, tissue scaffolds, and environmental factors, and thus can be used in developing and optimizing bioprocesses for culturing and engineering stem cells^[102].

Microfluidics and microbioreactors have also been studied recently for the drug screening based on stem cell proliferation and differentiation^[103]. The migratory behavior of cancer stem cells were evaluated in the compartmentalizing microfluidics by combining gradient generators, fluid handling, micro-electrodes and live cell imaging, which can be used for drug screening and disease diagnosis^[104]. Microbioreactors with 3-D fibrous matrices (Figure 4) were also developed to assess the cytotoxicity of drug and medium supplements^[14,87,101,105]. This 3-D model more resembled the *in vivo* microenvironment and thus the cell response to the drugs more reflected the response that could occur *in vivo*. For example, various Chinese herb medicines were tested in the 3-D microbioreactors seeded with mouse ESCs and cancer cells^[106], in

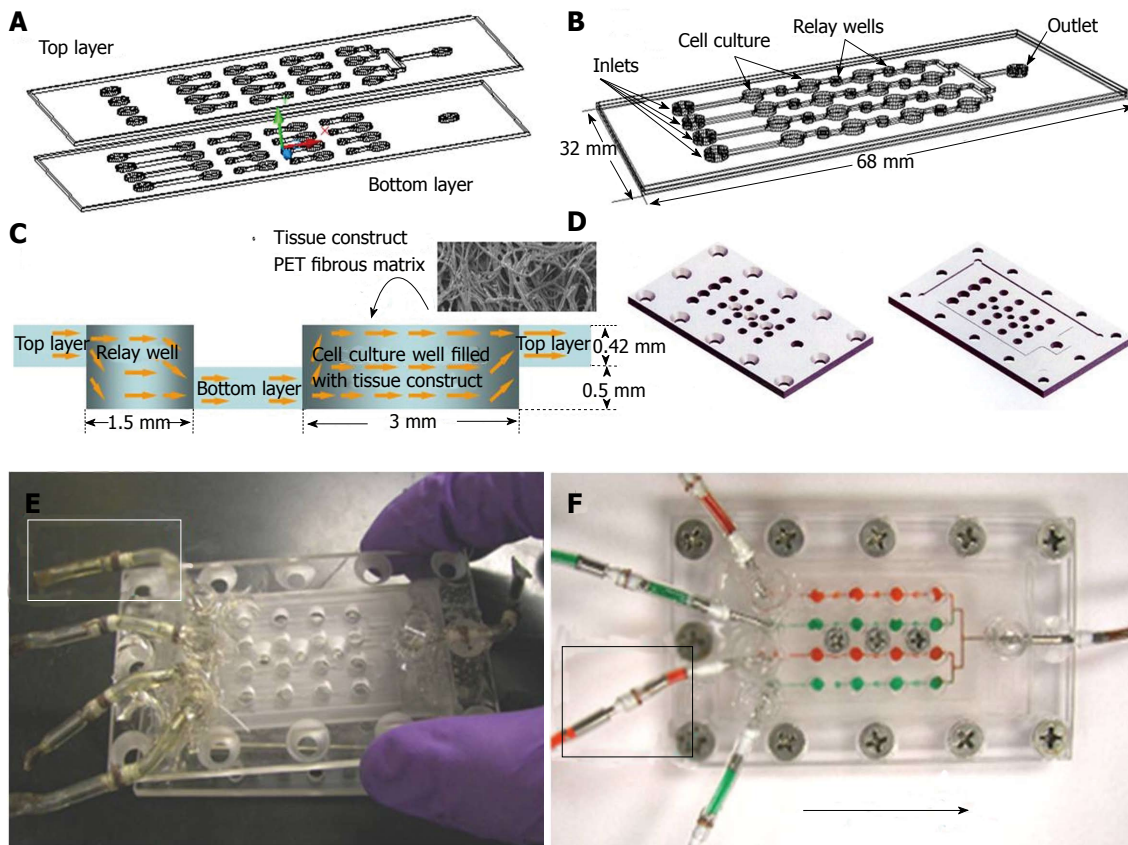


Figure 4 Perfusion bioreactor array for cell-based assay. A: Schematic drawing of the device composed of top and bottom layers; B: Perspective view of the assembled device to form microscale channels and wells; C: Design of individual cell culture well and relay well formed by two layers, with cell culture well that can be filled with any modular tissue engineering scaffold such as PET fibrous matrix; D: Perspective drawing of the top and bottom frames for frame-assisted assembly; E: Photograph of device in assembly with each inlet connecting to a flexible connector capped to prevent contamination (highlighter window); F: Photograph of assembled device at work with each inlet connected to an external tubing through a flexible connector (highlighter window). Reproduced from reference Wen *et al.*^[14].

which the sensitivity was greatly enhanced compared to 2-D screening platform. Thus the microfluidics and microbioreactors recreate the stem cell microenvironment while still allow the high throughput analysis, significantly improving the reliability of the screening outcomes.

Applications in regulating paracrine and autocrine signaling

Soluble growth factor secreted by stem cells is a critical niche factor for efficient stem cell differentiation. For examples, MSCs secreted various trophic factors to modulate the immune response and cell survival^[107]. PSCs have also been shown to self-regulate the expansion and differentiation through autocrine/paracrine signaling such as Wnt, lefty, and Activin A^[108-112]. For hematopoietic differentiation, the secreted VEGF, stem cell factor (SCF) and anti-apoptotic factors from ESCs stimulated the formation of colony-forming cells^[113]. Autocrine and paracrine signaling through transforming growth factor (TGF)- β regulates Smad crosstalk and controls the survival and repopulation ability of HSCs^[114]. The identified paracrine/autocrine signaling can be used to improve the stem cell differentiation by enhancing the positive signaling and inhibiting the negative feedback signaling.

Continuous flow microbioreactor arrays revealed the

effect of paracrine-dependent mechanism during human ESC differentiation into early mesoderm^[15]. The negative feedback loop to the mesoderm progenitors could be overcome by adding the glycogen synthase kinase (GSK)-3 β inhibitor CHIR99021 and the positive induction signals were enhanced by supplementing FGF-2. Endogenous Activin A may also be secreted during human PSC differentiation into early mesoderm cells, which requires the inhibition of this signaling to promote the mesodermal specification^[101]. Autocrine/paracrine signaling also supported PSC self-renewal. By increasing the flow rate to wash out the endogenous secreted factors, PSC spontaneous differentiation occurred^[108,115]. While microfluidics has been explored to study the autocrine/paracrine signaling of adherent stem cells^[108,110], its use in suspension stem cells is also possible in combination with other approaches such as pathway inhibition and fed-batch dilution^[110,116]. Cell communication and spatial ligand distribution in 3-D suspended cells are determined by cell organization, cell density and ligand diffusivity. The aggregate models have been shown to up-regulate factor secretion and provide spatial effect compared to monolayer cultures due to the enhanced cell-cell and cell-ECM interactions^[117,118]. For example, EB derived from human ESCs and iPSCs were seeded into the multi-wells which

prevented the EB washout^[10]. The revealing mechanism of autocrine/paracrine signaling enables the novel design of differentiation protocols and potential translation into large scale bioprocesses. Thus, microfluidics and micro-bioreactors can be used as powerful tools to better understand the autocrine/paracrine factors that could lead to more efficient stem cell engineering and differentiation.

CONCLUSION

Efficient stem cell differentiation into functional tissue-specific cells is one of the major challenges in stem cell engineering. Regulating the stem cell niches in bioreactors provides a platform to precisely control stem cell fate decision. The complexity of stem cell niches can be recreated in bioreactors by modulating the regulatory factors such as oxygen tension, the scaffolding materials and configurations, and by providing various stimulation forces. Microfluidics and micro-bioreactors increased the throughput of screening various microenvironmental factors and improved the understanding of local paracrine and autocrine signaling. Further studies are still required to recapitulate the *in vivo* stimuli and integrate various niche factors to interrogate the interactions among cells, ECMs, autocrine/paracrine signaling and physical forces. Overall, bioreactors provide the bridge from the fundamental mechanism to the enabling technology for stem cell-derived biomedicines and therapeutics.

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Molecular mechanisms of mesenchymal stem cell differentiation towards osteoblasts

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Abstract

Bone is a dynamic tissue that is constantly renewed by the coordinated action of two cell types, *i.e.*, the bone-resorbing osteoclasts and the bone-forming osteoblasts. However, in some circumstances, bone regeneration exceeds bone self repair capacities. This is notably often the case after bone fractures, osteolytic bone tumor surgery, or osteonecrosis. In this regard, bone tissue engineering with autologous or allogenic mesenchymal stem cells (MSCs) is been widely developed. MSCs can be isolated from bone marrow or other tissues such as adipose tissue or umbilical cord, and can be implanted in bone defects with or without prior amplification and stimulation. However, the outcome of most pre-clinical studies remains relatively disappointing. A better understanding of the successive steps and molecular mechanisms involved in MSC-osteoblastic differentiation appears to be crucial to optimize MSC-bone therapy. In this review, we first present the important growth factors that stimulate osteoblastogenesis. Then we review the main transcription factors that modulate osteoblast differentiation, and the microRNAs (miRs) that inhibit their expression. Finally, we also discuss

articles dealing with the use of these factors and miRs in the development of new bone MSC therapy strategies. We particularly focus on the studies using human MSCs, since significant differences exist between osteoblast differentiation mechanisms in humans and mice for instance.

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Key words: Mesenchymal stem cells; Osteogenesis; Runt-related 2; Wnt; MicroRNAs

Core tip: Several excellent reviews on the transcription factors involved in osteoblast differentiation have recently been published, but none also presented the microRNAs (miRs) that control the expression of these transcription factors. Moreover, most of these reviews mainly reported mouse studies but important differences are well acknowledged between humans and mice. For instance vitamin D3, an important hormone controlling bone homeostasis, has very different effect in these species. Therefore, in the present review we particularly focus on human cells to present the transcription factors and miRs controlling mesenchymal stem cells-osteoblastic differentiation.

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BONE REPAIR WITH MESENCHYMAL STEM CELLS

Historically, Friedenstein *et al*^[1] were the first to report the presence of fibroblastoid cells in the adult bone marrow that can make bone and reconstitute a hematopoietic

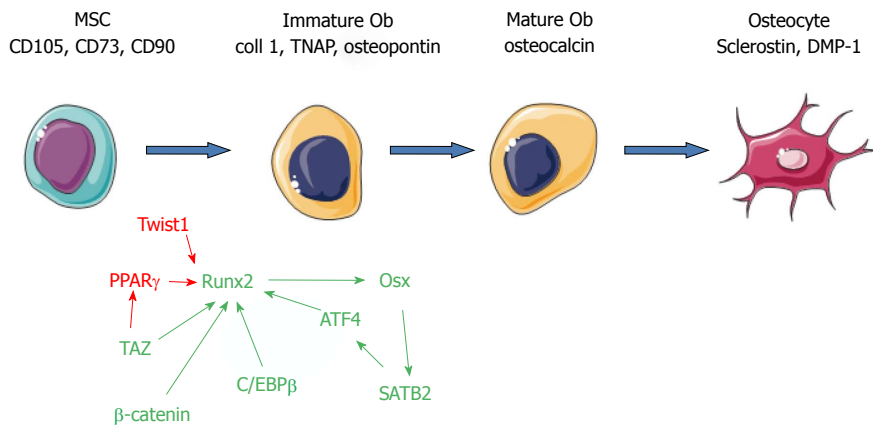


Figure 1 transcription factors involved in osteoblast differentiation from mesenchymal stem cells. Markers of differentiation are shown in black, stimulatory transcription factors in green, and inhibitory ones in red. MSCs: Mesenchymal stem cells; ATF4: Activating transcription factor 4; C/EBP: CCAAT/enhancer-binding proteins; Coll: Collagen; DMP-1: Dentin matrix protein-1; Ob: Osteoblast; Osx: Osterix; PPAR: Peroxisome proliferator activated receptor; SATB2: Special AT-rich sequence binding protein 2; TAZ: Transcription coactivator with binding capacity to PDZ motifs; TNAP: Tissue-nonspecific alkaline phosphatase; Runx2: Runt-related 2.

microenvironment when transplanted subcutaneously. These mesenchymal stem cells (MSCs) were later reported to contribute to various musculoskeletal tissues such as bone, cartilage, fat, muscle, ligament and tendon^[2]. In 2006, the International Society for Cellular Therapy proposed that cells with the following characteristics should be considered as MSCs, (1) cells adherent to plastic in culture; (2) presence of CD105, CD73 and CD90 but absence of CD34, CD45, CD14 or CD11b, CD79 α or CD19, and HLA-DR molecules; and (3) cells with the capacity to differentiate into osteoblasts, chondrocytes and adipocytes^[3]. However, although these criteria are widely accepted, they may still be imperfect. Indeed, the three markers are co-expressed in a wide variety of cells, and may therefore not be able to indentify a single MSC population *in vivo*^[4].

MSCs represent less than 0.01% of the bone marrow cell population. At birth, the frequency of MSCs has been reported as 1 MSC/10⁴ BM-mononuclear cells, decreasing to 1 MSC/10⁵ BM-mononuclear cells in teenagers to 1 MSC/2 \times 10⁶ BM-mononuclear cells in 80-year-old individuals^[5]. To overcome the drawbacks associated with MSC isolation from bone marrow, other sources have been contemplated. MSCs can indeed be recovered from several different locations such as adipose tissue^[6], dental pulp^[7] and umbilical cord^[8]. Recently, Sacchetti *et al.*^[9] reported CD146 high pericytes surrounding bone marrow vascular sinusoids can be considered as MSCs as they are self-renewing osteoprogenitors capable of ectopic bone formation. Finally, differences appear to exist between MSC populations from different tissues, which represents an additional challenge to devise a universal definition^[10].

MSC differentiation into osteoblasts can be achieved by adding vitamin D₃, ascorbic acid and β -glycerophosphate to the culture medium^[11]. Several laboratories use dexamethasone, a synthetic glucocorticoid, instead of vitamin D₃. Dexamethasone appears to optimize differentiation from MSCs, but not specifically to the osteoblast lineage^[12]. In osteogenic conditions, human MSCs secrete a matrix enriched in type I collagen which will be mineralized with apatite crystals upon activation of tissue-nonspecific alkaline phosphatase (TNAP) (Figure 1)^[13]. Osteoblasts also secrete a tissue-specific protein, osteocalcin,

recently shown to act as a circulating hormone involved in the control of insulin secretion and sensitivity^[14]. However, although this protein is a useful marker of osteoblast differentiation, it doesn't seem to impact bone formation. Eventually, some osteoblasts will become surrounded by a mineralized collagen matrix and further differentiate into bone-residing osteocytes, which secrete different proteins such as sclerostin, a canonical Wnt signaling inhibitor, and dentin matrix protein-1, a molecule controlling phosphatemia^[15].

MSCs have been implanted in association with different scaffolds to rebuild bone^[16,17]. Injection of MSCs has also been shown to correct bone defects. Notably, allogenic bone marrow transplants or injection of isolated MSCs in children with osteogenesis imperfecta (OI) have improved bone formation and function^[18,19]. However, although promising data were reported, many others led to contrasting if not disappointing results^[20]. In this regard, it appears crucial to better understand the molecular mechanisms of osteoblast differentiation from human MSCs. This will allow us to improve the bioactivity of injected MSCs or MSC-containing hybrid materials by stimulating their osteoblast differentiation. This may be achieved through genetic modification of MSCs. For instance, autologous MSCs may be modified to correct the abnormal collagen synthesis in patients with OI^[21]. Several excellent reviews on osteoblast differentiation have been published in recent years. To our knowledge however, none has focused on the interactions between transcription factors and microRNAs in human mesenchymal stem cells specifically. We believe that it is particularly important since significant differences are well acknowledged between osteoblastogenesis of human and mouse MSCs. For instance, while vitamin D₃ binds to a vitamin D response element (VDRE) in the osteocalcin promoter in humans and rats, the mouse osteocalcin promoter is devoid of any VDRE and vitamin D₃ exerts an indirect inhibitory effect on osteocalcin transcription^[22,23].

GROWTH FACTORS STIMULATING MSC-OSTEOBLASTIC DIFFERENTIATION

Two families of growth factors appear to stimulate osteo-

blast differentiation from MSCs: the Wnt (a portmanteau of Wingless and integration 1) family and the bone morphogenetic proteins (BMPs).

Wnt family members

Wnt proteins are a family of 19 highly conserved secreted glycoproteins that play essential roles during development and tissue homeostasis^[24]. Some Wnt proteins such as Wnt3a and Wnt10b bind to Frizzled receptors, and recruit the LRP5/6 coreceptors to activate the canonical signaling pathway, leading to glycogen synthase kinase- β inhibition, β -catenin stabilization, translocation into the nucleus and regulation of T-cell factor/lymphoid enhancer factor (TCF/LEF) transcriptional activity. Binding of Wnt proteins to LRP5/6 is inhibited by secreted factors such as Dickkopf-related protein 1 (Dkk1)^[24]. Dkk1 binds to LRP5/6 causing the receptor to attract Kremen, and this interaction promotes clathrin-mediated internalization thereby inactivating LRP5/6.

The importance of the canonical Wnt signaling in bone is well-acknowledged. Genetic reports established that Wnt/ β -catenin activity is essential for bone development^[25]. Deficiency of Dkk1 is associated with increased bone formation in mice and humans^[26]. Wnt10b may be particularly important for bone formation. Wnt10b is expressed in the bone marrow by osteoblast progenitors^[27], and transgenic overexpression of Wnt10b in mesenchymal cells leads to increased bone density and accelerated osteoblastogenesis *in vitro*, whereas Wnt10b^{-/-} mice have reduced trabecular bone^[28]. Moreover, Wnt10b seems to stimulate osteoblast functions through a positive autocrine loop^[29]. On the other hand, other recent findings indicate that canonical Wnt signalling inhibits osteoblast differentiation in human MSC cultures^[30-32]. These contrasting findings have been reconciled recently by Liu *et al*^[33] who found that Wnt/ β -catenin signalling favours osteogenic commitment in basal medium by inhibiting MSC commitment into adipocytes, but inhibits osteoblast differentiation in osteogenic conditions. This was confirmed by Kang *et al*^[34] who reported that Wnt10b induction of osteogenesis in mouse progenitors was due to inhibition of peroxysome proliferator-activated receptor (PPAR) γ and CCAAT/enhancer-binding protein (C/EBP) α activity. The mutual inhibition between β -catenin and PPAR γ will be discussed below.

Alternatively, non-canonical Wnt members may also be involved in the effects of TNF- α on ossification. In particular, Wnt5a seems to be the predominant Wnt variant expressed during osteoblastic differentiation of human MSCs^[35]. Wnt5a^{+/-} mice present a reduced bone mass phenotype with decreased osteoblast number^[36]. Wnt5a appears to stimulate osteoblast differentiation through an autocrine loop in human MSCs^[37,38]. Another non-canonical Wnt with a potential interest in bone repair is Wnt4. In two different models of craniofacial bone injury, Chang *et al*^[39] observed that human MSCs genetically engineered to express Wnt-4 enhanced osteo-

genesis and improved the repair of craniofacial defects in nude mice.

Bone morphogenetic proteins

BMPs are growth factors that belong to the transforming growth factor beta (TGF- β) superfamily^[40,41]. The term, bone morphogenetic protein was first introduced to describe the components in demineralized bone matrix that can induce ectopic bone formation when implanted intramuscularly or subcutaneously into rodents^[42,43]. To date, more than 20 BMP members have been characterized. As TGF- β , BMPs trigger cellular responses mainly through the Smad pathway^[44], although they can also activate the mitogen-activated protein kinase pathway^[45]. In the Smad pathway, type II and type I receptors with serine/threonine kinase activity and intracellular Smad proteins relay the signal from the cell surface to the nucleus. Three type II receptors can bind BMPs: type II BMP receptor, and type II and II B activin receptors (ActR-II and ActR-II B)^[40]. Three type I receptors for BMPs have also been characterized: type I A and I B receptors (BMP1A or ALK3 and BMP1B or ALK6), and type I A activin receptor (ActRIA or ALK2). The receptors activated by ligand binding phosphorylate a subgroup of receptor-regulated Smads (R-Smads including Smad 1, 5 and 8). The phosphorylated R-Smads then disassociate from their receptor and form complexes with the common partner Smad 4. Smad heterodimers then migrate into the nucleus where they associate with transcription factors to regulate gene transcription. This Smad signal is inhibited by Smad 6 and Smad 7, which block phosphorylation of R-Smads.

BMP factors are important in skeletogenesis^[40]. BMP-2 is expressed in areas surrounding cartilage condensations^[46,47], while BMP-4 is expressed in perichondrium^[47]. BMP-2 is also expressed in periosteal and osteogenic zones^[46]. Due to their effect on runt-related 2 (Runx2) and osterix expression^[48], BMPs are very potent inducers of mesenchymal progenitor cell differentiation into osteoblasts^[49]. Recombinant BMPs can be added in different materials such as in collagen sponges and calcium phosphate ceramics to be delivered *in situ* for clinical practice^[50-52]. In humans, recombinant human BMP-2 and BMP-7 have been approved for clinical use in orthopedic surgery for long bone open-fractures treated with intramedullary fixation and non-union fractures, and in spine surgery for spinal fusion in place of iliac crest bone graft^[53]. BMPs do not seem to accelerate fracture healing but tend to increase healing rates without requiring a secondary procedure^[54]. Nevertheless, several concerns today complicate the use of BMPs, such as heterotopic ossifications, immunogenic reactions or hardware failure^[54,55]. Moreover, the clinical interest of BMPs is limited to local applications, and BMPs may not represent an alternative treatment to systemic bone diseases such as osteoporosis. Systemic use of BMPs is limited by their non-skeletal effects, mitogenicity, and short half-life.

TRANSCRIPTION FACTORS INVOLVED IN MSC-OSTEOBLAST DIFFERENTIATION

Stimulatory transcription factors

β -catenin: As detailed above, β -catenin is potently activated in the canonical Wnt signaling pathway^[24]. In this pathway, unphosphorylated β -catenin molecules accumulate in the cytoplasm, translocate to the nucleus, and activate the transcription of downstream genes by binding to LEF/TCF transcription factors. Conditional deletion of β -catenin gene in Dermo-Cre or Prx1-Cre transgenic mice reveals its essential role in osteoblast differentiation^[25,56]. In addition, conditional deletion of β -catenin gene in Wnt1-Cre transgenic mice, in which Cre is expressed in neural crest cell precursors, results in loss of cranial bones derived from neural crest cells^[57]. Interestingly Runx2 is expressed in β -catenin deficient cells^[25,56], but is strongly enhanced by β -catenin/TCF1. It is required for osterix expression and osteoblast differentiation^[58] (Figure 1).

Runx2: Runx2 belongs to the Runx family, which consist of Runx1, Runx2 and Runx3. These transcription factors form heterodimers with Cbfb and bind to the consensus sequence TGPYGGPyPy^[59]. Runx2 is considered as the master osteoblast transcription factor (Figure 1). It was identified as a factor binding to an osteoblast specific cis-acting element in the promoter of the genes encoding for osteocalcin^[60]. Runx2 deficiency in mouse leads to the formation of a skeleton devoid of osteoblasts^[61,62]. In man, inactivating mutations in Runx2 leads to a skeletal dysplasia called cleidocranial dysplasia^[63]. Runx2 regulates many genes that determine the osteoblast phenotype. Runx2 is sufficient to induce the expression of many osteoblast markers, such as osteocalcin, in non-osteoblastic cells^[60]. However, Runx2 overexpression in osteoblasts severely reduces osteocalcin expression and osteoblast maturation^[64,65]. Therefore, whereas Runx2 is required to commit undifferentiated cells towards the osteoblast lineage, it appears to maintain these cells in an immature stage^[66].

In murine fibroblasts, the forced expression of Runx2 is sufficient to induce expression of osteoblast markers such as collagen type I, osteocalcin or bone sialoprotein. Adenoviral overexpression of Runx2 in mouse MSCs generated substantially more bone than control MSCs when implanted in subcutaneous tissue or in calvarial defects^[67]. Similarly, rat bone marrow stromal cells transduced with *Runx2* retroviral vector seeded onto 3D-fused deposition-modeled polycaprolactone scaffolds, produced biologically-equivalent mineralized matrices at nearly 2-fold higher rates than control cells^[68]. In human MSCs isolated from adipose tissue, electroporation of Runx2 stimulated osteoblast differentiation *in vitro* with increased expression of alkaline phosphatase and osteocalcin^[69].

Transcription coactivator with binding capacity to PDZ motifs: Transcription coactivator with binding capacity to PDZ motifs (TAZ) was originally identified during a series of control experiments in a proteomic screen

looking for 14-3-3-interacting proteins^[70]. TAZ contains a 14-3-3-binding motif, a single WW domain, an extended coiled-coiled region within a larger transcriptional regulatory domain, multiple sites of phosphorylation, and a C-terminal motif that can interact with PDZ-containing proteins^[71]. The WW domain of TAZ binds to the sequence motif Pro-Pro-X-Tyr. This motif can be found within the regulatory regions of a large number of transcription factors, including Runx2 and PPAR γ , as well as members of the Sox, and SMAD families, suggesting that TAZ may be involved in the regulation of MSC commitment and differentiation into osteoblasts, adipocytes and chondrocytes^[71]. The WW domain-containing molecule TAZ directly interacts with Runx2 and co-activates Runx2-dependent gene transcription^[72]. In contrast, TAZ binds to, and markedly inhibits, the ability of PPAR γ to drive the expression of adipocyte-associated genes such as adipocyte protein 2, and depletion of TAZ increases their adipocyte differentiation^[72]. The processes through which TAZ is induced and/or activated are poorly understood^[71]. TAZ levels increase substantially in MSCs induced to differentiate into osteoblasts with BMP-2, whilst conversely, they decrease during adipocyte differentiation^[71]. It was also reported that TNF- α stimulates osteogenesis in hMSCs from adipose tissue through NF- κ B activation and TAZ expression^[73]. However, the pathophysiological significance of this finding remains obscure. In mouse mesenchymal cells, high-throughput screening allowed to identify a chemical compound, so-called TM-25659, that enhances TAZ nuclear localization and osteoblast differentiation at the expense of adipocytes^[74]. Moreover, TM-25659 suppressed bone loss *in vivo* and decreased weight gain in an obesity model. Although this compound seems to have a favorable pharmacokinetic profile, work remains to be done to demonstrate its possible interest in clinical application.

Special AT-rich sequence binding protein 2: Special AT-rich sequence binding protein 2 (SATB 2) is a member of the family of special AT-rich binding proteins that binds to nuclear matrix attachment regions (MARs) and activates transcription in a MAR-dependent manner. SATB2 inactivation in man results in cleft palate^[75]. SATB 2^{-/-} osteoblasts are characterized by a decreased differentiation, illustrated by reduced bone sialoprotein (BSP) and osteocalcin expression^[76]. SATB2 can physically interact with both activating transcription factor 4 (ATF4) and Runx2 and enhance the transactivation function of both proteins^[76]. Overexpression of SATB 2 in mouse bone marrow stromal cells stimulates expression of osterix and BSP^[77]. Transplanted SATB 2-overexpressing adult stem cells genetically double-labeled with BSP promoter-driven luciferase and β -actin promoter-driven enhanced green fluorescent protein into mandibular bone defects accelerated new bone formation^[77]. In addition, SATB 2-overexpressing murine induced pluripotent stem cells^[78] show increased mineral nodule formation and elevated mRNA levels of key osteogenic genes, osterix, Runx2,

Bsp and osteocalcin^[79]. SATB 2-overexpressing induced pluripotent stem cells combined with silk scaffolds and transplanted into critical-size calvarial bone defects created in nude mice induced enhanced bone repair^[79].

Osterix: Besides Runx2, the second transcription factor absolutely required for osteoblast differentiation is Osterix (Osx, also known as Sp7). Osx is a zinc finger-containing transcription factor belonging to the SP family of transcription factors. Osx is specifically expressed in osteoblasts, and is required for bone formation^[80]. The fact that Runx2 is expressed in Osx-deficient mice combined with the absence of Osx in Runx2 null mice places Osx downstream of Runx2^[80]. Actually, Runx2 may induce Osx expression, through direct binding on its promoter^[81]. Interestingly, Osx binds to the promoter of *Satb 2* to increase the transcription of the *Satb 2* gene^[82]. Thus, part of the effects of Osx may rely on SATB 2 activity. Murine bone marrow stromal cells overexpressing Osx associated with type I collagen sponge as a carrier exhibited five times more amounts of newly formed calvarial bone than that the control group in adult mice^[83]. In addition, overexpression of Osx in human umbilical cord-derived MSCs result in increased alkaline phosphatase activity and osteocalcin expression, and enhanced bone regeneration in nude mice using polylactic-co-glycolic acid as a carrier^[84].

Smads: Runx2 cooperates with Smad (a portmanteau of Sma in *Drosophila* and Mad in *C. elegans*) 2 and Smad 5 to regulate bone-specific genes^[85,86]. These interactions appear to be important *in vivo*^[87-89]. Whilst Runx2 alone does not induce osteoblast differentiation, it synergizes with Smad 2 and Smad 5 to achieve this event. Mutant Runx2 with a truncated transcription activation domain fails to interact with Smad1 and consistently blocks BMP/Smad-induced osteoblast differentiation^[86]. In addition to Runx2, menin, the product of the multiple endocrine neoplasia type 1 gene, is required for BMP-induced osteoblast differentiation^[90]. Menin interacts with both Runx2 and Smad 1/5 in multipotential mesenchymal cells. When menin is knocked down, the cells fail to differentiate into the osteoblast lineage.

CCAAT/enhancer-binding proteins β : CCAAT/enhancer-binding proteins (C/EBPs) belong to the group of basic leucine zipper transcription factors. They are known to modulate both adipocyte and osteoblast differentiation. C/EBP β forms a homodimer or heterodimer complex with other C/EBP family members. C/EBP β is expressed before PPAR γ and induces it^[91,92]. More precisely, two main protein forms of C/EBP β , induced by alternative translation initiation, present opposite effects on adipogenesis^[91]. Whereas LAP, the main long isoform, is proadipogenic, the short one, LIP, acts as a dominant negative inhibitor of LAP. In murine mesenchymal cells, LIP inhibits adipocyte differentiation and preferentially induces osteoblast differentiation^[93]. C/EBP β promotes

osteoblast differentiation of mesenchymal cells in Runx2-dependent and -independent mechanisms^[93]. C/EBP β up-regulates Runx2 expression by directly binding to the Runx2 P1 promoter in mesenchymal, pre-osteoblastic, and osteoblastic cells^[94]. In addition, C/EBP β interacts with Runx2 and activates the transcription of the osteocalcin gene^[95]. C/EBP β heterodimerizes with activating transcription factor 4 (ATF4, presented below), another basic leucine zipper transcription factor crucial for osteoblast maturation. This complex transactivates osteocalcin-specific element 1 of the osteocalcin promoter^[96]. Absence of all C/EBP β isoforms results in decreased bone mass in mice, associated with impaired osteoblast differentiation and functional deficiency^[96]. These data suggest that C/EBP β activates osteoblastogenesis. However, before commitment C/EBP β may act as a transcriptional repressor of Runx2 and of osteoblast differentiation^[91,97]. Mechanistically, it has been proposed that once osteogenic differentiation is initiated, Smad3 expression increases, binds to C/EBP β , and blocks its inhibitory action on Runx2^[98].

Activator protein 1 proteins: Activator protein 1 represents heterodimeric transcription factors composed of members of the Jun and Fos family of basic leucine zipper proteins. Overexpression of Δ FosB or Fra1 leads to enhanced bone formation. Osteopetrosis in Δ FosB overexpressing mice is due to the inhibition of mesenchymal cell differentiation into adipocytes, leading to an increased number of osteoblasts^[99]. Moreover, conditional Fra1^{-/-} mice display reduced levels of several matrix proteins, such as osteocalcin^[100]. Finally conditional deletion of JunB causes bone defects with reduced osteoblast proliferation, and expression of osteocalcin and bone sialoprotein^[101].

ATF4: Mice deficient in ATF4 display a decreased bone formation, leading to a severe low bone mass phenotype^[102]. At the molecular level, ATF4 directly binds to the promoter of osteocalcin to activate transcription^[102]. This activation appears to rely on the physical interaction between ATF4, SATB2 and Runx2 at the promoter level^[103]. ATF4 may also cooperate with C/EBP β to activate transcription of the osteocalcin gene^[96]. Finally, ATF4 also plays indirect effects through its activation of amino acid transport^[104]. Indeed, osteoblasts from ATF4^{-/-} mice do not synthesize normal levels of type I collagen unless nonessential amino acids are added to the culture^[102].

Inhibitory TFs

Peroxisome proliferator-activated receptor γ : PPAR γ proteins are expressed in mice and humans as two different isoforms, PPAR γ 1 and PPAR γ 2, due to alternative promoter usage and alternative splicing. PPAR γ 1 is ubiquitously expressed whereas PPAR γ 2 expression is restricted to adipocytes^[105,106]. Homozygous PPAR γ -deficient ES cells fail to differentiate into adipocytes, but spontaneously differentiate into osteoblasts^[107]. Heterozygous

PPAR γ -deficient mice exhibit a high bone mass phenotype but normal osteoblast functions^[107]. PPAR γ 2 has been reported to bind to Runx2 and inhibit its transcriptional activity^[108]. Inhibition of PPAR γ with the pharmacological inhibitor GW9662 in human MSCs stimulates mineralization and bone formation *in vitro* and *in vivo*^[109,110]. Besides the inhibition of Runx2, PPAR γ inhibitory effects may also include β -catenin. Indeed, activated PPAR γ in mesenchymal cells induces the proteasomal degradation of β -catenin following direct interaction^[111]. Moreover, Lu *et al*^[112] reported that the PPAR γ inhibitor GW9662 significantly activates TCF reporter plasmid activity. Furthermore, Krause *et al*^[110] reported that GW9662 treatment of hMSCs resulted in β -catenin accumulation in the nucleus and PPAR γ nuclear export. However, it was recently suggested that whereas PPAR γ 2 pro-adipocytic activity relies on β -catenin inhibition, its anti-osteoblastic activity is independent of this interaction^[113].

On the other hand, a stimulatory role for PPAR γ in osteoblast differentiation has been reported. Overexpression of PPAR γ 2 in C3H10T1/2 mouse mesenchymal precursors do not only promote adipogenic differentiation, but also enhances osteogenic differentiation upon BMP-2 stimulation^[114]. Conversely, MSCs with PPAR γ 2 knockdown or mouse embryonic fibroblasts derived from PPAR γ 2^{-/-} mice exhibit a decrease in adipocyte differentiation, coupled with reduced osteoblastogenesis and decreased mineralization^[114]. In mouse MC3T3-E1 osteoblasts, activation of PPAR γ 1 with low doses of agonists stimulated alkaline phosphatase activity and mineralization^[115]. In hMSCs, two PPAR γ antagonists, BADGE and GW9662, as well as lentiviral knockdown of PPAR γ inhibited adipogenesis but had no effect on osteoblastogenesis^[116].

In conclusion, while most data seem to demonstrate an inhibitory effect of PPAR γ on osteoblastogenesis, several articles suggest that PPAR γ action on osteoblasts may actually be more ambiguous. Several mechanisms may account for these discrepancies. For instance, PPAR γ directly binds and inhibits Runx2^[108], and therefore inhibits MSC commitment into osteoblasts. On the other hand, since Runx2 appears to maintain osteoblasts in an immature stage^[66], PPAR γ may participate in osteoblast maturation. Besides Runx2, PPAR γ has also been shown to bind and inhibit β -catenin pro-osteogenic function^[111]. However, β -catenin and PPAR γ may not be systematically inhibitory because an elegant article recently showed that BMP-2 activated β -catenin/PPAR γ dimers have their specific transcriptional targets in endothelial cells^[117]. Since BMP-2 is a potent osteogenic factor, PPAR γ roles in osteoblasts may therefore be more subtle than commonly accepted.

Finally, PPAR γ activity is also dependent of a wide number of factors, such as 1,25(OH)₂ vitamin D₃ receptor, PPAR coactivator (PGC-1), the histone acetyltransferase p300, CREB binding protein, and steroid receptor coactivator-1^[118]; its effects on osteoblasts may thus vary as a function of cell differentiation, species and mode of

activation or inactivation. For instance, it was suggested that full but not partial agonist activation inhibits expression of osteoblast markers in human MSCs^[119].

Twist1: In mouse, there is a 4-5 d delay between the appearance of Runx2 and that of its target, osteocalcin. This delay seems to be due to the co-expression of Twist1^[120]. Twist1 is a basic helix-loop-helix transcription factor. Haploinsufficiency at the Twist1 locus causes Saethre-Chotzen syndrome, a form of craniosynostosis, *i.e.*, an increase in bone formation in the skull^[121,122]. Molecularly, Twist1 binds to the DNA binding domain of Runx2, and inhibits its transcriptional activity. Similarly, Twist1 also interacts with ATF4 and decreases its binding to the Osteocalcin promoter^[123]. As a consequence, osteoblast differentiation during development proceeds when and where Twist1 expression drops. In C3H10T1/2 mouse cell progenitors, silencing of Twist1 using short hairpin RNA expression enhanced osteoblast gene expression and matrix mineralization *in vitro*^[124]. In human MSCs, overexpression of Twist1 and Dermo-1 was associated with a decrease in the gene expression of osteoblast-associated markers, bone morphogenic protein-2, bone sialoprotein, osteopontin, alkaline phosphatase and osteocalcin^[125].

MICRORNAS INVOLVED IN MSC OSTEOBLASTIC DIFFERENTIATION

MicroRNAs

MicroRNAs (miRs) are small (19-23 nt) endogenous non-coding single-stranded RNA transcribed from both intergenic and genic regions of the genome^[126,127]. They are highly conserved molecules that control gene expression post-transcriptionally by binding to the 3'UTR of target mRNA. Near-perfect complementarity between the sequence of miR and its target results in the cleavage of target mRNA, whereas partial complementarity results in its translational inhibition^[128]. The biogenesis of these small regulatory RNA molecules starts out as primary transcripts termed pri-miR. The pri-miR is first processed in the nucleus by the RNase III enzyme DROSHA to produce pre-miRNAs. Once in the cytoplasm, pre-miRs are further processed by a second RNase III enzyme, DICER1 resulting in dsRNA miR complex, which unwound by the helicase activities of the Argonaute multiprotein complex known as the RNA-induced silencing complex (RISC). The preferred guide strand is incorporated into the RISC complex^[129]. MiR expression has both spatial and temporal specificity as well as tissue or cell specificity^[130]. Strikingly, bioinformatics analysis suggests that up to 30% of human genes may be regulated by miR^[131]. MiRs act as key regulators in diverse biological processes, such as early development, cell proliferation, differentiation, apoptosis, cancer and have the potential to control the expression of virtually any gene^[132]. Some miRs are directly involved in the formation of the human skeletal system. Thus, miRs have the great potential to

Table 1 Non-exhaustive list of microRNAs that have been reported to inhibit or stimulate osteoblast differentiation in mouse or human

miR	Species	Target	Effect	Ref.
MiR-206	Mouse	Connexin 43	Inhibitory	[129]
MiR-34	Mouse	Satb 2	Inhibitory	[130]
MiR-27a	Human	TNAP	Inhibitory	[131]
MiR-204	Mouse	Runx 2	Inhibitory	[132]
MiR-204/211	Human	Runx 2	Inhibitory	[143]
MiR-133	Mouse	Runx 2	Inhibitory	[133]
MiR-135	Mouse	Smad 5	Inhibitory	[133]
MiR-433	Mouse	Runx 2	Inhibitory	[134]
MiR-335	Human	Runx 2	Inhibitory	[142]
MiR-138	Human	FAK	Inhibitory	[144]
MiR-2861	Mouse	HDAC 5	Stimulatory	[136]
MiR-335-5p	Mouse	Dkk 1	Stimulatory	[137]
MiR-29a	Human	Dkk 1, Kremen 2	Stimulatory	[138]

miRs: MicroRNAs; FAK: Focal adhesion kinase; HDAC 5: Histone deacetylase 5; TNAP: Tissue-nonspecific alkaline phosphatase; Dkk 1: Dickkopf-related protein 1; Runx 2: Runt-related 2; Satb2: Special AT-rich sequence binding protein 2; Smad5: Portmanteau of Sma in *Drosophila* and Mad in *C. elegans*.

become a research focus for the prevention and treatment of skeletal diseases^[130].

MiRs and osteoblast differentiation

Conditional deletion of the miR processing enzyme Dicer in osteoblasts, chondrocytes, and osteoclasts has revealed their essential role in normal skeletal development and bone homeostasis^[133]. Differential expression of miRs has a major impact on the regulation of osteoblast differentiation^[134], where by various signaling pathways/transcription factors responsible for osteoblast differentiation can be modulated by miRs. An increasing number of miRs have been identified to negatively regulate osteoblast differentiation and bone formation by targeting important osteogenic factors and positively affect it by targeting negative regulators of osteogenesis.

Negative regulators: Many miRs were shown to act as inhibitors of osteoblast differentiation (Table 1). These include miR-206 by targeting *connexin 43* gene (*Cx43*)^[135] or MiR-34 that decreases *SATB2* accumulation^[136]. Additionally, Hsa-miR-27a and has-miR-489 down-regulate differentiation through repression of TNAP expression^[137]; miR-204 a negative regulator of Runx2 inhibits osteogenesis and promotes adipogenesis of mesenchymal progenitor cells and BMSCs^[138]. MiR-133 and miR-135 target *Runx2* and *Smad1/5* respectively in C2C12 mouse mesenchymal progenitors^[139]. MiR-433 suppresses BMP2-induced osteoblast differentiation via direct targeting of *Runx2* mRNA in C3H10T1/2 cells^[140]. Finally, some under-expressed miRs (hsa-miR-31, hsa-miR-106a, hsa-miR-148a and hsa-miR-424) in MSCs undergoing osteoblast differentiation have been predicted to target the mRNAs of *Runx2*, *Cbfb*, and *BMP3*; whereas hsa-miR-30c, hsa-miR-15b and hsa-miR-130b have been predicted to target MSC markers^[141].

Positive regulators: MiRs that may induce osteoblast differentiation include miR-2861, which promotes BMP2-induced ST2 osteoblast differentiation by repressing histone deacetylase 5 expression^[142] (Table 1). MiR-335-5p also enhances osteogenic differentiation by inhibiting Dkk1 expression, and consequently by activating Wnt signaling^[143]. Moreover, Kapinas *et al*^[144] have shown that miR-29a promotes osteoblast differentiation by down regulating the inhibitors of canonical Wnt signaling such as Dkk1, Kremen2, and secreted frizzled related protein.

MiRs and hMSC

Several miRs appear to significantly modulate osteoblast differentiation in mesenchymal precursors^[145]. Dicer or Drosha knockdown in human MSCs inhibits osteogenic differentiation (reviewed in^[146]). MiR expression patterns differ in MSC progenitors and fully differentiated cells, *e.g.*, osteoblasts, adipocytes and chondrocytes suggesting that these miRs are important in MSC lineage decisions. Indeed, high or low expression of particular miRs may be a prerequisite for the commitment and differentiation of MSCs into specific lineages (reviewed in^[147]). For instance, undifferentiated hMSCs isolated from various tissues were shown to express high levels of miR-335 while their differentiation resulted in a reduced expression of miR-335. The same miR as well as miR-204/211 impaired hMSC osteoblast differentiation by targeting *Runx-2*^[148,149]. In human MSCs, decreased expression of miR-138 has also been associated with osteogenesis, possibly by targeting focal adhesion kinase^[150]. Finally, and as presented above, MiR-148b, -27a, and -489 were found to play a critical role in early osteogenic differentiation of hMSC^[137].

CONCLUSION

In the last decade, we have considerably increased our knowledge on the molecular contributors to osteoblast commitment and maturation. Since the discovery of the key role played by Runx2 in 1997^[60], several other transcription factors have been demonstrated to modulate osteoblastogenesis. In addition, an increasing number of papers now indicate that the expression of these transcription factors is modulated by miRs, themselves being expressed under the control of the transcription factors they regulate^[151]. Many of the results that had been obtained with murine models have now been confirmed with human MSCs. Collectively, the better understanding of the interaction between transcription factors and miRs, and of their effect on osteoblast to genesis and osteoblast function, will help develop new strategies to improve diagnosis and treatment of bone diseases.

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Aiming to immune elimination of ovarian cancer stem cells

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Core tip: Ovarian cancer harbors, at a low frequency, cancer stem cells. Those cancer stem cells express stem cell specific antigens. Natural immunity against those antigens exists but is hampered by the suppressive microenvironment that the tumor creates. Erasing this suppressive microenvironment will make immunological elimination of those cancer stem cells is an attractive treatment option.

Di J, Duiveman-de Boer T, Figdor CG, Torensma R. Aiming to immune elimination of ovarian cancer stem cells. *World J Stem Cells* 2013; 5(4): 149-162 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v5/i4/149.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v5.i4.149>

Abstract

Ovarian cancer accounts for only 3% of all cancers in women, but it causes more deaths than any other gynecologic cancer. Treatment with chemotherapy and cytoreductive surgery shows a good response to the therapy. However, in a large proportion of the patients the tumor grows back within a few years. Cancer stem cells, that are less responsive to these treatments, are blamed for this recurrence of disease. Immune therapy either cellular or humoral is a novel concept to treat cancer. It is based on the notice that immune cells invade the tumor. However, the tumor invest heavily to escape from immune elimination by recruiting several immune suppressive mechanisms. These processes are normally in place to limit excessive immune activation and prevent autoimmune phenomena. Here, we discuss current knowledge about the immune (suppressive) status in ovarian cancer. Moreover, we discuss the immunological targets of ovarian cancer stem cells.

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Key words: Ovarian cancer; Cancer stem cell; Immune therapy; Immune suppression; Tumor microenvironment

EPITHELIAL OVARIAN CANCER

Ovarian cancer is the fourth leading cause of death from cancer in women and the leading cause of death from gynecological cancer. The lifetime risk to get this disease is 1 in 60 women in industrial countries but is less common in Asian and African women. Due to vague symptoms and adequate screening methods at the early stages, more than 60% of the patients are diagnosed at advanced stage. Most patients respond well to primary treatment, either cytoreductive surgery followed by chemotherapy or chemotherapy followed by surgical removal of remaining tumor foci. However, 80% of the patients diagnosed at late stage will eventually develop recurrent diseases, the survival is generally poor. The 5-year survival rates at stage III and IV are 29% and 13%, respectively. The relapse of tumor arises the question about the identity of the cells that give rise to the tumor and somehow escape from the first line treatment, reside in the body undetected, and finally initiate malignant tumor growth in a suitable microenvironment.

Despite intense efforts to improve chemotherapy, e.g., the introduction of paclitaxel, and to improve surgical techniques, over the past 20 years no significant progress has been made (Figure 1).

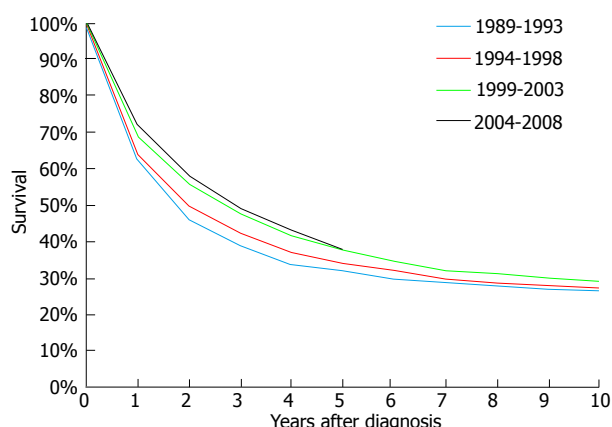


Figure 1 Survival of patients diagnosed with ovarian carcinoma. The percentage of survival after diagnosis is not significantly increased in the past 20 yr (Source from Integraal Kanker Centrum, The Netherlands).

Novel therapeutic approaches are urgently needed. Since ovarian cancer is immunogenic, immunotherapy should be further pursued and optimized. Stimulating the immune system to attack ovarian tumor is not a new concept, during the last 20 years numerous immunological modalities were involved in clinical trials in ovarian cancer treatment^[1]. Targeting a specific tumor antigen plays a decisive role in the success of immunotherapy.

CANCER STEM CELLS

Tumors are composed of phenotypically and functionally heterogeneous cells. There are two theories explaining how this heterogeneity arises^[2,3]. According to the stochastic model, tumor cells are biologically equivalent; virtually every tumor cell is able to generate new tumor cells. In contrast, the hierarchy model postulates the existence of tumorigenic as well as non-tumorigenic cells. Only a subset of cells can initiate tumor growth, and these cells are considered as tumor-initiating cells (TICs) or cancer stem cells (CSCs). CSC is a relatively rare cancer cell that has the ability of self-renewal giving rise to another malignant stem cell as well as a cell that undergoes massive proliferation and differentiation to give rise to the phenotypically and functionally more mature cancer cells^[4,5]. The similarities of CSCs and normal stem cells (NSCs) point to the origin of CSCs. There are two hypotheses^[6]. One states that CSCs can be derived from NSCs, so that they can make use of the already active self-renewal machinery. Another assumes that the CSCs can be derived from progenitor cells by regaining the self-renewal capability. NSCs possess several unique properties. Their self-renewal enables lifelong maintenance of all organs of the body. In most cases NSC divide slowly. For hematopoietic cells a doubling time of 30 d was reported^[7]. However, for intestinal cells a doubling time of less than 24 h was reported^[8]. Those fast regenerating organs have stem cells that are continuously dividing. One of properties of NSC is the expression of pumps of the ATP binding cassette (ABC) superfamily^[9-11]. Those pumps can remove

toxic components from the cell. Likewise CSC also expresses members of the ABC family^[10-19]. For melanoma ABC-B1 and ABC-B5 were reported while other tumors express other members^[12,13]. This endows CSC with a nasty property. The pump is able to remove cytotoxic drugs that are given to patients to kill the tumor. Indeed, a common property of CSC is their resistance against cytotoxic drugs, explaining the relapse that is seen in several patients. Traditional therapies that kill primarily non-tumorigenic cancer cells can shrink tumors, but will not cure the patient because the CSCs that survive the treatment will regenerate the tumor. By prospectively identifying and characterizing CSCs, it might be possible to identify more effective therapies^[20-24]. CSCs can be eliminated by direct killing, or force them to differentiated cells or by destroying their niche^[25]. Accordingly, targeting the CSCs has been put forward as such a new treatment modality for cancer immunotherapy^[26,27]. Several studies described in the literature provide several clues for optimizing the immunotherapy against ovarian cancer.

IDENTIFICATION AND CHARACTERIZATION

The first experimental evidence suggests the existence of CSC came from leukemia. Bonnet and co-workers demonstrated that human leukemias are driven by a small population of leukemic stem cells capable of transferring the disease to NOD/SCID mice^[28]. This concept was extended to solid epithelial tumors by Al-Hajj and co-workers, who demonstrated that a small population of cells within breast cancer with stem cell properties, bearing the surface marker CD24^{low}CD44^{high}^[4]. Subsequently, CSCs are identified and prospectively isolated from a variety of epithelial cancers, including pancreas, colon and prostate cancers^[29-40].

Ovarian CSC is responsible for ovarian tumor formation

The CSC hypothesis has recently also been explored in ovarian cancer. In 2008, Zhang *et al.*^[39] claimed that epithelial ovarian cancers derive from a subpopulation of CD44⁺CD117⁺ cells. Ferrandina and Curley independently found that CD133 expression defines a tumor initiating subpopulation of cells in human ovarian cancer^[41,42]. Gao and co-workers reported that CD24 could be utilized as a surface marker to enrich for ovarian CSCs^[32]. Ovarian CSCs were also detected in the so-called side population, which are tumorigenic and chemoresistant^[38,43,44]. Moreover, Stewart *et al.*^[45] established a quantitative assay that enables characterization of TICs from serous ovarian cancer, and they also found that the tumor initiating cell phenotype is heterogeneous across patients. And recently, a gene involved in maintaining stem cell pluripotency, Nanog, was proved to be expressed by ovarian tumor cells, and positive Nanog expression indicates poor progression of patients with ovarian serous carcinoma^[46].

As described above, increasing experimental evidence suggests that TICs may play a decisive role in the initia-

tion and progression of tumors^[4,29-31,35-39,46]. However, TICs with distinct tumorigenic abilities were identified^[31,47,48], as well as large variation in their frequency^[49,50]. TICs appear not to be a stable entity but show quite some plasticity^[2,51-54]. Recently, it was described that the TIC compartment can be subdivided into long-term TICs, tumor transient amplifying cells as well as delayed contributing TICs^[48]. Only the long-term TICs are capable of maintaining tumor formation in serial xenografts, and these cells are considered as cancer stem cells.

Phenotypic heterogeneity of ovarian CSCs

CSCs are operationally defined as tumor initiating cells because the CSC assays rely heavily on xenotransplantation^[55]. Although it was proven that frequency and tumorigenic ability of melanoma CSCs that can be detected after xenotransplantation were highly dependent on experimental design^[50,56], current studies on CSCs all use immunodeficient mice models to check whether putative CSCs can generate secondary tumors *in vivo*. And using this method, phenotypically diverse ovarian CSC populations have been characterized and isolated from both patient material and immortalized tumor cell lines with variable stem cell markers^[32,36,38,41,42,46,57,58]. However, due to the fact that a large number of cells was needed to establish a secondary tumor in immunodeficient mice, it is assumed that ovarian CSCs were just enriched in those cell populations^[59]. Also, it was questionable whether tumor cell lines can represent the status of primary tumor cells. Moreover, due to the heterogeneity among individuals, it is important to test CSC markers in significant numbers of patients.

The expression of well-known CSC markers, including, CD44, CD117, CD133, CD24, ABCG2 and aldehyde dehydrogenase (ALDH), on tumor and ascites derived cells from patients diagnosed with ovarian cancer is very diverse and is patient-dependent, and no correlation was found between marker expression and tumor histological subtype^[60]. In line with these data, another study investigated epithelial and mesenchymal markers expressed by primary ovarian tumors, and they also showed different phenotypic features and expression levels of those markers in different cellular subsets within tumors^[59]. Additionally, it has been reported that the CSC marker ALDH show distinct expression pattern in human epithelial cancers, and it can only be used to isolate CSCs for tumors whose corresponding normal tissues express low levels of ALDH^[61]. Also CD133 as a marker to identify ovarian CSCs has been questioned, since tumor initiating activities have been detected in both CD133⁺ and CD133⁻ fractions from primary ovarian masses, and CD133⁺ cell frequency varies between patients^[45]. Similar doubts of CD133 as a putative CSC marker has been reported in colon cancer and melanoma^[56]. Moreover, phenotypic heterogeneity of breast CSCs was also reported^[34,40,62]. Taken together, these data suggest that CSC phenotypes are heterogeneous, and experimental variables as well as xenograft recipients can dramatically influence CSC fre-

quency^[45]. So far a clear set of marker proteins remain to be identified to target ovarian CSCs.

For better recognition of CSCs, better experimental methods need to be established. One way to identify CSC is to focus on genes involved in stem cell pluripotency, because those genes may be involved in establishment of tumors and may be inherited by their malignant counterparts. Four genes are required for induction of pluripotent stem cells from mouse embryonic or adult fibroblasts *in vitro*, including Oct4, c-Myc, Sox2 and Klf4^[63]. A rare cell population, in ovarian tumor tissue as well as ascites, expressing Oct4, Nanog and c-Myc was found. Oct4 expression is crucial for the self-renewing and maintenance of pluripotent properties of embryonic stem (ES) cells^[64,65]. The expression of Oct4A indicates that the cells are undifferentiated^[66]. Recently, abnormal Oct4 expression level was correlated to several cancers^[67-69]. The two isoforms of Oct4, Oct4A and Oct4B, differ in their ability to confer self-renewal, only Oct4A can sustain stem cell properties^[70,71]. Several studies have shown that the different isoforms and Oct4 may lead to false positive signals during RT-PCR analysis^[72,73]. In order to rule out this, a primer set was described to distinguish the Oct4A from Oct4B and Oct4 pseudogenes^[73]. Oct4A mRNA expression was detected by us in ascites-derived tumor cells from all patients tested, regardless of histological subtypes. The c-Myc protein is normally expressed in the nucleus and is virtually undetectable in quiescent cells. It contributes to the long-term maintenance of the ES cell phenotype and is upregulated in many types of malignant human cancers^[74]. Moreover, Nanog also sustains ES cell pluripotency^[75]. Oct4 and Nanog were described to be higher expressed in side population cells obtained from ovarian cancer cell lines than the bulk of the cells^[76], confirming the expression of stem cell markers as described here. To sum up, expression of these genes suggests that those cells are the primitive CSC for ovarian cancer, because all genes needed for reprogramming to induce pluripotent stem are present in the same cell.

According to the hierarchy tumor model, the most "primitive" CSCs are able to self-renew, and develop into more differentiated cells like so-called progenitor cells or CSC-derived transit-amplifying cells, which are not able to self-renew but can generate new tumor cells to support tumor growth^[34,48]. In order to adapt to different host microenvironments, CSC-derived progenitors may differ in their phenotypes and functions and in turn differentiate into phenotypically and functionally heterogeneous tumor cells^[77]. And a different differentiation status might be generated also to adapt the complicated tumor growth environment^[78]. These indicate that CSCs and their progenies may differ between different patient tumors and may be able to change during tumor progression^[55]. Collectively, these data may explain why the expression of putative CSC phenotypes are heterogeneous among patients with ovarian cancer and why accumulating evidence shows that solid tumors are initiated by heterogeneous populations of CSCs, and each CSC subset responsible for distinct

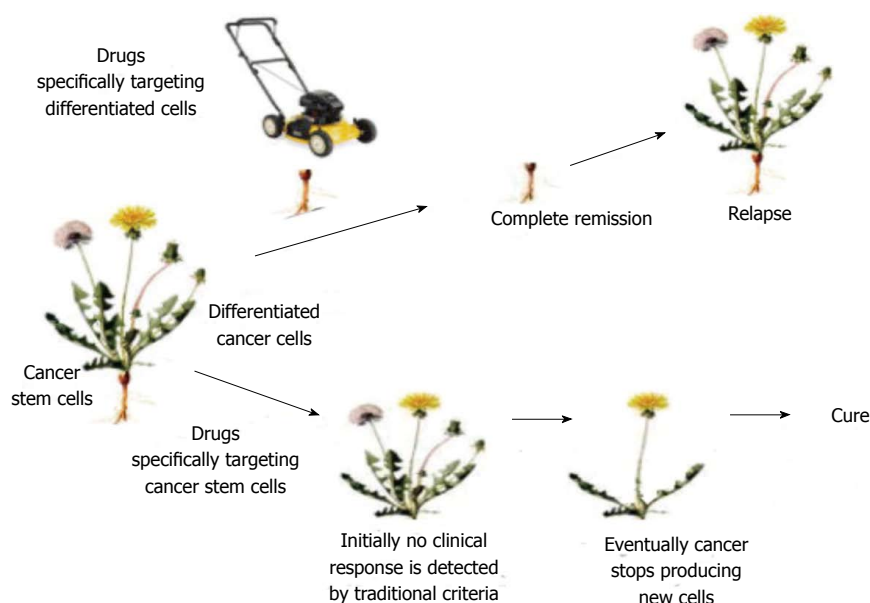


Figure 2 Killing the mature cancer cells leaves the root intact leading to regrowth of the tumor. Killing the root will exhaust the stem cell pool leading to eradication of the tumor. Reprinted from Jones *et al*^[154].

functions in tumor progression^[33,34,40,45,47,48,50,79-83] [Engh, 2011 #756].

Although CSC phenotypes are heterogeneous, current studies suggest ovarian tumor conforms to the CSC hypothesis^[45,59], and in this scenario, if the most primitive Oct4-expressing CSC population is eliminated specifically, the tumor will lose its feeding and eventually fade away (Figure 2).

Phenotypic plasticity of ovarian tumor cells

CSC may not be a stable entity. Plasticity describes the dedifferentiation potential of more differentiated cancer cells to acquire stem cell phenotype and characteristics, which further contribute to CSC heterogeneity, and which is an important determinant of the prognosis of tumors^[55,84,85]. Thus plasticity in CSCs and their progenies make the situation more complex^[51,59]. Two c-Myc expressing populations were found; one is only highly positive for c-Myc, the other also express Oct4. The relationship between these two subpopulations remains to be investigated. We argue that those intermediate c-Myc⁺ cells are more differentiated cells than c-Myc⁺ Oct4⁺ cells, since in some cases they were not able to survive in serum-free medium. Also, it is possible that the c-Myc⁺ cells somehow regain Oct4A expression and become a primitive CSC. In fact, phenotypic plasticity of ovarian tumor cells was detected under certain circumstances, *e.g.*, stress created by starvation or co-culture with either epithelial or mesenchymal cells *in vitro*^[59].

In line with this, plasticity has been described in other tumor stem cell studies, showing that non-tumorigenic cells can convert to a tumorigenic cell^[50,86,87]. For instance, knocking down of JARID1B in slow cycling melanoma cells exhausted the tumor, however, expression of JARID1B is dynamic since negative cells can become JARID1B positive^[47]. This indicates that the cancer cells might reversibly transit between tumorigenic and non-tumorigenic status, generate reversible heterogeneity^[85,88].

In addition to tumor cells, plasticity was also described in normal development procedures. Endothelial cells could simply be converted into multipotent stem-like cells by Transforming growth factor β 2 or Bone morphogenetic protein 4^[89]. Also in spermatogonial development more differentiated cells can go back to the stem cell state when the stem cell niche is emptied and the number of stem cells is decreased. In this way the normal number of stem cells is recovered by differentiated cells that regain stem cell properties^[90]. Plasticity would have major implications for the CSC model and for future therapeutic approaches, as discussed in^[52].

INTERPLAY BETWEEN TUMOR AND THE IMMUNE SYSTEM

The immune system affects cancer development and progression. Before the tumor cells cause clinically detectable disease, they have already resided in the body for a while. The immune system can recognize and interact with the transformed cells before and after the formation of tumormass; this process is termed “cancer immunoediting”. Cancer immunoediting consists of three distinct phases: elimination, equilibrium and escape^[91,92]. During the elimination phase, tumor specific immune cells and molecules are recruited to the tumor site and destroy the developing tumor cells. The equilibrium phase is a dynamic state; the interaction between tumor growth and immune prevention represents a type of tumor dormancy, in which tumor outgrowth is also limited by the immune system^[93]. Meanwhile, due to the immune selection, some malignant cell can acquire the ability to circumvent immune recognition, or no longer sensitive to immune effector mechanisms, and escape. And then their growth is no longer blocked by the host immunity anymore. In addition, the malignant tumor cells can even manipulate the immune system to promote their own growth^[91,92].

Immune elimination of tumors

The effectors mechanisms of both cell-mediated immunity and humoral immunity have been shown to kill tumors *in vitro*. In several cases also *in vivo* killing of tumor cells was observed. During the elimination phase of cancer immunoediting, different types of immune cells are recruited to the tumor site, including T cells, antibody-secreting B cells, different subsets of dendritic cells (DCs), tumor-associated macrophages (TAMs), myeloid-derived suppression cells (MDSCs), Th17 cells, natural killer (NK) cells, NK T cells and $\gamma\delta$ T cells^[94,95]. And those intratumoral T cells were functionally active since interleukin-2 (IL-2) and interferon- γ (IFN- γ) was produced, which may enhance T cell proliferation and anti-tumor immunity^[96,97].

An effective antitumor immune response is direct killing of tumor cells by CD8⁺ cytotoxic T lymphocytes (CTLs), which recognize tumor antigens presented by MHC I molecules. CD8⁺ T cell responses specific for tumor antigens may require cross-presentation of the tumor antigens by professional antigen presenting cells (APCs), such as DCs. Most tumor cells do not express the co-stimulatory molecules needed to initiate T cell responses or the class II MHC molecules needed to stimulate helper T cells that promote the differentiation of CD8⁺ T cells. It is possible that tumor cells or their antigens are ingested by host DCs, the tumor antigens are then processed inside the DCs, and peptides derived from these antigens are displayed bound to class I MHC molecules for recognition by CD8⁺ T cells. The APCs expressing co-stimulatory molecules that provide the signals needed for differentiation of naïve CD8⁺ T cells into anti-tumor effector CTLs, and the APCs express class II MHC molecules that may present internalized tumor antigens and activate CD4⁺ helper T cells as well. Once effector CTLs are generated, they are able to recognize and kill the tumor cells without a requirement for co-stimulation. CTLs mediate lysis of target cells by two major mechanisms, the predominant mechanism appears to be perforin-granzyme-dependent, and the other is FasL dependent^[98,99]. The ability of CTLs to provide effective anti-tumor immunity *in vivo* is most clearly seen in animal experiments. However, tumor-specific CTLs can be isolated from animals and humans with established tumors, such as melanomas^[100].

The importance of CD4⁺ helper T cells in tumor immunity is less clear. CD4⁺ cells may play a role in anti-tumor immune responses by providing cytokines for effective CTL development. In addition, CD4⁺ T cells specific for tumor antigens may secrete cytokines, such as tumor necrosis factor (TNF) and IFN- γ , that can increase tumor cell class I MHC expression and sensitivity to lysis by CTLs. IFN- γ may also activate macrophages to kill tumor cells. In addition to T cells, tumor-bearing hosts may produce antibodies against various tumor antigens^[101-104]. Whereas it has also been documented that CD4⁺ T cells can be more effective than CD8⁺ T cells in tumor killing in tumor bearing mice^[105]. Moreover, NK cells may kill many types of tumors, especially “missing” cells that have

reduced class I MHC expression and can escape killing by CTLs^[106,107]. CD4⁺ T cells cooperate with NK cells to accomplish the maximum tumor killing^[105]. Macrophages can kill many tumor cells more efficiently than they can kill normal cells^[108]. Several studies showed the existence of tumor infiltrating T cells in ovarian cancer associated with favorable clinical outcome^[109,110]. Distribution of tumor infiltrating lymphocytes (TILs) were studied in patients with late stage ovarian cancer, CD3⁺ T cells were detected in more than 50% of the patients and CD4⁺ and CD8⁺ T cells were either both present or absent. The presence of TILs correlates with a better 5 year survival as well as progression-free survival^[39]. It has also been documented that patients with higher TIL counts showed improved overall survival than patients with lower TIL counts^[111]. Moreover, Sato and co-workers demonstrated intraepithelial CD8⁺ TILs and the high CD8⁺ TIL/Treg ratio indicates better survival of ovarian cancer patients^[112].

Immune reactivity towards CSCs

When the immune system is directed to eliminate the CSC, it will also destroy CSC reverting from more differentiated progeny. We consider Oct4 as a suitable antigen for immunological targeting ovarian CSCs, since it is neither expressed in normal adult stem cells nor somatic cells. Once the progenitors re-express Oct4 and become CSCs, they can be recognized and eliminated by Oct4-reactive T cells. Removing of the CSCs from the pool will diminish the feeding of more mature tumor cells. Further understanding of the relationship between CSCs and their differentiated progenies can help us to develop better immunotherapeutic strategies that can prevent the emergence of tumor cell variants that are capable of generate a new tumor and metastases^[55,113].

OCT4-REACTIVE T CELLS ARE DETECTABLE

Naturally occurring T cells directed against tumor-associated antigens (TAAs) can be frequently detected in cancer patients (reviewed in^[114]). Amazingly, Oct4 reactive CD4⁺ as well as CD8⁺ T cells were detected in both healthy people and patients with ovarian cancer^[115]. This finding suggests that the host immune system has the ability to target the primitive ovarian CSCs. The frequency of Oct4 specific T cell was low in peripheral blood, while it was higher in the ascites of patients. This means those cells are either recruited to the tumor or proliferate upon exposure to Oct4. Moreover, lymphocytes isolated from ascites from patients with ovarian tumor contained Oct4 specific T-cells. It was shown that Oct4-reactive CD8⁺ T cells produce IFN- γ -inducible protein 10 (IP-10) and IFN- γ , and were capable of proliferation upon Oct4 peptide loaded or Oct4 mRNA pulsed dendritic cell stimulation. The CD8⁺ cytotoxic T cells were able to release lysosomal components as indicated by CD107a expression. Moreover, Oct4-reactive CD4⁺ T cells were also detected,

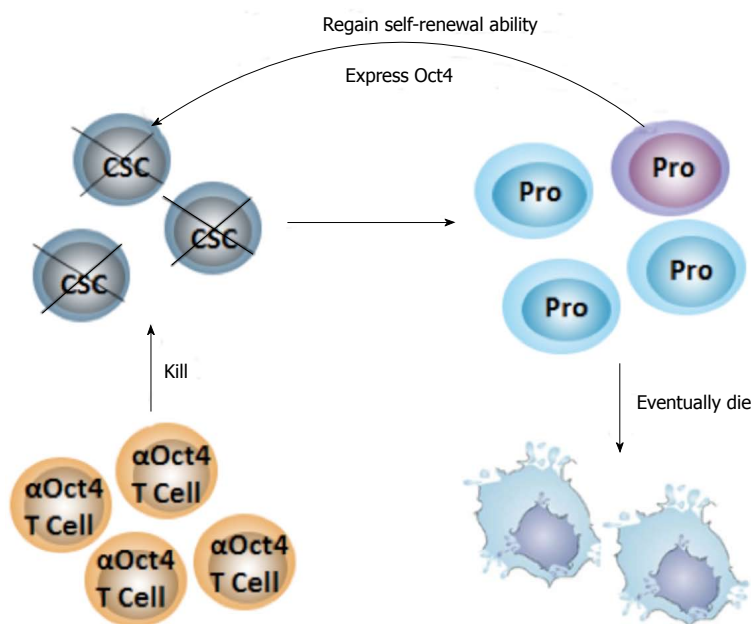


Figure 3 Hypothesis of specific targeting of primitive cancer stem cells. In a non-immunosuppressive tumor microenvironment, Oct4-specific T cells (α Oct4 T cell) can recognize the primitive cancer stem cells (CSCs), and destroy them. Progenitor cells (Pro) differentiate to more mature tumor cells and will eventually undergo apoptosis or necrosis. Once some progenitors regain the self-renewal machinery and re-express Oct4 to become a CSC, T cells will also eliminate it. In this way, the tumor loses its ability to generate new tumor cells.

and also capable of proliferating upon stimulation. These results proved the existence of anti-CSC specific T cells in patients with ovarian cancer.

Natural immunity against genes involved in pluripotency has been shown. Dhodapkar *et al.*^[115] claim the Oct4 responsive T cells were detected in PBMCs from 83% of healthy donors, although they showed the Oct4-specific cells were $CD4^+$ T cells. They also found 38% of patients with germ-cell tumors had measurable Oct4-specific T cell immunity at baseline, and after chemotherapy, 83% of the patients developed Oct4-reactive T cells. Also, it has been documented that $CD8^+$ Sox2-specific T cells were frequently detected in patients with monoclonal gammopathy of undetermined significance (MGUS). MGUS is a precursor lesion to myeloma, whereas Sox2-specific T cell immunity was not detectable in patients with myeloma^[116].

Taken together, these data indicate that the ovarian CSCs are prone to immunological attack because CSC specific T cells are present in the T cell repertoire (Figure 3). Meanwhile, this raises the question about why CSCs and their progenies escape from immune elimination, and why the already activated Oct4-reactive memory T cells do not kill those cells.

Immune escape by tumors

Many malignant tumors possess mechanisms that enable them to disturb the balance in the equilibrium phase and shift to escape phase, including down-regulation of MHC I expression on tumor cells, loss or hidden of tumor-antigen expression, production of immune suppressive molecules, and inhibition of co-stimulatory or MHC II molecules expression on APCs, leading to immunologic tolerance^[92,117,118]. Tumors escape not only from the host immune system, but also effectively benefit from infiltrating cells and create a microenvironment that favors its progression by modifying TIL functions^[119]. Ovarian

tumor can effectively create its suppressive microenvironment. Curiel *et al.*^[120] showed the first evidence that tumor associated $CD4^+CD25^+$ regulatory T cells (Treg) were correlated with a poor clinical prognosis of ovarian cancer. They showed the presence of Treg in both tumor tissue and malignant ascites, and also proved that tumor cells and microenvironmental macrophages produced the chemokine CCL22, which attracted Tregs to the tumor site. Tumor infiltrating Tregs suppress tumor-specific T cell immunity by blocking T cell proliferation as well as $IFN-\gamma$ and IL-2 production. Similarly, Woo *et al.*^[121] found that $CD4^+CD25^+$ Tregs contribute to $CD8^+$ T cell dysfunction by secreting the immunosuppressive cytokine transforming growth factor- β (TGF- β). Later on, forkhead box protein-3 (FoxP3) expressing Tregs were also detected and emerged as an independent prognostic factor for both poor progression-free and overall survival^[122]. Conrad *et al.* demonstrated that majority of these FoxP3⁺ Tregs accumulated nearby the tumor and also express inducible co-stimulator (ICOS)^[123]. The expansion and immunosuppressive function of these FoxP3⁺ICOS⁺ Treg cells are dependent on their interaction with plasmacytoid DCs (pDCs) which provide ICOS-ligand (ICOS-L) stimulation. The presence of immature pDCs was also found in the vicinity of ovarian tumor and associated with poor clinical outcome of patients with ovarian tumor^[124]. pDCs are recruited by CXCL12 produced by tumor cells and produce type I IFN in response to toll-like receptor (TLR) ligand triggering^[125,126]. In addition to $CD4^+$ Tregs, $CD8^+$ Tregs also exist in ascites produced by malignant ovarian tumor. Wei *et al.* showed that tumor pDCs induce suppressive $CD8^+$ Tregs in ascites. These $CD8^+$ Tregs inhibit T cell proliferation and $IFN-\gamma$ production, while they induce IL-10 production^[126]. Moreover, ovarian tumor infiltrating DCs express programmed death 1 (PD-1), which interacts with B7-H1 on tumor-associated macrophages. This reaction can lead to suppressed NF κ B

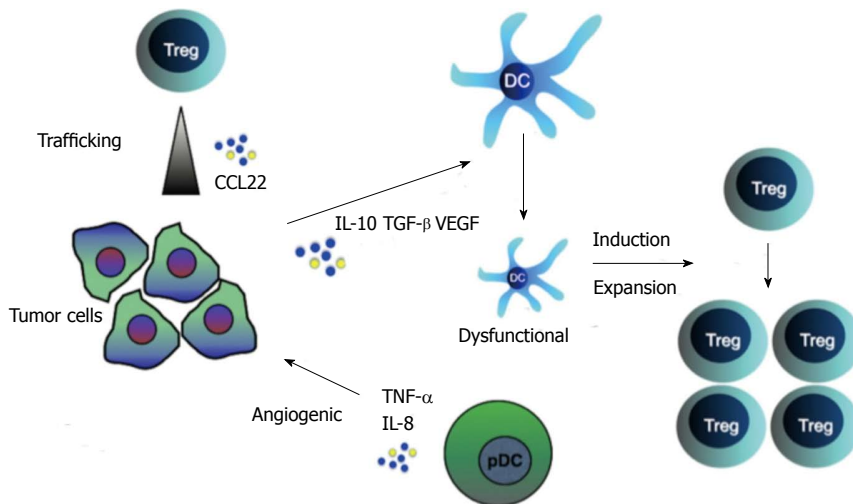


Figure 4 Immune-suppressive pathways in ovarian cancer. Tregs are attracted to the tumor environment by CCL22, secreted by the tumor. The tumor microenvironment expresses molecules that can convert functional antigen presenting cells (APCs) into dysfunctional ones. These dysfunctional APCs in turn stimulate Treg differentiation and expansion. pDCs are also present in the tumor environment and stimulate tumor growth by releasing tumor necrosis factor- α and interleukin-8. (modified from^[132]). pDCs also facilitate immunosuppressive function of FoxP3⁺ICOS⁺Treg^[123].

activation and downregulated co-stimulatory molecule expression on DCs^[127] (Figure 4).

Ovarian tumor infiltrating T cells are anergic

A remarkable characteristic of ovarian cancer is the typical metastasis behavior. Metastases are found but hardly in other organs. As the tumor spreads in a diffuse intra-abdominal fashion and even after recurrence, it is in most cases confined to the peritoneal cavity. There are several papers that report the presence of metalloproteases in ascites^[128-130]. Those enzymes are found in metastasizing tumors by chopping tissues to make room for the metastasis. Moreover, ovarian tumors orchestrate suppressive mechanisms that enable them to evade or resist host immune responses^[131-135]. The fact that CTLs against human tumors can be easily generated *in vitro* using peripheral blood lymphocytes indicates that the tumor microenvironment has immunosuppressive capacities^[131]. Tumor infiltrating immune cells together with fibroblasts and extracellular matrix form a scaffold supporting tumor cell expansion, contribute to establish an inflammatory milieu that nourishes the tumor and promotes its growth^[131,136]. And apparently, the weak anti-CSC immunity generated by Oct4-reactive T cells is counterbalanced (Figure 5). Collectively, this metastasis behavior suggest that as soon as tumor cells escape from the immune suppressive microenvironment in the peritoneal cavity and enter sites where full immune responses are possible in the periphery, they cannot survive^[132,134,137]. This opens enormous possibilities to treat patients by boosting the immune response.

The assumption that without this suppressive microenvironment the immune system is able to eradicate tumor cells needs further prove. Furthermore, as argued for immunotherapy, only boosting the antitumor immune response is not enough. It is of great importance to “repair” the already existing tumor specific T cells *in vivo*. It was found that ovarian tumor infiltrating lymphocytes fail to proliferate in response to CD3/CD28 stimulation and adding IL-2 cannot reverse this unresponsiveness. The inhibited T cell proliferation was due to reduced

cyclin E expression (unpublished data). So even though the host immune system can recognize the tumor, they lack the ability to eliminate it. The observed effects were reversible after culture of the cells *ex-vivo* for 10 d. This demonstrates that the impaired functions are reversible and can be repaired. The results are in line with recent findings from other groups proved that TIL isolated from melanoma, oral carcinoma, colorectal carcinomas were also functionally impaired, as manifested by decreased proliferative responses and decreased ability to mediate cytotoxicity^[138]. Abnormalities in signal transduction molecules associated with reduced expression of T-cell receptor (TCR) ζ chain^[139] and/or hampered Fas/FasL signaling pathway^[140]. Moreover, it has been shown that T cells isolated from ascites of patients with ovarian tumor were deficient in expression of ζ chain, lower basal levels of protein tyrosine phosphorylation, altered patterns of protein phosphorylation when stimulated via surface CD3 or CD16, and declined expression and kinase activity of p56^{lck}. These deficiencies in expression and function of signaling molecules were associated with reduced proliferation and an altered profile of cytokine secretion by the NK or T cells isolated from ascites and stimulated with IL-2 or by cross-linking of surface CD3^[141]. In addition, tumor-associated CD8⁺ T cells might be dysfunctional due to upregulation of programmed death 1 (PD-1) and T cell immunoglobulin and mucin-domain-containing molecule 3 (Tim-3)^[142,143]. We could not detect PD-1 expression in ascites-derived lymphocytes, however, both ascitic CD4⁺ and CD8⁺ cells showed upregulation of Tim-3 (Figure 6). These findings indicate that infiltrated immune cells are not only suppressed, but also impaired in their signaling pathways resulting from the yet unknown factors present in tumor associated ascites.

Furthermore, except for harming of immune cells, ovarian cancer cells also secrete immunosuppressive and pro-inflammatory cytokines into the tumor microenvironment to support tumor growth^[144,145]. Previous studies demonstrated that IL-6 is significantly increased in cyst fluid, serum as well as ascites of patient with advanced

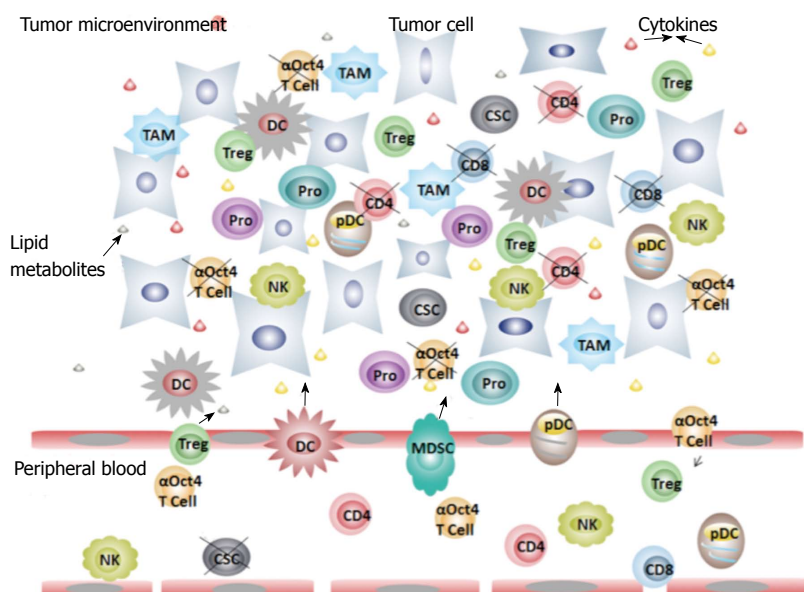


Figure 5 Dysfunctional immune system in peritoneal cavity of patients with ovarian cancer. Many types of immune cells are recruited to the ovarian tumor site, including regulatory T cells (Treg), dendritic cells (DC), tissue associated macrophages (TAM) myeloid derived suppressor cells (MDSC)^[155], plasmacytoid DCs (pDC), natural killer cells and T cells (CD4, CD8). Once being recruited, most cells function abnormally and become immune suppressive. T cells specific for Oct4 (α Oct4 T cell), CD4⁺ T cells, CD8⁺ T cells are damaged, due to dysfunctional DCs, pDCs and suppressive Tregs. Also the secretion of immune suppressive cytokines and lipid metabolites contribute to establish such an immunosuppressive tumor microenvironment, and may also be required for cancer stem cells (CSCs) maintenance. So even if the CSC is recognized, T cells lack the ability to eliminate it. Whereas such suppression mechanisms are not operative in the peripheral blood of the patients, once the CSC migrates to the peripheral, it is killed.

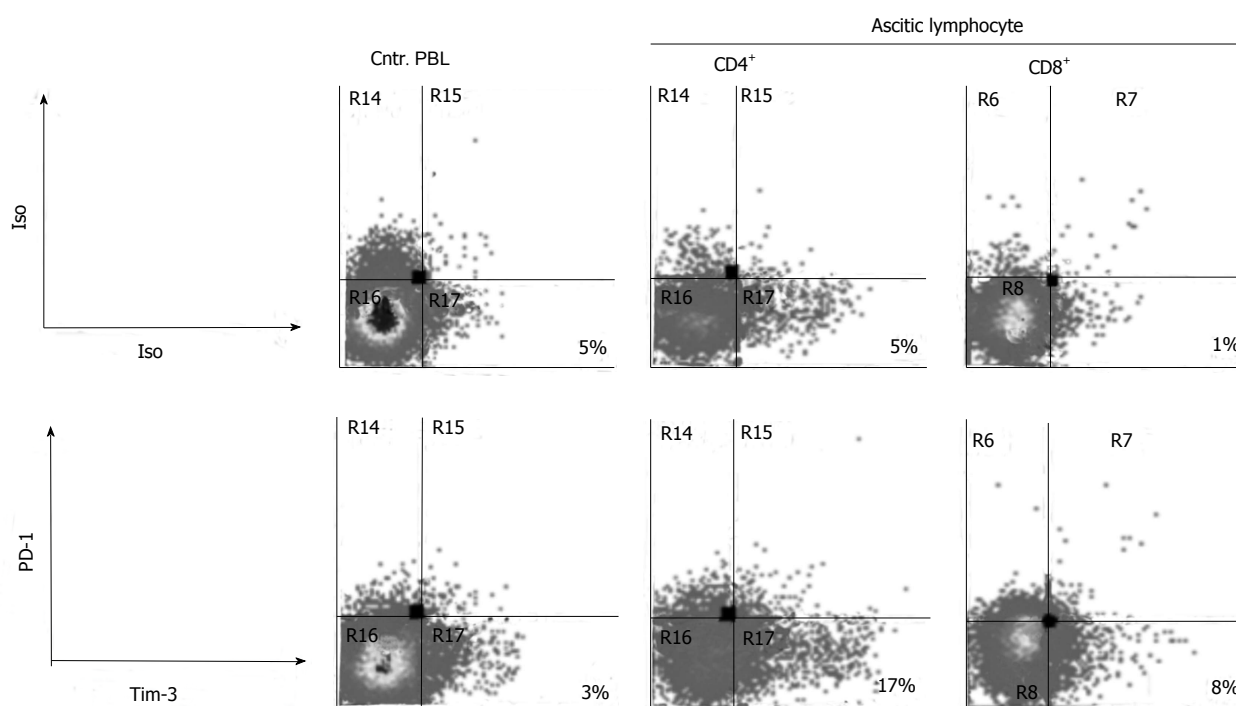


Figure 6 PD-1 and Tim-3 expression by ascitic lymphocytes from patients with ovarian cancer. PD-1 expression was undetectable in ascitic lymphocytes. Compare to isotype control, peripheral blood lymphocyte (PBL) from healthy express 2% Tim-3, ascitic CD4⁺ and CD8⁺ cells express four times more Tim-3 than control PBL.

ovarian cancer, and associated with poor prognosis^[144,146]. IL-6 is a pro-inflammatory cytokine. It has multiple effects on T cell function, and it has already been reported to be an important factor in promoting the progression of epithelial of ovarian cancer^[147]. IL-6 also plays a role in enhancing tumor growth by inducing abnormal c-Myc expression *in vitro*. It has been shown that IL-6 can induce c-Myc translation in multiple myeloma cells and meanwhile c-Myc is shuttled to cytoplasm by the RNA-binding protein, hnRNP A1^[148]. Our research demonstrated that c-Myc was expressed in both nucleus and cytoplasm in ovarian tumor tissue as well as ascitic cells,

while c-Myc is only expressed in the nucleus of normal stem cells. Similarly, except for being expressed in the nucleus, c-Myc was also detected in the cytoplasm of leukemia patients^[149]. Regulation of stem cell genes or even tumor development by cytokine indicates a strong correlation between the tumor and its microenvironment. Taken together, these results indicate that in addition to its suppressive property, the tumor successfully creates a favorable microenvironment to support tumor growth.

In conclusion, ovarian cancer is an extremely complicated disease, because the tumor growth might be driven by heterogeneous CSCs and multiple immunosuppressive

mechanisms are functional in the abdomen. To enable an immunological attack on CSC either the response has to be strengthened or the immunosuppressive milieu has to be reversed or both.

FUTURE PERSPECTIVES

For future studies, it is of great importance to investigate how somatic cells are reprogrammed *in vivo* to become malignant pluripotent cells, and how the self-renewal pathways are orchestrated in such transformed cells. Furthermore, it remains unclear why the pluripotent genes were upregulated in a small subset of tumor cells. We sequenced both Oct4 and c-Myc isolated from ovarian patient ascitic cells, however, no mutation was found (unpublished data). It is important to elucidate what went wrong in the self-renewal pathways in the patients and why. Understanding this might help to stop tumor growth before it happens.

Another challenge is how to boost the favorable host immune response in the suppressive tumor microenvironment and train the immune system to fight against ovarian cancer. To overcome this, it is of great importance to determine the mechanisms that contribute to protective immune responses against tumors and to enhance these effector mechanisms in a tumor specific way. And apparently, only boost the immune system is not enough to eliminate tumors, due to functional crippling of TILs.

Moreover, the role of ascites in tumor progression remains to be elucidated. Ascitic fluid is produced by ovarian tumor. The cellular fraction of ascites consists of tumor cells, lymphocytes and mesothelial cells; and the acellular fraction harbors cytokines, growth factors, bioactive lipids, angiogenic factors, and extracellular matrix constituents^[150-152]. Although the role of ascites as tumor cell microenvironment remains poorly understood, recent research suggests that it may affect cell growth, invasion and induction of resistance of ovarian cancer cells and thus may play a decisive role in ovarian tumor progression^[153].

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Regenerative therapy for neuronal diseases with transplantation of somatic stem cells

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Abstract

Pluripotent stem cells, which are capable of differentiating in various species of cells, are hoped to be donor cells in transplantation in regenerative medicine. Embryonic stem (ES) cells and induced pluripotent stem cells have the potential to differentiate in approximately all species of cells. However, the proliferating ability of these cells is high and the cancer formation ability is also recognized. In addition, ethical problems exist in using ES cells. Somatic stem cells with the ability to differentiate in various species of cells have been used as donor cells for neuronal diseases, such as amyotrophic lateral sclerosis, spinal cord injury, Alzheimer disease, cerebral infarction and congenital neuronal diseases. Human mesenchymal stem cells derived from bone marrow, adipose tissue, dermal tissue, umbilical cord blood and placenta are usually used for intractable neuronal diseases as somatic stem cells, while neural progenitor/stem cells and retinal progenitor/stem cells are used for a few congenital neuronal diseases and retinal degenerative disease, respectively. However, non-treated somatic stem cells seldom differentiate to neural cells in recipient neural tissue. Therefore, the contribution to neuronal regeneration using non-treated somatic stem cells has been poor and various differential trials, such as the addition of neurotrophic factors, gene transfer, peptide transfer for neuronal differentiation of somatic stem cells, have been performed. Here, the recent progress of regenerative therapies using

various somatic stem cells is described.

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Key words: Somatic stem cells; Transplantation; Regenerative therapy; Neuronal disease; Neuronal differentiation

Core tip: Pluripotent stem cells, which are capable of differentiating in various species of cells, are hoped to be donor cells in transplantation in regenerative medicine. Somatic stem cells with the ability to differentiate in various species of cells have been used as donor cells for neuronal diseases, such as spinal cord injury, cerebral infarction, amyotrophic lateral sclerosis, Parkinson's disease and multiple sclerosis. Here, the recent progress of regenerative therapies using various somatic stem cells is described.

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INTRODUCTION

Pluripotent stem cells, which are capable of differentiating in various species of cells, are hoped to be donor cells in transplantation in regenerative medicine. Human embryonic stem (ES) cells^[1] and induced pluripotent (iPS) cells^[2] have the potential to differentiate in approximately all species of cells. However, the proliferating ability of these cells is high and the cancer formation ability is also recognized^[2,3]. Ethical problems exist in using ES cells^[4], while iPS cells produced from the patients themselves have little ethical problems. Gene transfer, particularly oncogene transfer, is associated with DNA change and cancer formation^[2]. Omission of oncogene c-Myc from the defined four factors was tried and the cancer for-

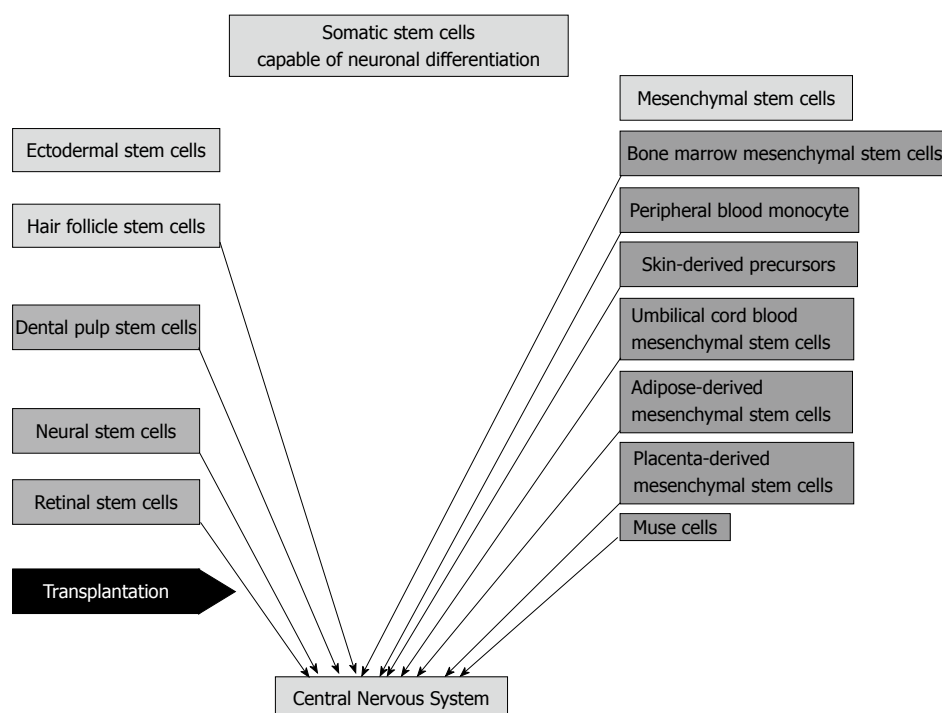


Figure 1 Somatic stem cells capable of neuronal differentiation. These cells are classified into two groups, ectodermal stem cells and mesenchymal stem cells. Ectodermal stem cells include hair follicle stem cells, dental pulp stem cells, neural stem cells and retinal stem cells, while mesenchymal stem cells include bone marrow mesenchymal stem cells, peripheral blood monocytes, skin-derived precursors, umbilical cord blood mesenchymal stem cells, adipose-derived mesenchymal stem cells, placenta-derived mesenchymal stem cells and Muse cells. These cells are candidates for donor cells for cell transplantation therapy for intractable neuronal diseases.

mation rate decreased^[5]. In addition, no integration of defined factors into the genome was tried and brought good results^[6]. However, cancer formation problems remain completely unsolved. It is probable that somatic stem cells reside in all organ tissues. In addition, truly pluripotent somatic stem cells, such as multilineage-differentiating stress enduring (MUSE) cells, are also probably harbored in all organ tissues^[7,8]. However, it has been reported that the capability of neuronal differentiation is recognized in only mesenchymal or ectodermal stem cells^[9,10]. Mesenchymal stem cells include bone marrow mesenchymal stem cells^[11], adipose-derived mesenchymal stem cells^[12], skin-derived precursors^[13], umbilical cord blood-derived mesenchymal stem cells^[14], placenta-derived mesenchymal stem cells, peripheral blood monocytes and MUSE cells^[7], while ectodermal stem cells include hair follicle stem cells^[15], dental pulp-derived stem cells^[16], retinal progenitor/stem cells and neural progenitor/stem cells^[17] (Figure 1). Although recent clinical trials of regenerative therapy for neuronal disease with transplantation of somatic stem cells has been performed with neural stem cells^[18,19], bone marrow mesenchymal stem cells^[20-25] and adipose mesenchymal stem cells^[26], most of them stay at the level of confirmation of safety, but the efficacy of the therapies has not been shown (Table 1). On the other hand, numerous studies of transplantation of somatic stem cells using neuronal disease models have been reported and most studies have confirmed it to be efficient for the repair of neuronal diseases^[27-34]. Ectodermal stem cells and mesodermal (mesenchymal) stem cells

potentially differentiate to neurons, while it seems that endodermal stem cells do not differentiate to neurons without dedifferentiation or induction to iPS cells. Being different from iPS cells, these stem cells do not basically transform or dedifferentiate to cancer cells. The clinical application of somatic stem cells has a greater advantage than iPS cells. The regenerative effect of transplantation of somatic stem cells is considered to be mostly derived from trophic factors secreted from somatic stem cells. It is reported that the transplantation effect of adipose-derived stem cells is greater than bone marrow mesenchymal stem cells because the former cells secrete more vascular endothelial growth factor (VEGF) or hepatocyte growth factor (HGF) than the latter^[35]. To survive as functional cells appropriate in the niche, it is necessary that transplanted cells differentiate to appropriate cells or somatic stem cells differentiate to appropriately functional cells before transplantation^[36]. Naïve somatic stem cells scarcely differentiate to appropriate cells in the niche. Therefore, for example, it is necessary that transplanted somatic neuronal cells in the nervous system are differentiated to neuronal cells. Here, I describe regenerative therapy for neuronal diseases with transplantation of somatic stem cells.

NEURAL STEM /PROGENITOR CELLS

It is difficult to obtain human neural stem/progenitor cells but they are easily obtained from human fetal brains without ethical problems. The use of these human cells is

Table 1 Clinical applications of somatic stem cells in the treatment of neuronal diseases

Kind of cell	Disease	Ref.
Neural stem cell	Pelizaeus-Merzbacher disease	[19]
	Neuronal ceroid lipofuscinosis	[18]
Bone marrow	Alzheimer's disease	[58]
mesenchymal stem cell	Parkinson's disease	[20,59,60]
	Amyotrophic lateral sclerosis	[61-71]
	Multiple sclerosis	[21,22]
	Cerebral infarction	[57,73,74]
	Spinal cord injury	[23-25,77,78]
Adipose mesenchymal stem cell	Parry-Romberg syndrome	[26]

accompanied with a great ethical problem^[37,38]. Previously, tissues of striatum and substantia nigra richly containing dopaminergic neurons were obtained from human fetal brain and were implanted into the striatum of Parkinson's disease patients. As a result, symptoms of a part of Parkinson's patients dramatically improved^[39,40]. However, these clinical trials were stopped due to the difficulty of obtaining fetal brain tissue and a great ethical problem in using an abortion fetus. Neural stem cells reside in the subventricular zone and hippocampus. It is more difficult to obtain autologous cells from the brain. Therefore, transplantation of autologous neural stem cells has not been tried for neuronal regeneration. In addition, few clinical applications of allogenic transplantation of human neural stem cells have been performed^[41,42].

In place of transplantation of human neural stem cells, activation of endogenous neural stem cells using fibroblast growth factor 2 (FGF-2), epidermal growth factor (EGF), erythropoietin and brain derived neurotrophic factor have been investigated^[41,42]. Murine or rodent neural stem/progenitor cells are frequently used for regenerative research. Transplantations of neural stem/progenitor cells have been used for a Parkinson's disease model^[36], cerebral infarction model^[43], spinal cord injury model^[27], retinal disease model^[44] and so on. However, transplantation of neural stem/progenitor cells without treatment is not useful for regeneration of neural tissue because non-treated neural stem/progenitor cells cannot survive in the recipient's neural tissue and in addition cannot differentiate to a neuron^[36]. Before transplantation, treatment of neuronal differentiation is effective for survival as a neuron. The addition of neurotrophic factors such as FGF8, sonic hedgehog and glial cell line-derived neurotrophic factor leads to neuronal differentiation^[45]. In addition, gene transfer to cells is useful for neuronal differentiation in neural stem/progenitor cells. Gene transfers of Math-1^[46], Ascl-1^[47], Nurr-1^[48] and von Hippel-Lindau (VHL)^[49] show neuronal differentiation in neural stem/progenitor cells. Neuronal differentiation of intracellular transfer of protein or peptide is also reported. Intracellular transfer of VHL peptide, consisting of an amino-acid sequence of binding sites to elongin C, is useful for neuronal differentiation in neural progenitor cells (Figure 2). VHL

peptide linked with protein transduction domain peptide shows high efficacy and rapid intracellular transduction. Transplantation of VHL peptide-treated neural stem cells promoted recovery in injured rat spinal cord^[27]. Clinical applications using human allogenic neural stem cells have been tried for neuronal ceroid lipofuscinosis^[41] and Pelizaeus-Merzbacher disease, both of which are hereditary intractable neuronal diseases^[42] (Table 1):

RETINAL PROGENITOR /STEM CELLS

Recently, retinal progenitor/stem cells (RSCs) have been identified in not only embryonic and newborn retina but also in adult ciliary epithelium (CE) of rodents and humans^[50-54]. Their niche has been suggested to be in the pigmented or nonpigmented epithelial layer of the ciliary margin at the peripheral edge of the retina. Since the majority of the differentiated cells were photoreceptor cells^[54], transplantation of RSCs has shown their potential as tools for cell replacement in retinal degenerative diseases.

BONE MARROW MESENCHYMAL STEM CELLS

Bone marrow mesenchymal stem cells are also called bone marrow stromal cells and have been reported to be able to differentiate cells of bone, cartilage, adipose tissue, liver and neural tissue^[55]. Transplantation of the bone marrow mesenchymal cells has been applied for cerebral infarction^[56,57]. These cells are transplanted via intravenous transfusion and a part of them have been demonstrated to penetrate the blood brain barrier (BBB), but these penetrated cells scarcely survive and function as neurons in the brain^[56,58]. Even if these cells do not differentiate to neural cells in the brain, these cells secrete neurotrophic factors which may have effects on neural tissue repair^[58]. When the bone marrow stromal cells are transferred with the gene of Notch intracellular domain and neurotrophic factors were added, these cells mostly differentiated to neurons^[55]. Clinical application with transplantation of bone marrow mesenchymal stem cells to neuronal degenerative disease patients of Alzheimer disease^[57], Parkinson's disease^[20,59,60], amyotrophic lateral sclerosis^[61-71] and multiple sclerosis^[21,22] have been tried, but those effects have not been fully established. Those induced neurons are transplanted to cerebral infarction model rats into the brain and the major part of the transplanted cells differentiated to neurons and the symptoms of the model rats improved^[72]. VHL peptide-transferred bone marrow stromal cells partially differentiate to neurons and transplantation of those induced neurons improved the behavior of the spinal cord injury rats^[28]. Human autologous bone marrow-derived mesenchymal stem cells have been transfused to brain ischemic disease patients^[73,74]. The results of the clinical trials appear to be feasible and safe and occasionally an improving effect is observed. Several human clinical applications for spinal

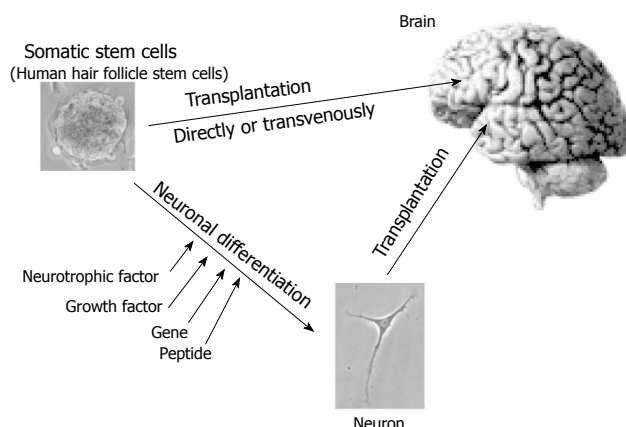


Figure 2 Transplantation of somatic stem cells into the central nervous system. Somatic stem cells (human hair follicle cell cells) forming a neurosphere, non-treated or neuronally differentiated by various methods, are directly or transvenously transplanted to brain.

cord injury with transplantation of bone marrow mesenchymal stem cells have been reported^[23-25,72-78] (Table 1). Among them, improvement of motor function and electrophysiological findings have been recognized^[25].

SKIN-DERIVED PRECURSORS AND HAIR FOLLICLE STEM CELLS

Skin-derived precursors (SKPs), which are also called dermal papilla stem cells, are reported to differentiate into various types of cells, including neuronal cells^[79-81]. Although these cells are considered to originate from mesenchymal tissue in dermis, they differentiate to not only mesenchymal-derived cells, such as smooth muscle cells and adipose cells, but also epithelial lineage cells, such as neurons, glia and keratinocytes. In addition, nestin-expressing hair follicle stem cells residing at the hair follicle bulge region in mice and at the outer root sheath of hair follicle beneath sebaceous glands in humans are reported to differentiate to epithelial lineage cells, including neuronal cells^[82,83], and they are also called neural crest stem cells^[84]. These cells might contribute to neuronal regenerative therapy, repairing not only peripheral nerves but also the central nervous system, including brain and spinal cord^[85,86] (Figure 2). It is reported that VHL peptide-transferred rodent SKPs were transplanted into cerebrum in Parkinson's disease rat models and they differentiated to dopaminergic neurons in the cerebrum with improvement of their symptoms^[29,30]. This report suggested that SKPs are hopeful sources of donor cells to transplant into the nervous system for neuronal regenerative therapy.

ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELLS

Adipose tissue-derived mesenchymal stem cells are similar to bone marrow-derived mesenchymal stem cells.

These cells differentiate to various types of cells, derived from not only mesenchymal organs but also epithelial and endogenous organs. Recently, directed differentiation of motor neuron cell-like cells from human adipose-derived stem cells was induced with retinoic acid and sonic hedgehog^[87,88], and the potential application for Huntington's disease or intracerebral hemorrhage is promising^[89,90]. The application to animal models and also the human clinical application have been tried using those cells, but their human clinical application is still limited^[91]. It is reported that adipose-tissue derived mesenchymal stem cells secrete trophic factors, such as VEGF and HGF, which contribute to repair for ischemic brain tissue^[35].

UMBILICAL CORD BLOOD-DERIVED MESENCHYMAL STEM CELLS

Human umbilical cord blood contains hematopoietic stem cells and mesenchymal stem cells. Umbilical cord blood-derived mesenchymal stem cells (UCBSCs) differentiate to neuronal cells and are clinically promising as a regenerative cell therapy for neuronal disease. It reported that neuronal differentiation of UCBSCs is mediated by protein kinase and that estrogen stimulates the neuronal differentiation of human UCBSCs^[92,93]. UCBSCs differentiated to dopaminergic neurons *in vitro*. Transplantation of those cells is applied to neuronal disease models^[94,95].

DENTAL PULP STEM CELLS

Dental pulp stem cells (DPSCs) are putatively neural crest cell-derived^[96] and thus differentiate to neurons^[97]. Therefore, these cells are promising as donor cells of neuronal regenerative cell therapy. Transplantation of DPSCs is applied to neuronal disease models such as spinal cord injury^[98]. It is suggested that implanted adult human dental pulp stem cells induce endogenous axon guidance^[99]. In addition, it is suggested that human DPSCs differentiate towards functionally active neurons in an appropriate environment^[16].

PLACENTA-DERIVED MESENCHYMAL STEM CELLS

Placenta-derived mesenchymal stem cells are from mesenchymal somatic stem cells and differentiate to cells of neuronal phenotype in the appropriate niche conditions^[100]. These cells differentiate to dopaminergic neuron-like cells *in vitro*^[101]. In addition, intracerebral transplantation of these cells has been reported^[102]. The transplantation of placenta-derived mesenchymal stem cells is promising for regenerative therapy for intractable neuronal diseases.

PERIPHERAL BLOOD MONOCYTES

Peripheral blood monocytes include mesenchymal stem

cells that are multipotential and capable of differentiating to neuronal lineage cells. These cells have the advantage of being obtained from an easily accessible minimally invasive procedure. With treatments of macrophage colony-stimulating growth factor and thereafter NGF, these cells express neuron specific enolase, neurofilament and microtubule associated protein 1-B that are neuronal markers^[103]. These cells differentiate to microglia that is supportive for neuronal tissue^[104] and are promising candidates as donor cells of autologous transplantation for neuronal regeneration.

MULTILINEAGE-DIFFERENTIATING STRESS ENDURING CELLS

Multilineage-differentiating stress enduring (MUSE) cells are pluripotent stem cells resembling ES or iPS cells^[105]. These cells are derived from skin fibroblast or mesenchymal stromal cells^[7,106]. Among stress (long-time heparin treatment) enduring fibroblasts, multilineage-differentiating stem cells were found. It is reported that these cells can differentiate to tri-dermal cells^[7]. They are promising as donor cells for regenerative cell therapy^[8]. Since they differentiated to neural lineage cells such as neuron and glia, they are hoped to be donor cells for neuronal regenerative cell therapy^[107]. MUSE cells are the most promising somatic stem cells and the obtaining method is established. The autologous transplantation of MUSE cells obtained from autologous fibroblast or mesenchymal stem cells is useful for neuronal regenerative cell therapy. The necessity of cell sorting using anti-SSEA-3 antibody is a limiting factor in generating MUSE cells^[7]. However, since the generative rate of MUSE cells is small but stable, use of MUSE cells is very promising as donor cells of transplantation of cell therapy for regeneration of neuronal disease.

ENDODERM-DERIVED SOMATIC STEM CELLS

Endoderm-derived somatic stem cells capable of neuronal differentiation are rare. When normal thyrocytes are cultured in non-serum small airway growth medium (SAGM) and their neuronal differentiation is induced, they express neuronal marker beta-III-tubulin^[108]. This result suggests that thyroid cells derived from endoderm are capable of differentiating to neurons. Although direct conversion of hepatocytes derived from endoderm to neurons using defined factors has been recently reported^[109], it is a report using a reprogramming method like iPS cells. Principally, it is likely that endoderm-derived stem cells are difficult to differentiate to neurons.

CONCLUSION

Cell transplantation therapy using somatic stem cells is very promising. At present, the kinds of clinically used

somatic stem cells are mostly limited to neural stem cells and bone marrow mesenchymal stem cells. Other somatic stem cells are scarcely used for clinical applications. However, therapeutic levels of somatic stem cell therapy still mostly stay at the confirmation of safety and feasibility. Undoubtedly, neuronal regenerative therapy with transplantation of somatic stem cells will be applied to intractable neuronal diseases and spread throughout the world in the future.

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MicroRNAs as novel regulators of stem cell fate

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Abstract

Mounting evidence in stem cell biology has shown that microRNAs (miRNAs) play a crucial role in cell fate specification, including stem cell self-renewal, lineage-specific differentiation, and somatic cell reprogramming. These functions are tightly regulated by specific gene expression patterns that involve miRNAs and transcription factors. To maintain stem cell pluripotency, specific miRNAs suppress transcription factors that promote differentiation, whereas to initiate differentiation, lineage-specific miRNAs are upregulated *via* the inhibition of transcription factors that promote self-renewal. Small molecules can be used in a similar manner as natural miRNAs, and a number of natural and synthetic small molecules have been isolated and developed to regulate stem cell fate. Using miRNAs as novel regulators of stem cell fate will provide insight into stem cell biology and aid in understanding the molecular mechanisms and crosstalk between miRNAs and stem cells.

Ultimately, advances in the regulation of stem cell fate will contribute to the development of effective medical therapies for tissue repair and regeneration. This review summarizes the current insights into stem cell fate determination by miRNAs with a focus on stem cell self-renewal, differentiation, and reprogramming. Small molecules that control stem cell fate are also highlighted.

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Key words: MicroRNA; Stem cell fate; Differentiation; Self-renewal; Reprogramming; Small molecule

Core tip: Stem cells are important in regenerative medicine applications due to their capacity to self-renew and differentiate into specific cell types. MicroRNAs (miRNAs) are short non-coding RNAs that negatively regulate gene expression at the post-transcriptional level. Recent studies suggest that miRNAs are key molecules in the regulation of stem cell fate decisions; this regulation is manifested as the fine tuning of cell- and tissue-specific gene expression. This review summarizes the current insights into stem cell fate determination by miRNAs and focuses on stem cell self-renewal, differentiation, and reprogramming. Small molecules that control stem cell fate are also highlighted.

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INTRODUCTION

Stem cells are a potential source for regenerative medicine and tissue engineering applications. These cells have the dual capacity to self-renew and differentiate into multiple distinct cell lineages^[1,2]. These cells are classified as embryonic stem cells (ESCs), non-embryonic adult stem cells, and induced pluripotent stem cells (iPSCs). ESCs are pluripotent cells produced within the inner cell mass

of a blastocyst stage embryo 4-5 d post-fertilization and can differentiate into all three germ layers: ectoderm, endoderm, and mesoderm^[3]. In contrast, adult stem cells are found in various tissues and organs, including the brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, and skin^[4]. Some adult stem cells are multipotent; they can produce a limited number of differentiated cell types from their specific tissue of origin. iPSCs are reprogrammed to be embryonic-like stem cells from adult somatic cells^[5,6].

Stem cell fate is controlled by transcription factors, epigenetic regulation, and non-coding RNAs^[7,8]. Transcription factors are well-known for regulating gene expression, by either directly or indirectly binding DNA elements, and for their role in epigenetic regulation, such as DNA methylation and histone modification. The control of gene expression also occurs during the post-transcription process. Recent findings have shown that small non-coding RNAs are involved in cell fate decisions, including the maintenance and differentiation of stem cells^[7,9].

MicroRNAs (miRNAs) are single-stranded, small non-coding RNA molecules. miRNAs modulate gene expression by either inhibiting mRNA translation or inducing mRNA degradation, which results from the complete or incomplete binding to the 3' untranslated region (3'-UTR) of specific mRNAs^[10,11]. More than 1000 different mature miRNAs have been discovered in humans, and they regulate one third of all protein-coding genes^[12,13]. Computational predictions of miRNA targets, functions, and expression, are accessible on multiple online prediction databases, such as TargetScan (<http://targetscan.org>), microRNA.org (<http://www.microRNA.org>), miRBase (<http://www.mirbase.org>), PicTar (<http://www.pictar.org>), and miRWalk (<http://mirwalk.uni-hd.de>)^[14,15]. One miRNA can target a large number of mRNAs, and/or many miRNAs can bind to one specific mRNA. This versatility may result in miRNAs mediating the effects of biological processes such as stem cell fate switches, proliferation, maintenance, and apoptosis. Interestingly, the first two miRNAs discovered, *lin-4* and *let-7*, were characterized during the developmental stage transition in *C. elegans*^[16,17]. By deleting enzymes involved in miRNA processing and maturation, namely, Dicer or Dgcr8, studies have shown that miRNAs are important in maintaining ESC pluripotency and differentiation capacity^[18-20]. miRNAs also play a role in the differentiation and self-renewal of mesenchymal stem cells (MSCs)^[21]. Many observations suggest that miRNAs critically regulate stem cell fate decisions, including self-renewal, differentiation into specific lineages, and reprogramming. Thus, this review focuses on miRNAs that are powerful regulators of stem cell fate. Furthermore, we discuss the potential of small molecules in regulating stem cell fate.

stem cells. Self-renewal is a process of symmetric division into two daughter cells. To self-renew, stem cells must proliferate without differentiating or becoming apoptotic to maintain their undifferentiated state^[22,23].

Cell division during self-renewal is achieved through regulated cell cycle events, such as the alternating activities of various D-type cyclins, cyclin-dependent kinases (CDKs), and E2F transcription factors. These cell cycle modulators and miRNA molecules are regulated during post-transcriptional modification^[10,24]. The transcription factors Oct4, Sox2, and Nanog are also important for the self-renewal of pluripotent cells^[7,25,26]. Oct4 and Nanog were the first transcription factors to be identified as necessary for the development and maintenance of ESC pluripotency. The expression of these factors is limited to pluripotent cell lines^[26-28]. Additionally, Oct4, Sox2, and Nanog have an autoregulatory feedback loop, which is an important feature of human ESCs^[29], and Sox2 implicitly interacts with Oct4^[30].

Oct4, Sox2, and Nanog may be upstream regulators of the miR-302-367 cluster of miRNA, which have been identified and differentially expressed in human ESCs^[31-33]. Conversely, miR-302-367 is required for Oct4, Sox2, and Nanog expression. Thus, miR-302-367 and the transcription factors (Oct4, Sox2, and Nanog) are tightly linked through an autoregulatory positive loop in pluripotent cells^[34,35]. Additionally, miR-302a promotes the G₁/S transition by repressing the translation of cyclin D1 in human ESCs^[36]. The inhibition of miR-302a causes an accumulation of pluripotent human ESCs in the G₁ phase^[36]. ESCs usually have a rapid G₁/S transition, which results in an extremely rapid proliferation rate (-10 h) compared to that of differentiated cells (more than 18 h)^[24]. The G₁/S transition is regulated by the cyclin D-Cdk4, 6 and cyclin E-Cdk2 complexes. The cyclin D-Cdk4, 6 complex is not present in mouse ESCs; however, the cyclin E-Cdk2 complex that induces S phase and DNA replication is present and active^[20,37]. *In vivo* experiments performed in a developing lung demonstrated that miR-302-367 decreased the expression of inhibitors of *cdkn1a* (p21) and *Rbl2*, inhibitors of the cyclin E-Cdk2 complex, which resulted in the formation of an undifferentiated multi-layered lung endoderm^[38]. Furthermore, in *Dicer*- and *Dgcr8*-knockout mice, ESCs exhibited reduced cell proliferation and an extended G₁ phase^[18,19].

Similar to miR-302-367, the miR-290-295 cluster is highly expressed in mouse ESCs, is regulated by Oct4, and binds Oct4, Sox2, Nanog, and Tcf3 to its promoters^[33,39]. The increased expression of the miR-290 family promotes the G₁/S transition, which enables rapid ESC proliferation and mediates the suppression of *cdkn1a*, *Rbl2*, and *Lats2*^[37]. Indeed, the miR-290 family functionally antagonizes differentiation-related miRNAs, such as the *let-7* family. The miR-290-295 cluster is rapidly downregulated during differentiation, which occurs with the restoration of *let-7* maturation. Increased *let-7* expression promotes differentiation by directly targeting pluripotency factors

MICRORNAS IN MAINTENANCE

Self-renewal and differentiation potential are hallmarks of

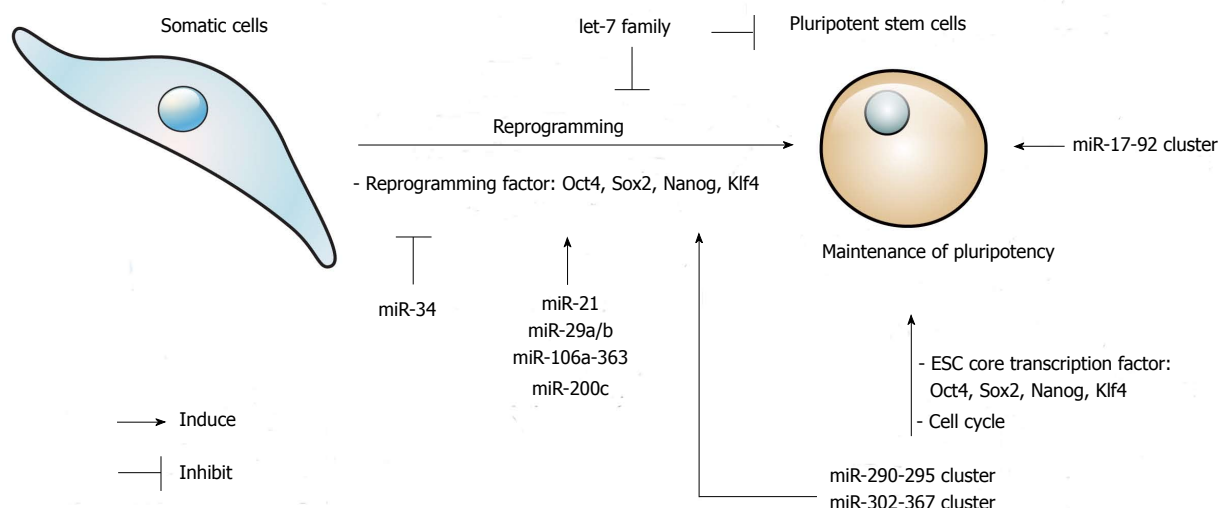


Figure 1 MicroRNAs regulate stem cell self-renewal and somatic cell reprogramming. ESC: Embryonic stem cell.

and ESC-enriched genes^[40].

Another important gene in stem cell maintenance is c-Myc, which is inhibited by let-7^[41]. In addition, c-Myc binds to the promoters of miR-141, miR-200, and miR-429. These miRNAs inhibit differentiation in mouse ESCs^[42]. Furthermore, c-Myc stimulates the expression of the miR-17-92 cluster in tumor cells^[43]. These miRNAs reduce the expression of the cell cycle control gene *Rb2*, which plays an important role in stem cell self-renewal^[44]. Moreover, miR-92b promotes the G₁/S transition through the repression of *cdkn1c* (p57, Kip2) in human ESCs^[45]. In fact, the miR-302-367, miR-290-295, and miR-17-92 clusters have been designated as ESC-specific cell cycle-regulating miRNAs (ESCC miRNAs) because they promote the G₁/S transition and cellular proliferation in ESCs^[37].

Compared to their role in ESCs, there is less evidence for the involvement of miRNAs in the self-renewal of somatic stem cells. The overexpression of miR-205 enhanced proliferation and expanded the population of progenitor cells by modulating PTEN, a tumor-suppressor gene^[46].

Therefore, stem cell self-renewal is tightly regulated through a complex network of core transcription factors, miRNAs, and the repression and/or promotion of differentiation mechanisms and pluripotent pathways, respectively (Figure 1).

MICRORNAS IN DIFFERENTIATION

Vascular differentiation: endothelial cells, vascular smooth muscle cells, and cardiomyocytes

Some studies indicate that miRNAs affect the vascular development or differentiation of stem cells, and others provide detailed reviews of the effect of miRNAs on endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and cardiomyocytes^[47-49] (Figure 2).

Endothelial cell differentiation

The first evidence for the regulation of endothelial cell

functions by miRNAs came from observations that dicer knockout mice displayed defects in embryos and yolk sacs during vasculogenesis and early angiogenesis^[50]. Dicer, accompanied by the altered expression of vascular endothelial growth factor (VEGF), fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor; FLT1), kinase insert domain receptor (a type III receptor tyrosine kinase; KDR), and tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie-1), plays an essential role in endothelial development.

Increased expression of miR-126 was first identified in Flk-1⁺ mesoderm populations derived from mouse ESCs^[51]. Two additional studies, performed with zebrafish and mice, demonstrated that miR-126 is essential for vessel integrity and endothelial function regulation but that it is not required to control the differentiation of ESCs to ECs^[52,53].

The expression of miRNAs associated with angiogenesis (let-7b, let-7f, miR-126, miR-130a, miR-133a, miR-133b, miR-210, and miR-296) was enhanced in day 10 differentiated cells compared to pluripotent human ESCs^[54]. Increased expression of the let-7 family during differentiation occurred by directly targeting pluripotency factors and ESC-enriched genes^[40]. Specifically, let-7f contributed to the angiogenic sprouting of ECs *in vitro*^[55]. The other upregulated miRNAs, miR-130a, enhanced angiogenesis by modulating GAX (growth arrest-specific homeobox) and HOXA5 (homeobox protein Hox-A5), which are anti-angiogenic homeobox transcription factors^[56]. Additionally, miR-210 was shown to be required for angiogenesis by targeting EphA2^[57], and miR-146b, miR-197, and miR-625 expression was enriched in CD31⁺ endothelial populations derived from mouse ESCs^[52]. Although the function of these miRNAs has been studied in cancer cells^[58-60], their role in the differentiation and functionality of ECs remains unknown.

The miRNA miR-181a promotes the reprogramming of lymphatic ECs toward a blood vascular phenotype^[61].

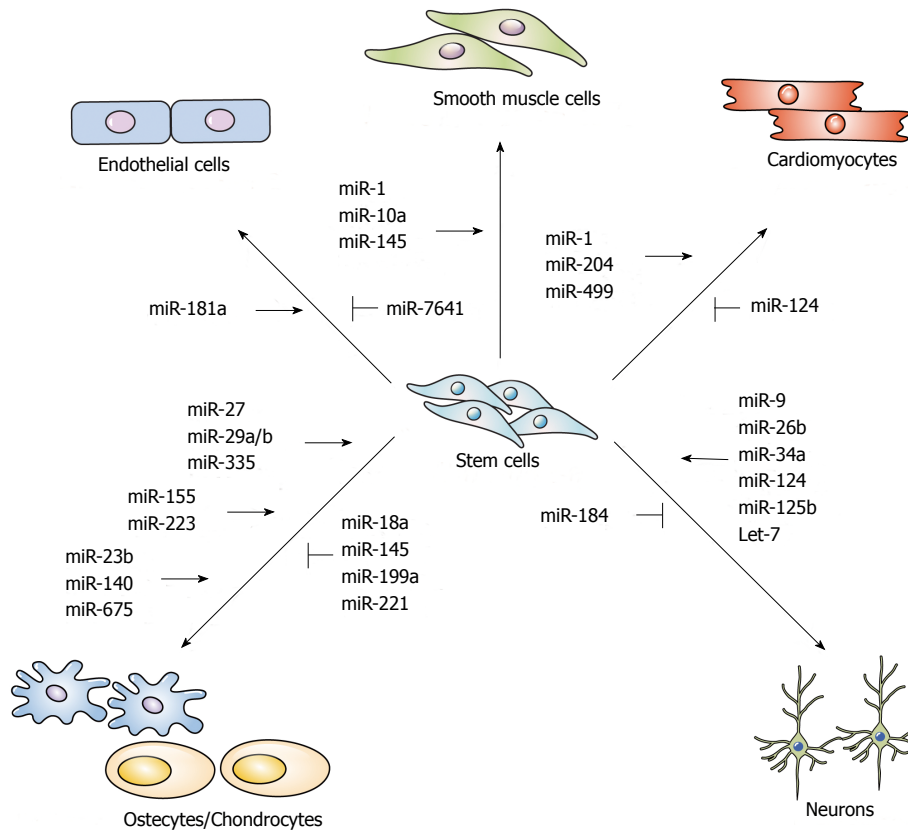


Figure 2 MicroRNAs are key regulators in stem cell differentiation.

The binding of miR-181a, to the 3'UTR of Prox1 (prospero homeobox 1, a key gene involved in lymphatic EC identity) results in inhibited expression. In human ESCs, miR-99b, miR-181a and miR-181b regulated the mRNA and protein expression of EC-specific markers, increased nitric oxide production, and improved therapeutic neovascularization *in vivo*^[62]. In addition, the expression of miR-7641 was downregulated during the endothelial differentiation of human ESCs. The overexpression of this miRNA significantly suppressed the expression of CXCL1 (a member of the CXC chemokine family)^[63]. CXCL1, which is involved in EC biogenesis and angiogenesis, is known to promote neovascularization by binding G-protein-coupled receptors^[64,65].

Smooth muscle cell differentiation

The miRNAs miR-143 and miR-145 are abundantly expressed in smooth muscle tissue. These miRNAs promote smooth muscle cell (SMC) differentiation from neural crest stem cells and are upregulated during differentiation, which is consistent with early expression patterns in the aorta of developing mouse embryos^[66-68]. Recently, it was discovered that miR-145 also promotes SMC differentiation from human ESCs^[69]. The expression of miR-143 and miR-145 is controlled by serum response factor (SRF), myocardin (MYOCD), and the following miRNA target transcription factors: KLF4, ELK1, and angiotensin-converting enzyme (ACE)^[66-68]. Other targets of miR-145 are Oct4, Sox2, and Klf4, which are

transcription factors for the self-renewal of pluripotent cells. These miRNAs are involved in regulating cell fate decisions across different lineages^[70]. A loss of miR-145 induced a different SMC phenotype, which was similar to the proliferating SMCs found in vascular lesions, but did not affect SMC differentiation^[66,67]. A reduction in neointima formation after vessel injury was observed in miR-145^{-/-} mice and, to a lesser extent, in miR-143^{-/-} mice^[68]. However, the overexpression of miR-143 and miR-145 also decreased neointima formation in a rat model of acute vascular injury^[71]. These data suggest that miR-143 and miR-145 are vital to SMC differentiation *in vitro*, but are not essential for SMC differentiation during embryonic development *in vivo*.

Another study showed that the increased expression of miR-10a during the *in vitro* differentiation of mouse ESCs to SMCs occurred via the post-transcriptional inhibition of histone deacetylase 4 (HDAC4)^[72]. The inhibition of miR-10a impairs SMC differentiation.

The miRNA miR-1 is involved in cardiomyocyte differentiation, cardiac hypertrophy, and apoptosis; however, recent studies suggest that it also plays a role in SMC differentiation^[73]. During the differentiation of mouse ESCs to SMCs, the expression of miR-1 steadily increased. Loss-of-function approaches using inhibitors against miR-1 resulted in the downregulation of SMC-specific markers and a decrease in the population of derived SMCs, indicating that miR-1 is required for the SMC lineage differentiation of ESC cultures. Previously identified

as a miR-145 target, KLF is a target for miR-1.

Cardiomyocyte differentiation

The miRNAs miR-1 and miR-133 were first described as critical regulators for muscle proliferation and skeletal muscle^[74] and cardiac muscle^[51] differentiation. Both miR-1 and miR-133 promote mesoderm formation from ESCs; however, these miRNAs have opposing functions during differentiation to cardiac muscle progenitors^[51,74-76].

These miRNAs, miR-1-1 and miR-1-2, are specifically expressed in cardiac and skeletal muscle precursor cells and direct transcriptional targets, such as SRF, myogenic differentiation 1 (MyoD), and myocyte enhancement factor 2 (Mef2)^[77]. An increased expression of miR-1 in mice led to embryonic developmental arrest at day 13.5, which resulted in a decreased population of proliferating ventricular cardiomyocytes^[77]. Hand2, a transcription factor that regulates ventricular cardiomyocyte expansion, is a direct target of miR-1^[77]. However, the targeted deletion of one of the two miR-1 genes (miR-1-2) located in muscle-specific miRNAs revealed numerous dysfunctions in the heart, including defective morphogenesis, electrical conduction, and unregulated cell-cycle control^[76]. Additionally, *Drosophila melanogaster* miR-1 modulates cardiogenesis and muscle-gene expression^[75]. Ivey *et al.*^[51] described that miR-1 acts as a repressor of non-muscle genes and that the overexpression of miR-1 upregulates Nkx2.5, an early cardiac marker, to promote cardiac differentiation. Notch ligand Delta-like 1 (Dll-1) is a target of miR-1^[51]. In human ESC-derived embryoid bodies, miR-1 also increased the expression of myosin heavy chain (MHC) genes^[78]. Additionally, miR-1 increased the expression of cardiomyocyte-specific genes and enhanced cardiomyocyte differentiation from human-derived cardiomyocyte progenitor cells by targeting HDAC4^[79]. Interestingly, the transplantation of murine ESCs overexpressing miR-1 into the border zone of infarcted mouse hearts prevented ischemia-induced apoptosis^[80]. In addition, miR-1 facilitates the electrophysiological maturation of ESCs^[81]. Furthermore, when miR-1 was transfected into fibroblast cells, gene expression profiles shifted toward that of muscle-like cells^[82]. Recently, miR-1 induced the expression of several cardiomyocyte markers, including Nkx2.5, GATA-4, cTnT, and CX43, *via* the downregulation of Hes-1, the downstream target molecule of the Notch pathway in MSCs^[83].

Although miR-1 and miR-133 are bicistronic^[76,84], they have opposing actions. The deletion of *miR-133a* genes causes lethal ventricular-septal defects, and results in the ectopic expression of smooth muscle genes. Therefore, miR-133a regulates the proliferation of cardiomyocytes by SRF and cyclin D2 activity^[84]. Specific cardiac markers were downregulated in miR-133-overexpressed mouse and human ESCs^[51,85], and miR-133 induced the proliferation of myoblasts by repressing SRF^[74]. A recent study revealed that miR-133 inhibited the proliferation of the prostate cancer cell lines PC3 and DU145 by targeting the epidermal growth factor receptor (EGFR)^[86].

Concurrently, our group also discovered that miR-133a expression increased during differentiation and that the overexpression of miR-133a promoted cardiac differentiation in human MSCs by targeting EGFR^[87].

Increased miR-499 expression was discovered in adult cardiac progenitor cells and human ESCs^[78,79]. This miRNA is encoded by an intron of MHC^[88] and shares many predicted targets with miR-208, which plays a crucial role in the stress-adaptation of the adult heart. The overexpression of miR-499 reduced the proliferation and enhanced the differentiation of human cardiomyocyte progenitor cells and ESCs through targeting Sox6, which is expressed in heart and skeletal muscle^[79]. The miRNA miR-499 has also been shown to play a role in myocyte lineage differentiation and the generation of mature working cardiomyocytes *in vitro* and after infarction *in vivo*^[89]. Both Sox6 and regulator of differentiation 1 (Rod1) are targets of miR-499. In addition to ESCs, cardiac stem cells, and cardiomyocyte progenitor cells, a recent study showed that the overexpression of miR-499 in rat MSCs induced cardiac differentiation through the Wnt/ β -catenin signaling pathway^[90].

Additionally, miR-204 is required for human cardiomyocyte progenitor cell differentiation, which occurs through targeting ATF-2^[91], whereas miR-124 inhibits the cardiomyocyte differentiation of MSCs by targeting STAT3^[92]. Finally, the deletion of the miR-17-92 cluster led to very specific defects in the development of the heart^[93]; however, the function of the miR-17-92 cluster in cardiac differentiation and development remains unclear.

Neuronal differentiation

Neural stem cells (NSCs) give rise to neurons, astrocytes, and oligodendrocytes and play an important role in embryonic development and the maintenance of the adult central nervous system (CNS)^[94]. The differentiation of NSCs is tightly associated with multiple signaling pathways: the Wnt signaling pathway regulates NSC proliferation and differentiation^[95], the transcription factors Neurog2 and Tbr2 are linked to NSC differentiation^[96]; the orphan nuclear receptor TLX is necessary for adult NSC proliferation^[97]; and the methyl CpG binding protein 2 (MeCP2), methyl-CpG binding protein 1 (MBD1), and histone-lysine N-methyltransferase Ezh2 are related to adult neurogenesis^[98,99]. In the mammalian brain, some miRNAs expression is tissue-specific, such as the let-7 family, miR-124, and miR-9, which regulate neurogenesis^[100,101]. Brain-specific miR-124 is upregulated during CNS development and the neuronal differentiation of the adult subventricular zone (SVZ)^[102,103]. During neurogenesis, the suppression of RE-1-silencing transcription repressor (REST) induces the expression of miR-124, which represses JAG1, Dlx2, and Sox9. In addition, laminin γ 1 and integrin β 1, which are expressed in neural progenitors but inhibit neuronal differentiation, are also targeted by miR-124 and lead to neurogenesis^[104]. The miRNA miR-9 is also highly expressed in the brain and is involved in modulating the balance between

NSC self-renewal and differentiation *via* negative TLX expression^[105]. The overexpression of miR-9 promotes neural differentiation but downregulates TLX. Let-7d, a member of the let-7 family, also targets TLX, promotes neurogenesis, and reduces NSC proliferation^[106]. Let-7a is a downstream molecule of tripartite motif-containing protein 32 (TRIM32); therefore, let-7a is also required to induce NSC differentiation^[107]. The overexpression of TRIM32 induces neuronal differentiation, whereas the inhibition of TRIM32 preserves the self-renewal capability of neural progenitor cells. The miRNA miR-137 is essential for embryonic NSC fate decisions; the overexpression of miR-137 inhibits NSC proliferation and induces accelerated differentiation by suppressing histone lysine-specific demethylase 1 (LSD1), a co-transcription factor of TLX^[108]. Additionally, miR-137, which mediates epigenetic proteins such as MeCP2 (a DNA methyl-CpG-binding protein), Ezh2, and Polycomb group (PcG) protein, regulates the balance of NSC proliferation and differentiation in adult neurogenesis. A reduction of miR-137 expression promotes differentiation, whereas the overexpression of miR-137 increases adult NSCs proliferation^[98]. Similar to miR-137, miR-184 is associated with controlling the balance between the proliferation and differentiation of adult NSCs. Upregulated miR-184 targets methyl-CpG binding protein 1 (MBD1) and Numblike (Numb), which are related to NSC differentiation in the adult brain, to induce cell proliferation and reduce the differentiation of adult NSCs^[99]. In neural stem/progenitor cells (NSPCs) isolated from adult mice, the miR-106b-25 cluster (miR-106b, miR-93, and miR-25) regulates NSPC proliferation and differentiation. The miRNA miR-25 targets insulin/insulin-like growth factor-1 (IGF) signaling pathways. The expression of miR-106b-25 is mediated by FoxO3, a member of the FoxO family of transcription factors that is important for the maintenance and differentiation of NSCs^[109]. Recently, it was determined that miR-34a is involved in NSC differentiation; miR-34a promotes Notch signaling by repressing Numb, a negative regulator of Notch signaling that inhibits neuronal differentiation^[110]. Additionally, miR-26b activates neurogenesis by suppressing Ctdsp2 protein expression^[111,112]. By targeting Nestin, miR-125b promotes NSPC differentiation and migration while inhibiting NSPC proliferation^[113] (Figure 2).

Osteoblast, osteoclast, and chondrocyte differentiation

The skeleton consists of osteoblasts and osteoclasts in bone tissue and chondrocytes in cartilage tissue^[114]. Increasing evidences suggests that miRNAs are an integral part of regulating bone and cartilage formation, metabolism, homeostasis, osteogenesis, and chondrogenesis^[115,116].

Osteoblast differentiation from bone marrow stromal cells undergoes three stages: pre-osteoblast (proliferation), osteoblast/pre-osteocyte (matrix maturation), and osteocyte (mineralization)^[117]. Each cell type expresses different genes and factors; therefore, miRNAs may be selectively expressed in particular stages during osteogenesis. At

different stages of osteoblast differentiation, miR-29 has multiple distinct functions. For example, miR-29b initiates the osteogenic pathway by repressing anti-osteogenic factors, such as HDAC4, TGF- β 3, activin A receptor type IIA (ACVR2A), beta-catenin-interacting protein 1 (CTNNBIP1), and dual-specific phosphatase (DUSP2). Collagen type I (COL1A1) directly targets miR-29b. During mineralization, when collagen accumulation is at a steady state, high endogenous levels of miR-29b downregulate the mRNA expression of COL1A1^[118]. In addition, miR-29 suppresses osteonectin (secreted protein acidic and rich in cysteine, SPARC) during matrix maturation and the mineralization phase during late differentiation^[119]. Although collagens and osteonectin play an important role in bone mass and osteogenesis, the inhibition of these proteins by miR-29b prevents sclerotic bone formation and increases bone structure stability^[117]. Moreover, canonical Wnt signaling is involved in osteoblast differentiation; a high level of β -catenin is required for osteogenesis. Therefore, targeting the Wnt pathway by miRNAs has been shown to contribute to osteogenesis^[120]. The miR-29 family also targets Wnt signaling-mediated proteins; the expression of miR-29 is increased by Wnt activation during osteoblast differentiation. Additionally, miR-29a negatively regulates the Wnt receptor complex Dickkopf-related protein 1 (Dkk1), Kremen2, and secreted frizzled related protein 2 (sFRP2)^[121], whereas miR-29b downregulates the β -catenin inhibitor CTNNBIP1^[118]. Both miR-27 and miR-335 are upregulated during osteogenesis and target the APC gene and Dkk1, a negative regulator of Wnt signaling, respectively, which leads to osteoblast differentiation^[122,123].

Only a few miRNAs contribute to osteoclast differentiation. In particular, miR-223 is regulated by transcription factor PU.1. An increased expression of miR-223 and receptor activator of nuclear factor- κ B (RANK) is observed in bone marrow derived osteoclast precursors after induction by M-CSF^[124]. miR-223 regulates NFIA, a suppressor of osteoclastogenesis, which leads to the upregulation of the M-CSF receptor^[125]. A key regulator in the maturation of hematopoietic cells to macrophages, miR-155 has been studied as another osteoclastogenic miRNA^[126]. miR-155 represses MTF, a necessary transcription factor for osteoclast differentiation, to inhibit osteoclastogenesis^[127].

Cartilage tissue forms bone *via* the endochondral process of ossification. The loss of miRNAs in cartilage accelerates the differentiation of mature hypertrophic chondrocytes and abnormal bone growth^[128]. Cartilage-specific miR-140^[129] is related to palatogenesis, which mediates platelet-derived growth factor D (PDGFD) signaling in zebrafish^[130], craniofacial development and endochondral bone formation *via* targeting HDAC4^[131] and inhibits BMP signaling in mouse models^[132]. HDAC4 and BMP signaling pathways contribute to chondrocyte hypertrophy and osteoblast differentiation and can be negative effector of osteogenesis. The miRNA miR-675 can promote chondrogenic differentiation by inducing the

expression of cartilage-specific collagen type IIa through the positive regulation of cartilage-specific Sox9^[133]. The chondrogenic differentiation of MSCs is induced by miR-23b, which negatively inhibits of protein kinase A signaling^[134]. In addition, miR-18a, miR-199a, miR-145, and miR-221 have been identified as negative regulators of chondrogenesis. To repress chondrogenesis, miR-18a directly targets the CCN family protein 2/connective tissue growth factor (CCN2/CTGF)^[135]. Similarly, miR-199a, a bone morphogenic protein 2-responsive miRNA, significantly inhibits early chondrogenesis by targeting Smad1^[136]. In addition, miR-145 targets Sox9, a key transcription factor for chondrogenic differentiation^[137,138], and miR-221 negatively regulates Mdm2 and therefore prevents the degradation of Slug protein, which is involved in chondrogenesis inhibition^[139] (Figure 2).

Other types of differentiation

Despite the multi-lineage differentiation potential of stem cells, little is known about the differentiation of stem cells to other cell types than those described above. For example, the hepatic differentiation of human umbilical cord lining-derived MSCs (hUC-MSCs) and liver-derived progenitor cells (LDPCs) is regulated by miR-542-5p and miR-146a^[140]. The miRNA miR-182 is involved in the differentiation of inner ear stem/progenitor cells into hair-like cells *via* the repression of Tbx1^[141]. Pancreatic transcription factor Ptf1a is specifically expressed at different stages during pancreatic development; low levels of Ptf1a enhance the differentiation of pancreatic progenitor cells to endocrine cells, whereas high levels of Ptf1a are involved in exocrine cell differentiation. The endogenous expression of Ptf1a is regulated by miR-18a^[142]. During the adipogenic differentiation of mouse ESCs, the expression of miR-10b, miR-15, miR-26a, miR-30a-5p, miR-30c, miR-98, miR-99a, miR-103, miR-143, miR-148a, miR-152, miR-224, miR-422b, and miR-let-7b increased, whereas the expression of the miR-17-92 cluster was downregulated^[143]. Myeloid differentiation is promoted by PU.1 transcription factor, and the overexpression of the miR-23a cluster in hematopoietic progenitor cells suppresses B-cell development^[144]. Furthermore, miRNAs are involved in the differentiation of diploid spermatogonia to haploid spermatozoa. The miRNA miR-34c is highly expressed in the late stages of spermatogenesis, which induces the upregulation of germ cell-specific genes^[145].

MICRORNAS IN REPROGRAMMING

In 2006, the astonishing research of Yamanaka demonstrated that somatic cells such as mouse fibroblasts, can be reprogrammed to a pluripotent state using only four transcription factors: Oct4, Sox2, Klf4, and c-Myc^[5]. These reprogrammed fibroblasts are referred to as iPSCs, and they are functionally and molecularly similar to ESCs. After one year, the same group induced human iPSCs in a similar manner as the mouse iPSCs^[146]. These

initial studies introduced the somatic cell reprogramming strategy. Although the method of transcription factor-mediated reprogramming is simple, problems such as time, low efficiency, and the possibility of tumorigenesis remain unsolved^[147]. To improve the quality of generated iPSCs, researchers have focused on using miRNAs, which are associated with regulating the epigenome. Because the ectopic expression of transcription factors during reprogramming is related to epigenetic changes, miRNAs are considered an attractive alternative for somatic cell reprogramming^[35] (Figure 1).

To improve the efficiency of iPSC generation, reprogramming barriers must be overcome. The reprogramming process undergoes two phases: the early phase (initiation phase) and the late phase^[8]. The early phase is a pre-pluripotent state involving increased cell proliferation and a change into an epithelial-like cellular state called the mesenchymal-epithelial transition (MET)^[148]. This phase is regulated by p53-induced cell-cycle repression and the TGF- β -accelerated epithelial-mesenchymal transition (EMT). The late phase is the transition of pre-iPSCs by inducing pluripotency-related genes, such as Nanog, Sox2, and Lin28, and establishing the pluripotency network^[8]. Thus, reducing these barriers by utilizing miRNA-mediated epigenetic and transcriptional regulation enhances reprogramming efficiency and generates functional cells that resemble ESCs^[8,148,149].

The first attempt to reprogram focused on miRNAs that were highly expressed in ESCs and governed pluripotency but were absent in fibroblasts. Among members of the miR-290-295 family, miR-291-3p, miR-294, and miR-295, in combination with Oct4, Sox2, and Klf4, increased the reprogramming efficiency of mouse fibroblasts^[150]. In human somatic cells, miR-302a-367 and/or miR-371-373 (mouse homolog miR-290-295), in combination with Oct4, Sox2, Klf4, and c-Myc, enhanced the efficiency of reprogramming by inhibiting TGF- β -induced EMT^[151]. During the early reprogramming stage, miR-17-92, miR-106b-25, and miR-106a-363 clusters, which share the seed sequences of the miR-302 cluster, were shown to be highly induced^[152]. The overexpression of the miR-106a-363 and miR-302-367 clusters promoted a distinct increase in iPSCs generated from mouse fibroblasts. This increase was achieved by targeting TGF- β type II receptor with Sox2, Klf4, and Oct4, which accelerated MET^[153]. In addition, the activation of BMP signaling induced the expression of the miR-205 and miR-200 family and enhanced the MET^[154]. Therefore, the TGF- β and BMP signaling pathways are critical mechanisms that induce MET and promote reprogramming. Further investigation of somatic reprogramming is possible using only miRNAs to directly promote reprogramming events. Recently, Anokye-Danso and coworkers reported that the transfection of miR-302 and miR-367 clusters successfully reprogrammed mouse and human somatic cells to iPSCs without the use of exogenous transcription factors^[155]. Interestingly, the direct transfection of mature biomimetic miRNAs, such

Table 1 Small molecules in stem cell fate and somatic cell reprogramming

Chemical	Effect (Target)	Result	Ref.
PD0325901	MEK inhibitor	Promotes mouse ESC self-renewal	[161]
CHIR9902	GSK-3 inhibitor	Enhances human ESC survival	[161-164]
Y27632	ROCK inhibitor	Induces human ESC differentiation into endothelial cells and neural tissues	[167,168]
Thiazovivin	TGF- β receptor inhibitor (SMAD signaling inhibitor)	Somatic cell reprogramming	[170-175]
SB431542	HDAC inhibitor		
VPA	HMT inhibitor		
BIX-01294	DNMT inhibitor		
RSC133			
5-Aza			
SB431542	TGF- β receptor inhibitor		
PD0325901	MEK inhibitor	Promote HSC self-renewal	[177-179]
TSA	HDAC inhibitor		
Trapoxin			
Chlamydocin			
SR1	AHR antagonist		
PGE2	PG pathway		
Pyrvinium	Wnt inhibitor	Promote MSC self-renewal	[191,192]
SKL2001			
H-89	PKC inhibitor	Induces human MSC differentiation into chondrocytes	[130,180]
Katogenin	Filamin A	Induces human MSC differentiation into osteoblasts	[181,182]
Purmorphamine	RUNX2 activator	Induces rat MSC differentiation into hepatocytes	[183]
CW008	cAMP/PKA/CREP pathway agonist		
SJA710-6			
PMA	PKC activator	Induces rat MSC differentiation into cardiomyocytes	[184,185]
LY294002	PI3K/AKT inhibitor	Inhibits mouse MSC differentiation into adipocytes	[186,187]
CHIR9902	GSK-3 inhibitor	Induces human MSC differentiation into adipocytes	
Troglitazone	PPAR γ agonist	Induces human MSC differentiation into neural-like cells	[188-190]
SB431542	SMAD inhibitor		
LY94002	PI3K/AKT inhibitor		

ESC: Embryonic stem cell; HSC: Hematopoietic stem cell; MSC: Mesenchymal stem cell; MEK: Mitogen-activated protein kinase kinase; GSK-3: Glycogen synthase kinase 3; ROCK: Rho-associated protein kinase; TGF- β : Transforming growth factor beta; HDAC: Histone deacetylases; HMT: Histone methyltransferases; DNMT: DNA methyltransferases; AHR: Aryl hydrocarbon receptor; PG: Prostaglandins; PKC: Protein kinase C; RUNX2: Runt-related transcription factor 2; cAMP: Cyclic adenosine monophosphate; CREP: cAMP response element-binding protein; PI3K: Phosphoinositide 3 kinase; AKT: Protein Kinase B; PPAR γ : Peroxisome proliferator-activated receptor gamma.

as miR-200c and the miR-302-369 family, promoted the reprogramming of mouse and human somatic cells. This method does not require lentiviral vectors for gene transfer^[156].

Contrary to the aforementioned examples, some miRNAs must be suppressed to enhance reprogramming. For example, let-7 miRNAs are negative regulators of the

potent reprogramming factor Lin28. The inhibition of let-7 miRNAs leads to the dedifferentiation of somatic cells to iPSCs, which induces cell proliferation and pluripotency genes^[40]. Another important miRNA barrier for reprogramming is the p53-mediated pathway. The p53-mediated pathway induces the expression of the miR-34 family and the suppression of the pluripotency factors Nanog and Sox2^[157]. The genetic deletion of miR-34a increased the efficiency and kinetics of reprogramming and established pluripotency at a late stage. Additionally, the suppression of p53, by overexpressing miR-138^[158] or repressing miR-21 and miR-29a, enhanced reprogramming^[159]. The expression of endogenous miRNAs is regulated by transcription factors^[160]. The expression of miR-29b is directly regulated by Sox2 during iPSC generation and miR-29b is an essential facilitator for Oct4, Klf4, Sox2, and c-Myc (or Oct4, Klf4, and Sox2) mediated reprogramming^[161].

Reported reprogramming factors Oct4, Klf4, Sox2, and c-Myc have demonstrated that miRNAs play a crucial role in regulating stem cell fate events, such as reprogramming, differentiation, and self-renewal. However, some questions pertaining to the mechanisms of reprogramming remain unresolved. Addressing these questions will provide further understanding of reprogramming and will promote the development of iPSC generation technologies and stem cell therapies.

SMALL MOLECULES AND STEM CELL FATE

Stem cell fate is regulated by both intrinsic/extrinsic regulators and the extracellular niche. Because these regulators have limitations, such as efficiency and selectivity for controlling stem cell fate, a new strategy is to use of small molecules^[162] (Table 1). Compared to genetic manipulations, small-molecule approaches have a number of advantages: 1) the biological effects of small molecules are rapid, reversible, and dose-dependent; 2) small molecules have specific targets in signaling pathways or epigenetic mechanisms; and 3) a variety of chemical libraries provide data for the functional optimization of small molecules^[163]. Recently, many small molecules have been identified and characterized that can manipulate stem cell fate, including self-renewal, lineage-specific differentiation, and somatic cell reprogramming^[35,164].

The self-renewal capacity of mouse ESCs is maintained by PD0325901 (MEK inhibitor) and CHIR99021 (GSK3 inhibitor) without feeder cells or exogenous cytokines^[165]. The molecules Y-27632 and thiazovivin (ROCK inhibitor) enhance the survival of human ESCs^[166-168], whereas a combination of PD0325901, CHIR99021, and Y-27632 supplemented with bFGF supports the maintenance of human ESCs^[169]. Because the lineage-specific commitment of stem cells provides possible therapeutic applications, studies that control stem cell differentiation have been consistently reported.

Wnt signaling modulators promote cardiomyocyte generation in zebrafish embryos and murine ESCs^[170], and the inhibition of TGF- β receptor by SB431542 induces the endothelial cell differentiation of human ESCs^[171]. The inhibition of SMAD signaling by noggin and SB431542 directs the differentiation of human ESCs to neural tissues^[172].

ESCs have the ability to propagate indefinitely and to differentiate into any cell type; however, ethical issues regarding the use of ESCs still remain. Therefore, tissue-specific adult stem cells and the ability to reprogram somatic cells have fascinated researchers^[164,173]. Ever since Yamanaka demonstrated that Oct4, Sox2, Klf4, and c-Myc can convert mouse fibroblasts into induced pluripotent stem cells (iPSCs)^[5], the study of reprogramming has accelerated with the use of epigenetic process modulators, which target histone deacetylase (HDAC)^[174,175], histone acetyltransferase (HMT)^[176,177], and DNA methyltransferase (DNMT)^[176,178]. Recently, a chemical cocktail including HDAC inhibitors and other kinase inhibitors enhanced the reprogramming efficiency of human fibroblasts^[175,179].

Hematopoietic stem cells (HSCs) are related to the hematopoietic lineage, and cell phenotypes include macrophages, erythrocytes, dendritic cells, T-cells, B-cells, and NK-cells^[180]. The fate of HSCs is regulated by small molecules that promote self-renewal^[181-183]. Using small molecules, multipotent MSCs can differentiate into various non-hematopoietic cells, such as chondrocytes^[134,184], osteoblasts^[185,186], hepatocytes^[187], cardiomyocytes^[188,189], adipocytes^[190,191] and neuronal-like cells^[192-194]. Additionally, the maintenance of MSCs is associated with the Wnt signaling pathway^[195,196].

Although chemical approaches are a very young field in stem cell research, these small molecules exhibit a similar biological outcome to that achieved with the use of miRNAs in stem cell fate regulation^[35]. Recently, small molecules have been correlated with endogenous miRNA expression and function^[197-202]. Therefore, identifying the relationship between miRNAs and small molecules could provide new insights for drug development for regenerative medicine and elucidate detailed mechanisms of miRNA expression and function in the control of stem cell fate.

CONCLUSION AND FUTURE DIRECTIONS

Increasing evidence has demonstrated that miRNAs are promising regulators of stem cell fate. The current strategy in stem cell biology can elucidate the links between miRNAs and stem cell fate determination. Although miRNAs strictly regulate the multiple molecular signaling pathways and transcription factors that control stem cell fate, some significant issues have not received adequate attention. Current challenges focus on verifying the downstream targets of miRNA; however, the study of miRNA upstream targets is virtually nonexistent. In addition, the correlation between miRNAs is not well understood. Small molecules not only modulate stem cell

fate but also regulate miRNA synthesis and the function of transcription factors and miRNAs. The challenge of identifying the relationship between miRNAs and small molecules is still at an initial stage. Complementary to conventional and interdisciplinary strategies, including miRNAs and/or chemical manipulation techniques in the regulation of stem cell self-renewal, tissue- or organ-specific differentiation, and iPSC generation provides a powerful tool to identify the underlying cellular mechanisms of stem cell biology and isolate the therapeutic agents required for clinical applications such as cell therapy and regenerative medicine.

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Epithelial-mesenchymal transition - activating transcription factors - multifunctional regulators in cancer

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Abstract

The process of epithelial to mesenchymal transition (EMT), first noted during embryogenesis, has also been reported in tumor formation and leads to the development of metastatic growth. It is a naturally occurring process that drives the transformation of adhesive, non-mobile epithelial like cells into mobile cells with a mesenchymal phenotype that have ability to migrate to distant anatomical sites. Activating complex network of embryonic signaling pathways, including Wnt, Notch, hedgehog and transforming growth factor- β pathways, lead to the upregulation of EMT activating transcription factors, crucial for normal tissue development and maintenance. However, deregulation of tightly regulated pathways affecting the process of EMT has been recently investigated in various human cancers. Given the critical role of EMT in metastatic tumor formation, better understanding of the mechanistic regulation provides new opportunities for the development of potential therapeutic targets of clinical importance.

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Key words: Epithelial-to-mesenchymal transition; Metastatic growth; Embryonic signaling pathways; Transcription factors; Cancer

Core tip: This review article discusses the mechanistic regulation of embryonic signaling pathways with a spe-

cial focus on epithelial mesenchymal transition (EMT)-activating transcription factors in cancer progression *via* modulating the process of EMT. Deciphering this mechanism may lead to the design of cancer therapies by altering the balance between the process of EMT/mesenchymal epithelial transition in cancer stem cells and thereby clinically treat the cancer.

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INTRODUCTION

Epithelial-mesenchymal transition is naturally occurring and a vital process during embryogenesis, adult tissue repair and maintenance. It is characterized by specific gene expression pattern changes, loss of adherent tight junctions that keep epithelial cells in contact with their neighbors, gain of mesenchymal phenotype, including fibroblastoid morphology, and increased mobility potential to distant locations.

Based on the biological context, epithelial mesenchymal transition (EMT) is classified into three types^[1]. Type 1 EMT is associated with the transition of epithelial cells to motile mesenchymal cells during implantation, embryo formation, gastrulation, neural crest delamination, in the development of placenta, somites, heart valves, urogenital tract and secondary palate, as well as during branching morphogenesis of different organs. Primary mesenchymal cells act as progenitors and *via* the process of mesenchymal epithelial transition generate secondary epithelia in mesodermal and endodermal organs. Type 2 EMT describes the events in wound healing, tissue regeneration and organ fibrosis where tissue fibroblasts

are generated from epithelial or endothelial cells. Type 3 EMT has been described in epithelial cancer cells where the cells dedifferentiate and acquire a mesenchymal phenotype. These cells invade and metastasize through the circulation and generate a metastatic lesion at distant tissues or organs by MET^[2]. The three types of EMT program are considerably distinct biological processes but tumor cells take over the developmental pathways for metastatic dissemination through well conserved or similar genetic controls and biological mechanisms. Besides acquiring a mesenchymal property, cancer cells with EMT phenotype exhibit more aggressive behavior, including resistance to drugs, stresses and apoptosis, inhibition of senescence, immune evasion and acquisition of stem cell-like features. All these dramatic changes in tumor cells allow them to infiltrate surrounding tissues, metastasize at distant locations and promote cancer progression.

Growing evidences in the last few years document the key role of EMT-activating transcription factors (ATFs) in oncogenic transformation. They override cancer safeguard programs against cancer like apoptosis, senescence, regulate cancer cell stemness, determine resistance to chemotherapy and promote tumor angiogenesis.

EMT-ACTIVATING TRANSCRIPTION FACTORS

Recent research documents the involvement of EMT in the induction of cellular traits associated with the metastatic progression of cancer. EMT is characterized by the downregulation of epithelial markers/tight junction components, desmosomes, cytokeratins and gain of mesenchymal markers like reorganization of the cytoskeleton (*e.g.*, switch from cytokeratins to vimentin), and the synthesis of extracellular matrix components and metalloproteases^[3]. Loss-of-function mutations and promoter hypermethylation could downregulate E-cadherin expression and function in a number of carcinomas, but modulation of EMT during embryogenesis and cancer progression mostly involves the participation of EMT-ATFs. In addition to a mesenchymal switch, these factors control the entire EMT program and endow cancer cells with stem-like characteristics. These migrating cancer stem cells are not only important in the genesis of primary tumors, but also enhance metastasis and possibly the root cause of tumoral chemoresistance and recurrence^[4].

Molecular reprogramming occurring during EMT is triggered and orchestrated by various EMT-ATFs, including the Snail family of zinc-finger transcription factors: Snail1 (Snail), Snail2 (Slug) and Snail3 (Smuc); the two-handed zinc-finger factors of d-crystallin/E2 box factor (DEF1) family proteins DEF1/zinc-finger E-box-binding homeobox (ZEB)1 and Smad-interacting protein (SIP)1/ZEB2; the basic helix-loop-helix factors (Twist1 and Twist2); E12/E47 and Tbx3^[5-8]. These factors act as molecular switches, respond to the known signaling pathways and regulate the EMT program. These transcription

factors recognize the E-box DNA sequences in the promoter region of E-cadherin, recruit cofactors and histone deacetylases and thereby repress its expression^[9].

The expression of other epithelial molecules, including claudins, occludins and mucin1, are suppressed by Snail while the genes associated with the mesenchymal and invasive phenotype are induced. Snail has been linked with tumor grade, metastasis, recurrence and poor prognosis, and suppresses tumor suppressor Raf kinase inhibitory protein (RKIP), an inhibitor of nuclear factor kappa B (NF- κ B)^[10,11]. Snail and Twist further cooperate in inducing the expression of ZEB1 and act as key regulators in the process of EMT. These oncogenic factors when overexpressed in neoplastic cells make them more aggressive and promote the development of metastatic properties. Studies in cell lines and xenograft mice models verify EMT-ATFs' functions in cancer and set them not only as important diagnostic and prognostic biomarkers, but also as potential therapeutic targets. In view of the expanding portfolio of EMT-ATFs as multifunctional regulators in the hallmarks of cancer, it will be important to review their mechanisms of actions.

REGULATORY MECHANISMS OF EMT-ATFS

ZEB1 and ZEB2

The ZEB family comprises of zinc finger/homeodomain proteins-ZEB1 and ZEB2, well conserved among species, interacts with other transcriptional regulators and their activities are modulated by post-translational modifications like SUMOylation by Pc2 or acetylation by p300/pCAF and phosphorylation. These proteins trigger an EMT by repression of epithelial markers and activation of mesenchymal properties.

Growth and steroid hormones; hypoxia inducible factor-1 α (HIF-1 α) in hypoxic conditions; inflammatory cytokines; ligands [*e.g.*, fibroblast growth factor (FGF), insulin growth factor-1, platelet derived growth factor receptor]; downstream signals frequently activated in tumors like Ras-ERK2-Fra1, NF- κ B and JAK/STAT3; classical signaling pathways-transforming growth factor β (TGF β)/smad; Wnt and Notch directly activate the expression of ZEB factors. They are also regulated by miR-200a-microRNA that inhibits ZEB factors in a reciprocal negative feedback loop and finally induces the emergence of mesenchymal-epithelial transition phenotype in various cancers, extensively reviewed by Sánchez-Tilló *et al*^[12].

Snail, Slug and Smuc

All three members of the Snail family, Snail, Slug and Smuc, share the SNAG domain at N terminal region and zinc finger cluster at C terminal region that binds to E-boxes in the regulatory regions of target genes. Snail-mediated histone modifications (deacetylation, methylation and demethylation) cooperate to repress E-cadherin but this mechanism is still not completely known. Post-transcriptional modifications alter protein stability and in-

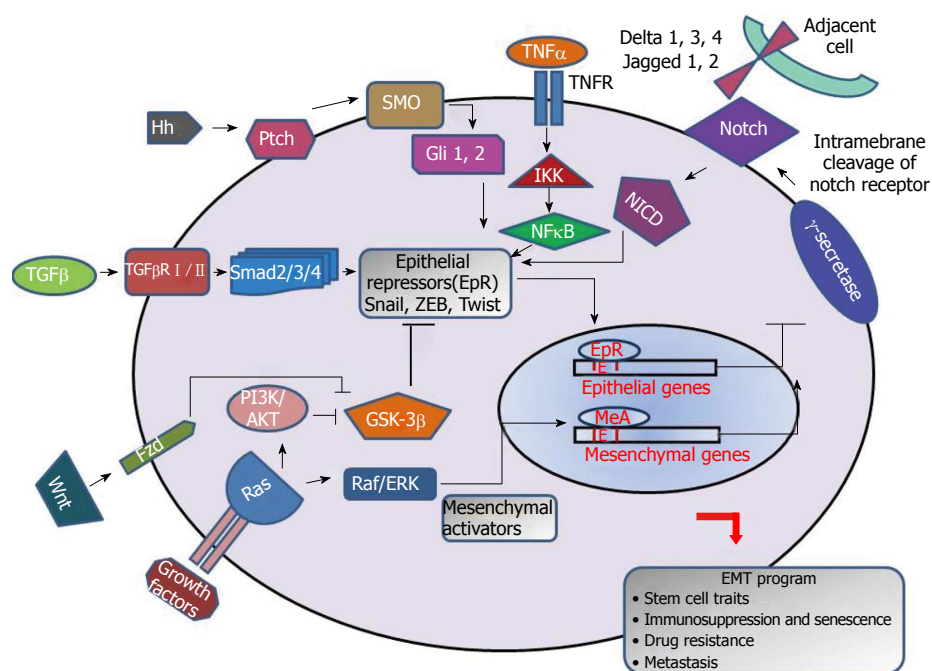


Figure 1 Schematic illustration of embryonic signaling pathways mediating epithelial-to-mesenchymal transition. Wnt, Notch, hedgehog, transforming growth factor β (TGF β) along with other growth factors of cytokines transduce signal cascades, modulate the expression of epithelial-to-mesenchymal transition (EMT) regulators and allow them to translocate to nucleus. They act as epithelial repressors (EpR) and/or mesenchymal activators (MeA) and bind with E box of promoter regions of epithelial genes and mesenchymal genes respectively. These complexes have an inductive effect on EMT program by repressing epithelial genes and activating mesenchymal genes. IKK: I κ B kinase; NF- κ B: Nuclear factor kappa B; SMO: Smoothened; TNF- α : Tumor necrosis factor- α ; PI3K: Phosphatidylinositol 3 kinase; GSK3 β : Glycogen synthase kinase 3 β ; Fzd: Frizzled.

tracellular localization of Snail1 and Snail2 and modulate their transcriptional activities. Signals, including TGF β , Notch, tumor necrosis factor- α (TNF- α), EGF, FGF, Wnt, Shh, SCF/c-kit, hypoxia and estrogens, regulate Snail proteins, not only during development but also in cancer cells^[3].

Twist1 and Twist2

Basic/bHLH domain in Twist1 and Twist2 mediates their binding to DNA and homo/ hetero-dimerization. Twist box at the C terminal end is involved in both transcriptional activation and repression^[13,14]. Binding of Twist factors to other transcriptional regulators, post-translational modifications and choice of partner for dimerization regulate the expression of target genes. Twist1 interaction with components of the NuRD complex, polycomb repressor complexes PRC1 and PRC2 at the E-cadherin promoter is required for E-cadherin repression. In addition, binding of Twist1 to the H4K20 methyltransferase SET8 represses E-cadherin and activates N-cadherin^[15,16].

Homo- or heterodimerization of Twist proteins depends on the availability of E12 where Twist/E12 heterodimers can both activate or repress transcription. Post-translational modification like phosphorylation of the bHLH domain of Twist not only alters dimerization partner choice but also binding affinity for DNA^[13]. Classical EMT-inducing pathways, such as TGF β , Wnt, hypoxia and ligand binding activation of receptor tyrosine kinases and inflammatory cytokines receptors, activate Twist factors and have significant implications in tumor invasion and angiogenesis^[14,17].

Pathways implicated in modulating the EMT/MET program and tumor progression are further reviewed with a special focus on the involvement of transcription factors in the complex network of signaling pathways.

SIGNALING PATHWAYS INDUCING EMT IN CANCER

The Hh, Notch, Wnt and TGF- β signaling pathways interact through cross talk and serve to increase cellular diversity to extracellular stimuli, elicit EMT response by mobilizing embryonic transcription factors, reprogramming the epithelial cell to acquire both progenitor-like, pro-motility and mesenchymal features (Figure 1). Identification and understanding the interlinked cross-talk in cancer cells which provide tumor cells with an additional mechanism to evade chemotherapy may allow designing more effective anti-tumor combinational therapies.

TGF β pathway

TGF β signaling has been implicated in the process of EMT, embryogenesis and cancer pathogenesis. During early stages of tumor growth, TGF β acts as a tumor suppressor and induces growth arrest and apoptosis. In later stages of tumor progression, TGF β and its receptors along with other receptor tyrosine kinases (RTKs) regulate transcription by Smad dependent/independent TGF β receptor signaling pathways, initiate cancer growth and metastasis. T β R I and T β R II, serine-threonine kinase receptors after binding with TGF β ligand, activate Smad2 and Smad3. Activated Smad2/3 forms complexes with Smad4, translocated into the nucleus, and interact with various transcription factors/coactivators to regulate target gene transcription^[18]. Many EMT promoting transcription factors like Snail (Snail1 and Snail2/Slug), Twist (basic helix- loop- helix), six family homeobox and ZEB (ZEB1 and ZEB2/SIP1) are induced by TGF β . These transcription factors interact with Smads and result in the formation of EMT promoting Smad complexes. This is followed by suppression of transcription of E-cadherin, occludin and claudin, and promotion of

tumor growth^[19,20]. TGF β signaling activates Smad independent pathways-phosphatidylinositol 3 kinase (PI3K), Akt, mitogen activated protein kinase and small GTPases of the Rho family, which function along with Smad dependent pathways to modulate the transcription of EMT regulators-Snail, Twist and ZEB. TGF β also collaborate with other signaling pathways: Notch, Wnt/ β catenin, NF κ B, RTKs, PDGF/PDGF receptor autocrine loop to facilitate an EMT like switch, allow efficient cell migration and invasion of metastatic cancer cells^[21-25].

Notch pathway

Four Notch receptors (Notch1-4) bind with the five ligands (Jagged1, 2 and Delta like1, 3, 4), initiate Notch signaling between adjacent cells, followed by intramembrane cleavage of Notch receptor by γ -secretase, release of Notch intracellular domain and translocation to the nucleus to generate a transcription factor complex with transcriptional regulators CSL (RBPJk), mastermind like 1 and histone acetyltransferase p300/CBP. Notch target genes *Myc*, cell cycle regulator p21, hairy/enhancer of split (HES) and the HES related repressor (HERP, HRT and HEY) families are activated but insufficient to induce EMT and hence are coordinated with other signaling pathways. TGF β increases Notch activity through Smad3, upregulate Jagged1 and HEY1 and thereby Slug expression, and suppress E-cadherin. The Wnt1 transformed cells enhance expression of the Delta like1, 3 and 4 ligands, needed for tumorigenic phenotype. Notch controls Snail expression either directly or by indirect mechanisms which operate *via* increased expression of lysyl oxidase (LOX) by recruiting HIF-1 α to LOX promoter. This stabilizes the Snail protein, induces EMT and cancer metastasis^[2].

Wnt/ β catenin pathway

Wnt signaling can be either canonical/ β -catenin or non-canonical. Activation of canonical signaling is initiated by binding of Wnt ligands (Wnt1 and Wnt3a) to seven transmembrane domain receptor Frizzled (Fzd) and the lipoprotein receptor related protein complexes. Activation of Wnt signaling leads to the inhibition of destruction complex which consists of adenomatous polyposis coli, glycogen synthase kinase 3 β (GSK3 β), Axin and casein kinase-1 α . This inhibition of destruction complex stabilizes β -catenin and allows its accumulation/ translocation to the nucleus followed by its interaction with the lymphoid enhancer factor/T cell factor complex leading to targeted gene transcription. Fifty percent of breast carcinomas show β -catenin accumulation within the nucleus or cytoplasm and it is correlated with a poor prognosis. EMT program is activated by induced expression of intracellular protein Axin2 that stabilizes Snail and by blocking the activity of GSK3 β ^[26,27]. In addition to Wnt/ β -catenin canonical pathway, Wnt ligands can activate EGFR signaling through Fzd, whereas EGFR can activate β -catenin, a downstream effector of the Wnt pathway, *via* the RTKPI3K/Akt pathway, and plays a critical

role in cell proliferation and oncogenesis^[28]. Induction of Wnt signaling, EMT and stem cell-like properties, including the CD44^{high}/CD24^{low} signature by silencing or loss of the Wnt antagonist secreted frizzled-related protein 1, has been reported in numerous types of human cancer, including colon, breast, melanoma and prostate carcinomas.

Hedgehog pathway

Hedgehog signaling not only modulates tissue polarity but also maintains stem-cell characteristics. Binding of Hh ligands, like Sonic, Desert and Indian to Patched, a 12-pass transmembrane protein, results in the de-repression of smoothened (SMO) and its translocation to the primary cilium, internalization and activation. The zinc-finger transcription factors GLI-1, -2 and -3 are activated, leading to transcription of GLI target genes^[29]. Hh signaling regulates EMT by inducing the expression of a repressor of E-cadherin, Snail1. Decreased E-cadherin expression and induced expression of mesenchymal cell markers including Snail has been observed during tumor development and progression. SHH-Gli1 signals are reported to promote EMT by mediating a complex signaling network, including TGF β , Ras, Wnt, growth factors, PI3K/AKT, integrins, transmembrane 4 superfamily and S100A4 in pancreatic cancer cells. Colorectal xenografts with high metastatic potential, epithelial morphology and EMT-associated markers are examined to have high GLI-1 expression, while SMO antagonists/inhibitors in pancreatic cancer cell lines block EMT and metastasis^[30,31].

TNF- α and NF- κ B pathway

TNF- α acts as a tumor promoting factor which signals through two distinct cell surface receptors, TNFR1 and TNFR2. TNF receptor associated factor and receptor interacting protein (RIP) are recruited by TNFR1 associated death domain protein, which in turn recruits I κ B kinase (IKK) complex and is activated in a RIP dependent manner. Inhibitory protein I κ B gets phosphorylated by IKK complex and promotes its rapid ubiquitination and proteasome mediated degradation, thus releasing NF- κ B. Upon translocation of free NF- κ B to nucleus, many transcription factors, such as Snail, Slug, Twist, and ZEB1/ZEB2 involved in EMT, are induced and mesenchymal marker vimentin and matrix metalloproteinases (MMPs), such as MMP2 and MMP9, are activated^[32,33]. Recently, a circuitry between RKIP, a metastatic suppressor, NF- κ B and Snail has been identified where overexpression of Snail in tumors inhibits RKIP and induces EMT^[34].

Receptor tyrosine kinase pathway

Growth factors, such as hepatocyte growth factor, epidermal growth factor or FGF, activate extensive cross talk between signaling pathways and play a key role in determining the balance between epithelial and mesenchymal traits in cancer cells. They transduce signals *via* constitutive activation of receptor tyrosine kinases (RTKs) and their downstream signaling effectors, such as MAPK

Table 1 Increased expression of epithelial mesenchymal transition-activating transcription factors inducing various hallmarks of cancer

Hallmarks of cancer	Increased expression of EMT-ATFs	Cancer type	Ref.
Increased migratory potential and invasion	Snail, Slug	TNBCs	[42]
	ZEB1, ZEB2	HNSCCs	[43]
	Snail, Slug	Pancreatic cancer	[44]
	Snail	Ovarian cancer	[45]
	Snail1, Slug	Lung cancer	[46]
	SIP1	Intestinal type gastric cancer	[47]
	Snail	Endometrial cancer	[48]
	Twist1	Melanoma	[49]
	ZEB1	Colon cancer	[50]
	SNAI1 and Twist1	Colorectal adenomas	[51]
Angiogenesis	Snail and ZEB1	Colon cancer	[52]
	Twist1	Breast cancer	[53]
	Snail, Slug, and ZEB1	Breast cancer	[54]
Chemo/radioresistance	Snail, Slug	Breast cancer	[55]
	Twist1	Cervical cancer	[56]
	Snail	Head and neck cancer	[57]
	Snail2	Colorectal cancer	[58]
	Snail	Lung cancer	[59]
	SIP1	Intestinal type gastric cancer	[47]
Resistance to anoikis	Snail	Pancreatic cancer	[60]
	ZEB1, Snail1, Slug	Colorectal cancer	[61,62]

EMT-ATFs: Epithelial mesenchymal transition-activating transcription factors; TNBCs: Triple-negative breast cancers; HNSCCs: Head and neck squamous cell carcinomas; ZEB1: zinc-finger E-box-binding homeobox.

or PI3K, affect the expression of EMT regulators, control cytoskeletal organization and confer epithelial cells with an increased rate of proliferation^[2]. Activation of Ras mediated by growth factors also cooperates with TGF β to induce Snail1 expression. Ras-activated MAPK promotes EMT and metastasis *via* increasing Twist1 serine 68 phosphorylation and stabilization in breast tumor cells. Inhibition of the ERK-MAPK pathway has been reported to restore E-cadherin expression in cells with moderate levels of Ras signaling. Despite having ability to destroy epithelial cell polarity and tight junction assembly, these oncogenic pathways whose signaling involves RTKs fail to induce EMT and mesenchymal migratory phenotype. Interplay of multiple signaling pathways has been reported to sufficiently elicit EMT in various carcinomas.

EMT AND CANCER STEMNESS

Cellular traits associated with the metastatic progression of cancer are believed to be induced by EMT. In recent years, multiple research reports are being added up to prove the significant involvement of interlinked network of signaling pathways *via* induction of EMT-ATFs in EMT control programs. The origin of cancer stem cells is controversial and it is not clear whether different cancer stem cells arise from multipotent tissue stem cells or from reprogramming of differentiated cells that revert to a stem cell-like phenotype. Research advancements suggest that invading carcinoma cells which function as migrating CSCs have undergone an EMT process. The acquisition and maintenance of stemness in non-tumorigenic, immortalized human mammary embryonic cells, along with the capacity to form mammospheres, self-renewal and increasing tumorigenicity in xenotransplants

by overexpressing EMT-ATFs has been observed^[35-37]. Higher expression of EMT-ATFs in colorectal and ovarian cancers correlates with the derepression of stemness gene signature further suggests the origin of CSCs from the dedifferentiation of non-stem cancer cells rather than proliferation of existing CSCs. Experimental evidences in ovarian cancer cells suggest that increased expression of Snail1 and Snail2 mediate chemo and radioresistance by inducing expression of stem-like promoting genes, such as Nanog, kruppel-like factor 4 and T cell factor 4, and by suppressing p53-mediated apoptosis^[38,39]. EMT induction and acquired stem-cell like characteristics in pancreatic tumor cells and breast cancer cells are linked with gemcitabine and tamoxifen resistance respectively. Table 1 sites current studies on the connection between the increased expression of EMT-ATFs and various hallmarks of cancer induced by them in human cancer.

Experimental studies in human cancer cell lines explore the possibility of cancer cells transition between tumorigenic and non-tumorigenic states and this balance can be altered by modulating the EMT-ATFs expression. Since the drug resistance to apoptosis exhibited by cancer cells due to induced EMT is critical for the ability of cells to survive the passage from primary tumors to the sites of dissemination, potential reversal of EMT by silencing EMT master regulators could restore the drug sensitivity. This opens up new therapeutic strategies to reverse metastable EMT by stabilizing the non-invasive epithelial phenotype and restore sensitivity to cytotoxic agents and thereby clinically treat the cancer^[40,41].

CONCLUSION

Reactivation of an embryonic development program re-

ferred to as EMT is the main cause for the metastatic dissemination of cancer cells from the primary tumor. It is a highly conserved morphogenic process, associated with the loss of epithelial cell markers, apicobasal polarity, cell-cell contacts and gain of mesenchymal phenotypes with increased invasive characteristics. Transcription factors regulating the process of EMT belong to the ZEB, Snail and Twist families and are tightly controlled at transcription, translational, protein stabilization and epigenetic levels. Activation of these factors by complex network of dynamic signaling pathways are implicated in the cancer stem cell property, immune suppression, increased resistance to radio/chemotherapeutic drugs and cancer recurrence. Deciphering the mechanistic regulation between EMT, increased metastatic potential, expanding subpopulation of cancer stem cells and chemoresistance may lead to improvements in cancer therapy. Potential implications of various anticancer agents in altering the levels of master regulators of EMT switch, killing cancer stem cells by sensitizing them to radio/chemotherapeutic drugs, as well as increasing response to DNA damage and reprogramming the sensitivity of tumor cells to apoptosis, may provide a novel therapeutic strategy in the treatment of cancers.

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Mobilization of CD34⁺CD38⁻ hematopoietic stem cells after priming in acute myeloid leukemia

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Abstract

AIM: To evaluate quantitatively and qualitatively the different CD34⁺ cell subsets after priming by chemotherapy granulocyte colony-stimulating factor (\pm G-CSF) in patients with acute myeloid leukemia.

METHODS: Peripheral blood and bone marrow samples

were harvested in 8 acute myeloid leukemia patients during and after induction chemotherapy. The CD34/CD38 cell profile was analyzed by multi-parameter flow cytometry. Adhesion profile was made using CXC chemokine receptor 4 (CXCR4) (CD184), VLA-4 (CD49d/CD29) and CD47.

RESULTS: Chemotherapy \pm G-CSF mobilized immature cells (CD34⁺CD38⁻ population), while the more mature cells (CD34⁺CD38^{low} and CD34⁺CD38⁺ populations) decreased progressively after treatment. Circulating CD34⁺ cells tended to be more sensitive to chemotherapy after priming with G-CSF. CD34⁺ cell mobilization was correlated with a gradual increase in CXCR4 and CD47 expression, suggesting a role in cell protection and the capacity of homing back to the marrow.

CONCLUSION: Chemotherapy \pm G-CSF mobilizes into the circulation CD34⁺ bone marrow cells, of which, the immature CD34⁺CD38⁻ cell population. Further manipulations of these interactions may be a means with which to control the trafficking of leukemia stem cells to improve patients' outcomes.

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Key words: Acute myeloid leukemia; Leukemia stem cell; Immunophenotype; Priming; Timed sequential chemotherapy

Core tip: Timed sequential chemotherapy and priming with hematopoietic growth factors have been recently used in the treatment of acute myeloid leukemia in order to mobilize more leukemic cells in the cell cycle and therefore improve the cytotoxic effect of chemotherapy. In this paper, we looked the impact of this type of treatment in a small series of patients on the mobilization of different subsets of CD34⁺ cells involving "bulk" leukemic cells and more "immature" leukemic cells.

Plesa A, Chelghoum Y, Mattei E, Labussière H, Elhamri M, Cannas G, Morisset S, Tagoug I, Michallet M, Dumontet C, Thomas X. Mobilization of CD34⁺CD38⁻ hematopoietic stem cells after priming in acute myeloid leukemia. *World J Stem Cells* 2013; 5(4): 196-204 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v5/i4/196.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v5.i4.196>

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder that originates from leukemia stem cells (LSCs) with the ability to generate an excessive amount of malignant myeloid blasts. Despite therapeutic advances in younger adults, relapses remain a major issue^[1]. Chemotherapeutic regimens markedly reduce tumor burden, but only target the “bulk”, non-clonogenic cells and spare LSCs, which allow for recrudescence of leukemia^[2]. Interactions of the leukemic cells with the bone marrow (BM) microenvironment via specific receptor and adhesion molecules, such as the CXCR4-stromal cell-derived factor-1 (SDF-1) axis, are in part responsible for chemotherapy resistance. AMLs with high CXCR4 cell surface expression and therefore a high tendency of stromal protection have been shown to have a poor prognosis^[3,4].

The cell cycle is a critical regulator of the processes of cell proliferation and growth. One strategy to increase the cytotoxicity of cycle-dependent antileukemic agents is to enter more leukemic cells into the cell cycle. Timed sequential chemotherapy (TSC) is based on the findings that the initial cytoreductive drug induces the remaining malignant cell cohort to enter a proliferative state at a predictable time following drug administration^[5]. Combination with hematopoietic growth factors (HGFs) has also been developed to enhance the efficacy of cytotoxic agents^[6,7]. Granulocyte colony-stimulating factor (G-CSF) stimulates very immature progenitors and cleaves SDF-1^[8], inducing stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating its receptor CXCR4^[9]. Such an approach could theoretically target the self-renewal machinery of LSCs by inducing the quiescent LSCs into the cycle and circulation. However, a better understanding of those mechanisms is warranted.

Basically, low numbers of immature hematopoietic cells are released into the peripheral blood (PB). Treatments, including HGFs and/or TSC DNA-damaging agents, known as priming, can cause a marked increase in hematopoietic stem cell mobilization^[10]. The aim of the present study was to investigate BM and PB leukemic blasts in the CD34 versus CD38 bidimensional space in order to evaluate quantitatively and qualitatively the different CD34⁺ cell subsets during and after priming by TSC ± G-CSF as induction chemotherapy in adult AML patients.

MATERIALS AND METHODS

Patients and treatments

PB and BM samples were procured from a total of 8 AML patients (Table 1). All studied patients were treated according to the Acute Leukemia French Association (ALFA)-0702 trial^[11]. Diagnosis was morphologically proven according to the French-American-British classification^[12]. The study protocol was approved by the Human Ethics Committee of our institution and was conducted in accordance with the Declaration of Helsinki. All patients gave written informed consent prior to registration on the study. This trial was registered at www.clinicaltrials.gov as No. NCT00932412. All patients received TSC induction and 5 of them were also primed by G-CSF (filgrastim). Morphological complete remission (CR)^[13] and risk classification, based on cytogenetics and molecular marker analyses, were defined as previously described^[7,14].

AML cells and flow cytometry analyses

PB and BM samples were harvested in all patients at different times: at diagnosis (T0), at the end of the first sequence of chemotherapy (4 d) (T4), at the beginning of the second sequence of chemotherapy (8 d) (T8), at the end of chemotherapy (10 d) (T10), during aplasia (15 d) (T15), and at the time of cell recovery (between 28 and 35 d) (TR).

Surface and intracellular antigen detection were performed by multi-parameter flow cytometry (MFC). Briefly, the CD34/CD38 cell profile was analyzed in one single tube by a multi-parameter combination using CD7, CD13, CD33, CD34, CD38, CD45 and CD19. Analyses were performed, using FACS Diva software (BD Bioscience). Instrument set-up was routinely optimized by analyzing Calibrite beads- Rainbows 8 picks beads and CST beads system. Adhesion profile was made using CXCR4 (CD184), VLA-4 (CD49d/CD29) and CD47. Cells were incubated with the appropriate combination of MoAbs (1×10^6 total cells per tube), washed and then analyzed by flow cytometry. The required minimal number of CD34⁺ events was set at 20 and the total number of events ranged from 100000 to 500000. Immunoglobulin G (IgG) isotype staining was used as a negative control for both CD38 and adhesion markers expression for ratio median fluorescence intensity (rMFI) evaluation. Discriminating the apoptotic/necrotic status of each subpopulation was made possible by using fluorescein isothiocyanate (FITC)-labelled annexin V and propidium iodide (PI), as previously described^[15].

The gating strategy was based on CD45^{low}/SSC total blast and CD34⁺CD45^{low} cell populations gated from total FSC/SSC viable cells. Three populations of CD34⁺ cells were distinguished based on differential expression of the CD34 and CD38 antigens^[16]: A first cell population, which expressed a great amount of the CD34 antigen, lack of CD38 (CD34⁺CD38⁻) and often con-

Table 1 Characteristics at diagnosis and outcome of the 8 acute myeloid leukemia patients

Pts	Age (yr)	Sex	Cytogenetics	CD34 ⁺ (%) ¹	FAB	Molecular Biology ²	WBC (x 10 ⁹ /l) ²	Risk-group	G-CSF priming	BM 15 d	CR	Consolidation and DFS
1	59	M	Normal	85	M5	Flt3-ITD neg CEBPA pos	55.1	Favorable intermediate	Yes	No blast	Yes	Chemotherapy 15 ⁺ mo
2	35	M	Normal	85	M2	Flt3-ITD neg CEBPA pos	4.8	Favorable intermediate	Yes	No blast	Yes	Chemotherapy 16 ⁺ mo
3	56	F	+8, del (20) (q11; q13)	93	M1	Flt3-ITD pos	12.6	Poor intermediate	Yes	ND	Yes	Chemotherapy 3 mo
4	19	M	Normal	67	M6	Flt3-ITD pos MLL pos	0.9	Unfavorable	Yes	ND	Yes	AlloSCT 6 mo
5	55	M	Normal	8	M1	Flt3-ITD pos NPM1 pos	147.3	Poor intermediate	Yes	ND	Yes	AlloSCT 14 ⁺ mo
6	55	F	Normal	3	M5	Flt3-ITD neg Evi-1 pos	3.7	Unfavorable	No	5% blasts	Yes	AlloSCT 14 ⁺ mo
7	44	M	Complex	94	M0	Flt3-ITD neg NPM1 neg	1.4	Unfavorable	No	> 5% blasts	No ³	AlloSCT 11 mo
8	45	F	-7, +21	83	M4	Flt3-ITD neg NPM1 neg	2.4	Unfavorable	No	No blast	Yes	AlloSCT11 ⁺ mo

¹Expression of CD34 by leukemic cells; ²Molecular biology and WBC count at diagnosis; ³Morphological complete remission was subsequently achieved by salvage therapy combining idarubicin with high-dose cytarabine. Patients prognosis was defined according to the following classification based on cytogenetics and molecular marker analyses. Karyotype abnormalities that involved core binding factor (CBF) leukemias [*t* (16; 16) (p13; q22), inv (16) (p13; q22), or *t* (8; 21) (q22; q22)] with or without other cytogenetic abnormalities were considered favorable cytogenetics. Monosomies or deletions of chromosomes 5 and 7; abnormalities of the long arm of chromosome 3 (or Evi-1 gene mutation); 11q23 abnormality (or MLL gene mutation); or complex cytogenetic abnormalities (defined as at least five unrelated cytogenetic clones) were considered unfavorable risk factors. Other cytogenetic abnormalities and cytogenetically normal (CN)-AML were designated intermediate risk factors. Intermediate-risk cytogenetics was further subdivided into a favorable intermediate risk group [CN-AML with nucleophosmin (*NPM1*) or CCAAT/enhancer-binding protein-*(CEBPA)* mutations and no *FLT3-ITD*] and a poor intermediate risk group (other patients). Induction chemotherapy consisting of a TSC including a first sequence combining daunorubicin, 60 mg/m² per day IV on 1–3 d, and cytarabine, 500 mg/m² per day IV over the same period. The second sequence, administered after 4 d free interval, consisted of daunorubicin, 35 mg/m² per day, IV on 8 d and 9 d, and cytarabine, 1000 mg/m² per 12 h on 8–10 d. Five patients received G-CSF (filgrastim) priming (5 µg/Kg per day) on 1 to 10 d. Three patients were not primed with G-CSF. AlloSCT: Allogeneic stem cell transplantation; BM: Bone marrow; CR: Morphological complete remission; DFS: Disease-free survival; F: Female; FAB: French-American-British morphological classification; M: Male; neg: Negative; pos: Positive; Pts: Patients; WBC: White blood cell; G-CSF: Granulocyte Colony-Stimulating Factor.

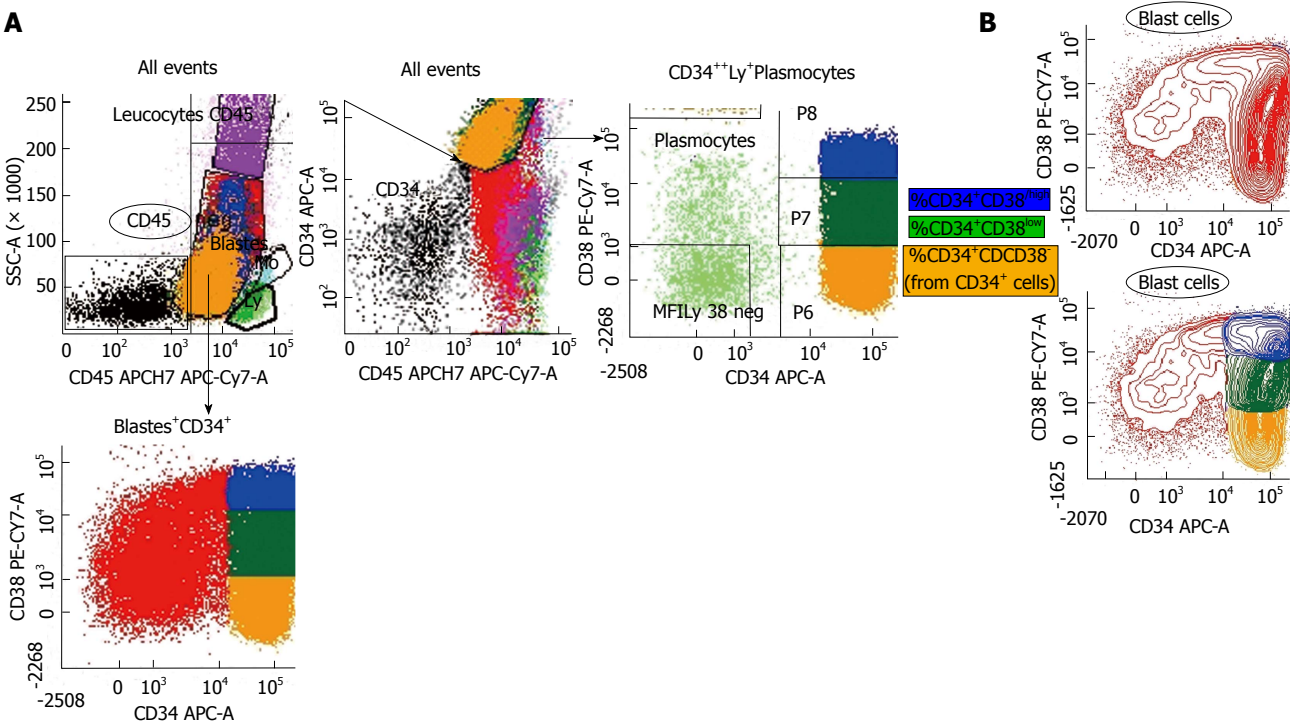


Figure 1 Multicolor flow cytometry (patient # 3): (left) Strategy used for gating the blast cell population and CD34⁺CD38⁻ cell subpopulations (red: immature CD45^{low} SSC blast cells, blue: CD34⁺ cells, green: lymphocytes, blue cyan: monocytes, violet: granulocytes, black: CD45⁺ cells (erythroblasts); CD34⁺ cells were separated into different stem cell fractions based on their CD38 antigen expression: A first cell population expressing a great amount of the CD34 antigen and lacked of CD38 (CD34⁺CD38⁻), a second cell population characterized by a great amount of the CD34 antigen and by a low density of CD38 antigen (CD34⁺CD38^{low}), and a third cell population characterized by a large density of CD38 antigen and of CD34 antigen (CD34⁺CD38⁺); (right) The two density plots indicate CD45^{low}/SSC blast cells (in red) and CD34⁺CD38⁻ subpopulations (CD34⁺CD38^{low} in green and CD34⁺CD38⁺ in blue).

tained very few events requiring to be tightly clustered in a FSC/SSC and CD45/SSC plot; a second population characterized by a great amount of the CD34 antigen and by a low density of CD38 antigen (CD34⁺CD38^{low});

and a third population characterized by a large density of CD38 and CD34 antigens (CD34⁺CD38⁺). Figures 1 and 2 are typical examples of an analysis. CD38 was expressed as percent positively stained cells within CD34⁺

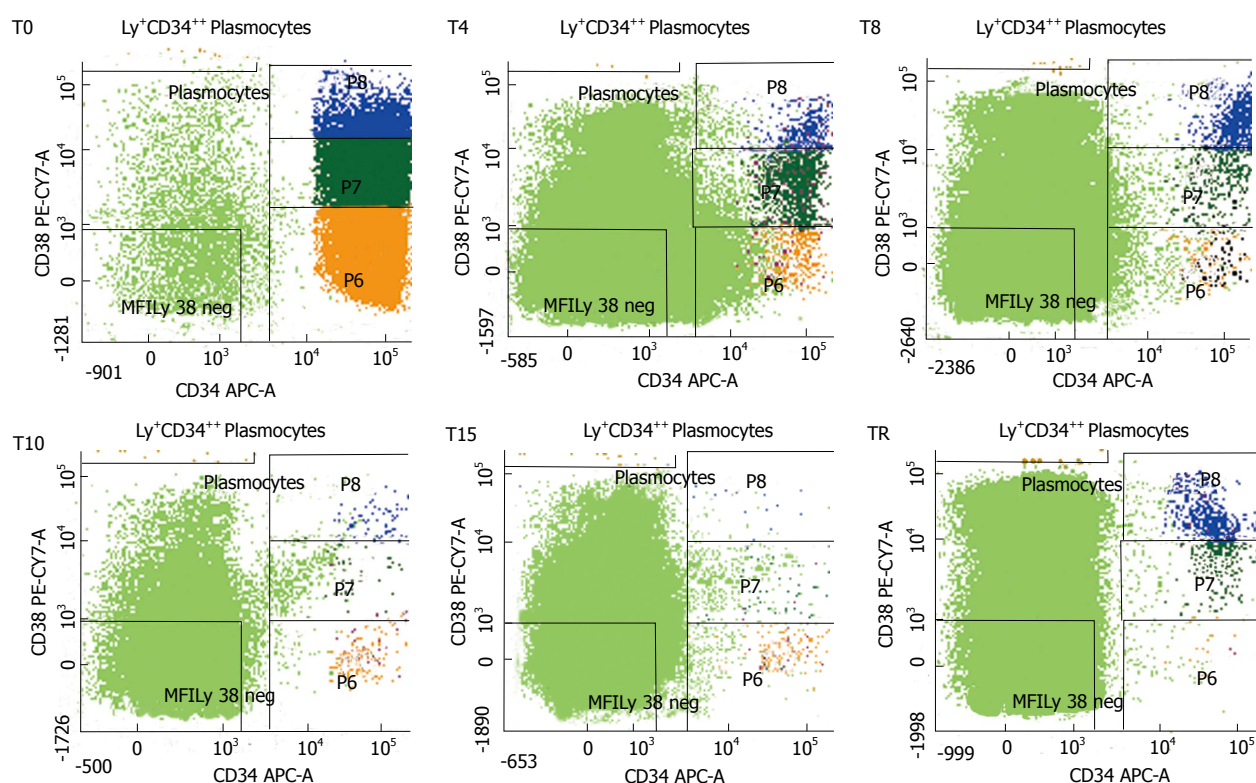


Figure 2 Characterization of the different stem cell fractions. A typical example of analysis (patient 3) performed at different times: at diagnosis (T0), at the end of the first sequence of chemotherapy (4 d) (T4), at the beginning of the second sequence of chemotherapy (8 d) (T8), at the end of chemotherapy (10 d) (T10), during aplasia (15 d) (T15), and at the time of cell recovery (TR). CD34⁺ cells were separated into different stem cell fractions based on their CD38 antigen expression: A first cell population expressing a great amount of the CD34 antigen and lack of CD38 (CD34⁺CD38⁻); a second cell population characterized by a great amount of the CD34 antigen and by a low density of CD38 antigen (CD34⁺CD38^{low}); and a third cell population characterized by a large density of CD38 antigen and of CD34 antigen (CD34⁺CD38⁺).

populations as well as intensity of fluorescence signal quantified as rMFI_{CD38} from CD34⁺ gated cells and from CD45^{low}/SSC total blast cells. The adhesion markers CXCR4 and VLA4 were also expressed as rMFI. Regarding CD47 expression, a normalized rMFI was used and defined as followed: (median expression of CD47 in blasts-median expression of IgG1 in blasts)/(median expression of CD47 in lymphocytes-median expression of IgG1 in lymphocytes).

Statistical analysis

Descriptive statistical values included median, mean \pm SD and range. For most of the analyses, BM cell samples from the 8 patients were pooled as well as all PB cell samples from the same 8 patients. Statistical significance was assessed using the ANOVA test, assuming equal variance for comparison of 2 groups. Correlations between the expressions of cell surface antigens were given by the linear Pearson test.

RESULTS

Expression of CD34 during and after chemotherapy

At T0, the average proportion of CD34⁺ cells in BM was 32.06% \pm 28.96% (median, 21.47%) and 15.33% \pm 23.89% (2.84%) in PB. At T0, most CD34⁺ cells cor-

responded to the “bulk” leukemia cell population. The CD34⁺ cell population decreased progressively in BM (4.6% \pm 9.54%; 0.31% at T15) corresponding to the decrease in leukemic blasts after chemotherapy, and increased again lightly at the time of cell recovery (1.25% \pm 0.84; 1.22%), corresponding mainly to the emergence of normal progenitor cells. In PB, a nadir of CD34⁺ cells was observed at T15 (0.51% \pm 0.87%; 0.15%), while the percentage of CD34⁺ cells remained low at TR (0.10% \pm 0.07%; 0.16%), confirming undetectable levels of immature cells in PB.

Expression of CD38 antigen on CD34⁺ cells

Three populations of CD34⁺ cells were distinguished on differential expression of the CD38 antigen. The CD34⁺CD38⁻ population appeared in a restricted light-scattering region. When considering CD34 and CD45 expressions, these cells projected initially mainly in the CD34⁺CD45^{low/+}, and then appeared in the CD34⁺CD45^{-/low} area after cell mobilization. The CD34⁺CD38^{low} cells were mainly found in the CD34⁺CD45^{low/+} area, but also involved the CD34⁺CD45^{-/low} area. With respect to light-scattering properties, the cell population indicated by CD34⁺CD38⁺ was more heterogeneous but projected almost exclusively in the CD34⁺CD45^{low/+} area. At T0, the smallest popula-

Table 2 Circulating CD34⁺ cell subsets before, during and after intensive chemotherapy

Time	CD34 ⁺ vs CD38		
	CD34 ⁺ CD38 ⁻	CD34 ⁺ CD38 ^{low}	CD34 ⁺ CD38 ⁺
At diagnosis (T0)			
Median	7.32%	35.45%	56.74%
Mean ± SD	15.40% ± 19.21%	32.35% ± 20.41%	53.81% ± 35.37%
At 4 d (T4)			
Median	12.82%	39.15%	37.30%
Mean ± SD	21.17% ± 22.68%	34.01% ± 23.08%	45.11% ± 37.48%
At 8 d (T8)			
Median	15.99%	26.21%	36.81%
Mean ± SD	30.14% ± 29.51%	30.28% ± 20.87%	39.06% ± 32.98%
At 10 d (T10)			
Median	41.35%	22.99%	18.90%
Mean ± SD	48.18% ± 26.79%	27.86% ± 18.65%	24.29% ± 22.73%
At 15 d (T15)			
Median	73.56%	26.18%	3.93%
Mean ± SD	66.42% ± 20.18%	27.33% ± 9.26%	8.05% ± 12.24%
At recovery (TR)			
Median	5.37%	32.76%	63.42%
Mean ± SD	10.63% ± 13.81%	29.53% ± 11.23%	61.79% ± 18.39%

TR: Time of cell recovery.

tion (CD34⁺CD38⁻) lacked the CD38 antigen, while the larger population (CD34⁺CD38⁺) expressed the greatest amount of the CD38 antigen. The average proportions of the CD34⁺CD38⁻, CD34⁺CD38^{low} and CD34⁺CD38⁺ populations in BM and PB were 3.48% ± 5.66%, 12.62% ± 7.31%, 84.65% ± 11.91%, 15.40% ± 19.21%, 32.35% ± 20.41% and 53.81% ± 35.37%, respectively. Chemotherapy ± priming with G-CSF mobilized immature cells (CD34⁺CD38⁻ population), while the more mature cells expressing CD38 (CD34⁺CD38^{low} and CD34⁺CD38⁺ populations) decreased progressively after treatment. At T15, the average proportions of the CD34⁺CD38⁻, CD34⁺CD38^{low} and CD34⁺CD38⁺ cell populations in PB were 66.42% ± 20.18%, 27.33% ± 9.26%, and 8.05% ± 12.24%, respectively. In BM, the CD34⁺CD38⁻ and CD34⁺CD38^{low} populations were increased at T15, while the CD34⁺CD38⁺ population was decreased: 22.02% ± 17.11%, 48.96% ± 16.20%, and 29.91% ± 26.69%, respectively. Proportions of CD34CD38 cell fractions returned to baseline at TR (Table 2).

Effect of priming with G-CSF on CD34⁺ cell populations

CD34⁺ cells in PB tended to be more sensitive to chemotherapy after priming with G-CSF (mean ± SD: 0.21% ± 0.18%; median: 0.17% at T10) than without G-CSF (5.17% ± 8.47%; 0.31%). The evolution of the three subsets of CD34⁺ cell populations over time were confirmed when considering the absolute number of cells. The CD34⁺CD38^{+/low} cell population, containing the leukemia bulky cell population, followed the evolution of white blood cell count with a massive decrease during and after chemotherapy corresponding to cell lysis and a slight increase at the time of evaluation corresponding to cell recovery. At T15, the absolute number of the more mature cell population (CD34⁺CD38⁺) tended to be lower after priming with G-CSF ($5.1 \times 10^6/l \pm 4.1$) than without

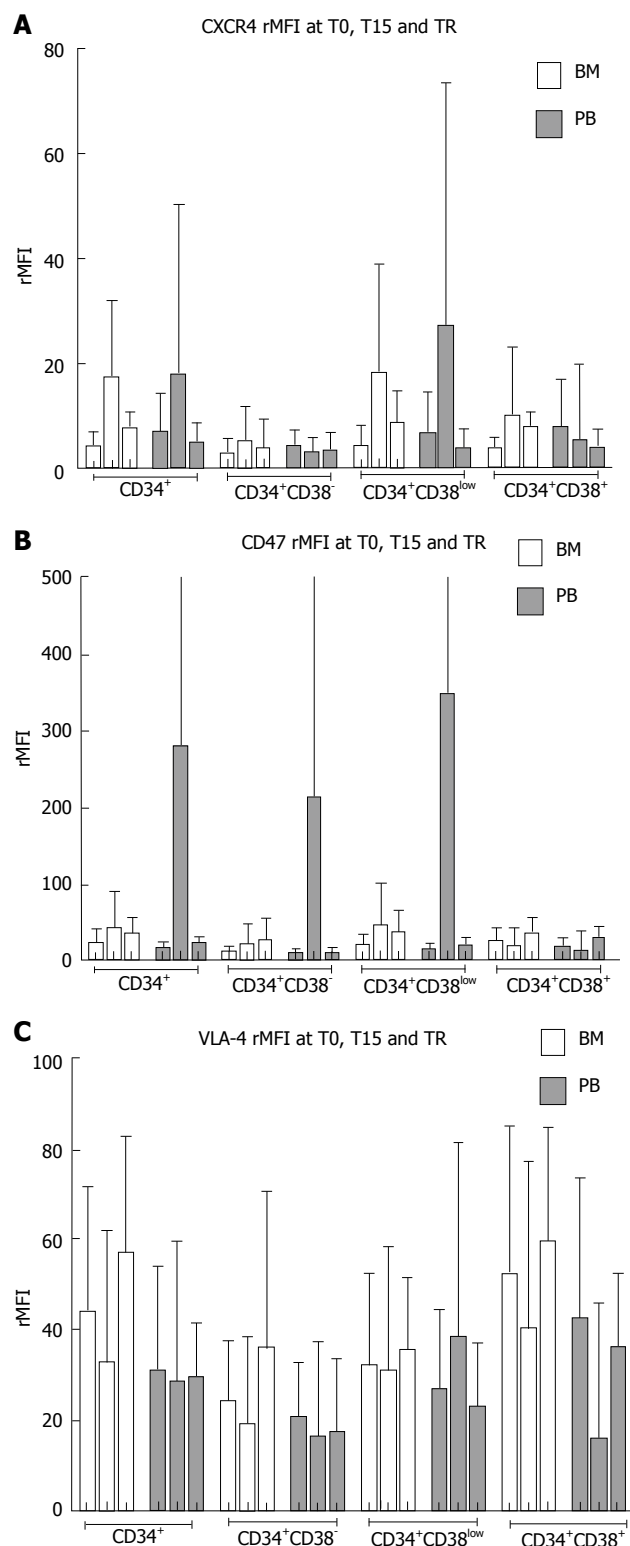


Figure 3 Evolution of CXCR4, CD47, and VLA-4 ratio median fluorescence intensity on CD34⁺ cells and CD34⁺CD38 cell sub-populations in bone marrow and peripheral blood. A: CXCR4, B: CD47, C: VLA-4. PB: Peripheral blood; BM: Bone marrow; rMFI: Ratio median fluorescence intensity.

G-CSF ($19.3 \times 10^6/l \pm 15.2$), suggesting a lytic impact of priming on leukemic cells. Reversely, the most immature subset (CD34⁺CD38⁻) increased progressively during and after chemotherapy corresponding to mobilization

Table 3 Mean percentage of viable, apoptotic and necrotic cells in peripheral blood and bone marrow samples at T0, T15 and time of cell recovery

Cell population	T0 (BM)	T0 (PB)	T15 (BM)	T15 (PB)	TR (BM)	TR (PB)
Immature cells						
Viable cells	75.5 ± 17.6	52.0 ± 21.2	18.5 ± 3.5	14.5 ± 12.0	37.0 ± 16.9	34.0 ± 14.1
Apoptotic cells	17.5 ± 17.6	19.5 ± 21.9	33.0 ± 7.0	27.5 ± 23.3	34.5 ± 13.4	24.5 ± 7.7
Necrotic cells	3.0 ± 1.4	28.0 ± 0.0	45.0 ± 11.3	54.0 ± 38.1	25.0 ± 2.8	38.5 ± 24.7
CD34 ⁺ cells						
Viable cells	77.5 ± 20.5	59.5 ± 33.2	34.0 ± 19.7	24.5 ± 3.5	65.0 ± 25.4	29.0 ± 19.7
Apoptotic cells	20.5 ± 20.5	34.5 ± 28.9	31.0 ± 29.6	38.0 ± 15.5	32.0 ± 22.6	44.0 ± 1.4
Necrotic cells	2.5 ± 0.7	5.0 ± 2.8	35.0 ± 8.4	34.0 ± 21.2	3.0 ± 2.8	24.0 ± 19.7
CD34 ⁺ CD38 ⁻ population						
Viable cells	68.0 ± 19.7	59.5 ± 34.6	52.5 ± 24.7	21.5 ± 10.6	51	32.5 ± 30.4
Apoptotic cells	27.5 ± 24.7	38.0 ± 33.9	33.5 ± 23.3	60.0 ± 2.8	47	52.5 ± 16.2
Necrotic cells	0.5 ± 0.7	2.0 ± 1.4	13.5 ± 2.1	18.0 ± 14.1	1	22.5 ± 0.7
CD34 ⁺ CD38 ^{low} population						
Viable cells	72.5 ± 26.1	48.0 ± 43.8	15.5 ± 6.3	15.0 ± 7.0	33.5 ± 2.1	30
Apoptotic cells	25.5 ± 26.1	45.0 ± 38.1	34.5 ± 28.9	38.5 ± 20.5	54.0 ± 5.6	49
Necrotic cells	2.0 ± 1.4	7.0 ± 5.6	49.5 ± 33.2	46.5 ± 27.5	12.5 ± 3.5	18
CD34 ⁺ CD38 ⁺ population						
Viable cells	78.0 ± 19.7	70.5 ± 30.4	21.5 ± 7.7	27.5 ± 6.3	67.0 ± 25.4	44.5 ± 36.0
Apoptotic cells	19.0 ± 19.7	23.5 ± 27.5	26.0 ± 26.8	20.5 ± 20.5	31.0 ± 24.0	30.5 ± 0.7
Necrotic cells	2.0 ± 0.0	5.0 ± 2.8	53.0 ± 18.3	42.0 ± 43.8	1.5 ± 0.7	28.0 ± 39.5

BM: Bone marrow; PB: Peripheral blood; TR: Time of cell recovery.

with the highest absolute count for the CD34⁺CD38⁻ cell subset in PB at T15 ($179.4 \times 10^6/l \pm 79.7$) and decreased thereafter suggesting partial lysis, migration to BM and/or differentiation through the CD34⁺CD38⁺ cell pool. When considering the CD34⁺CD38⁻ population, the increase in cell percentage between T0 and T15 was 24 fold higher after priming with G-CSF than without priming with G-CSF, suggesting a higher level of cell mobilization.

Expression of CXCR4 and adhesion molecules on CD34⁺ cell subsets

Figure 3 summarizes the evolution of CXCR4 (Figure 3A), CD47 (Figure 3B) and VLA-4 (Figure 3C) rMFI on CD34⁺ cells and on CD34⁺CD38⁻ cell sub-populations in BM and PB during the induction course. CXCR4 and CD47 rMFI on CD34⁺ BM and PB cells increased with chemotherapy \pm priming by G-CSF, while VLA-4 remained stable. The evolution of CXCR4 and CD47 expression was correlated in both BM ($r = 0.64$) and PB ($r = 0.55$). From T0 to T15, CD47 expression increased 10 fold more on PB CD34⁺ cells than on BM cells with a mean expression of $280.2 \text{ vs } 43.6$, while increased expression of CXCR4 was similar. When considering CD34⁺ cell subsets according to CD38 expression, CD47 significantly increased at T15 mainly in PB for the CD34⁺CD38⁻ and CD34⁺CD38^{low} populations, while mean rMFI did not change for the CD34⁺CD38⁺ population. CXCR4 significantly increased at T15 in both PB and BM mainly for the CD34⁺CD38^{low} cell population, while mean rMFI did not change for the CD34⁺CD38⁻ and CD34⁺CD38⁺ populations.

Identification of apoptotic and necrotic cells

The fluorescence parameters allowed characterization of necrotic (PI⁺ annexin V-FITC⁺ cells), apoptotic

(PI⁻ annexin V-FITC⁺ cells) and viable cells (PI⁻ annexin V-FITC⁻ cells) (Table 3). The evolution over time of mean proportions of apoptotic and necrotic cells was similar in BM and PB for all cell subsets: a decrease of viable cells was noted after chemotherapy, while a massive increase in apoptotic and necrotic cell populations was observed (Figure 4). However, the percentage of cells with apoptotic/necrotic status tended to be higher in PB. At T15, the percentage of necrotic cells in both PB and BM was higher in the more mature cell populations (CD34⁺CD38^{low} and CD34⁺CD38⁺) than in the immature CD34⁺CD38⁻ population. However, survival tended to be promoted by G-CSF, as indicated by a decrease of annexin V-FITC⁺ cells in patients primed with G-CSF. The percentage of apoptotic cells did not differ among patients primed with G-CSF and those not primed: $51\% \pm 21\% \text{ vs } 48\% \pm 21\%$ in the whole CD34⁺ cell population. Although not statistically significant, the percentage of necrotic cells tended to be lower in patients primed with G-CSF: $12\% \pm 6\% \text{ vs } 27\% \pm 21\%$ in the whole CD34⁺ cell population ($P = 0.2$).

DISCUSSION

The pursuit of the best chemotherapy regimen for AML continues in an attempt to improve CR proportions and long-term survival. The LSC model has implications for the development of new therapeutic strategies. LSCs respond to depletion of the leukemia cell mass that occurs when anti-proliferative drugs are administered. It is therefore suggested that one way to eliminate dormant LSCs is to find the window in which they cycle and then kill them^[17]. Priming may modulate cell cycle kinetics of AML blasts and render them more susceptible to phase-specific agents. This has been shown *in vitro*^[18] and *in vivo*

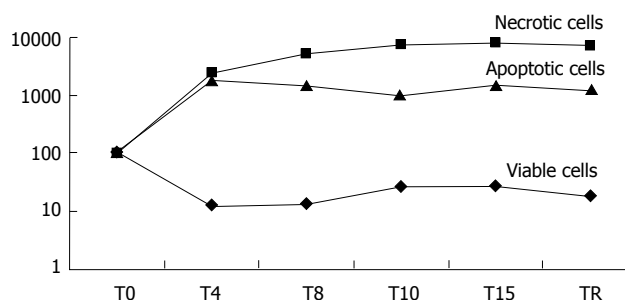


Figure 4 Kinetics of viable, apoptotic and necrotic cells in peripheral blood according to the different times of study (patient 8). Values were given comparatively to those observed at the time of diagnosis, assuming that the observed value at T0 corresponded to 100%.

in leukemic mice^[19], and more recently in clinical trials for younger adults with AML^[6,7].

Our study showed that CD34⁺ cells were significantly affected by chemotherapy in both BM and PB. However, treatment mainly impacted on the more mature cell populations, as demonstrated by the decrease of the CD34⁺CD38^{+/low} cell population corresponding to “bulk” leukemic blasts. LSCs represent only a small fraction of malignant cells^[20] and are believed to be restricted to the Lin⁻CD34⁺CD38⁻ fraction^[21,22], which was insignificant at T0 in most of our patients. However, this cell subset was able to proliferate and to egress from BM to PB after chemotherapy \pm G-CSF, and partly differentiates through the more mature CD34⁺CD38^{+/low} or CD34⁺CD38⁺ cell phenotypes, before returning to its initial value after leukocyte recovery. However, LSCs can arise from the malignant transformation of a normal stem cell that has accumulated oncogenic insults over time, or from a more differentiated cell that develops the capability for continual self-renewal^[23]. The phenotype of LSCs is therefore heterogeneous and can vary even within a single sample. Some AML have LSCs exclusively in the CD34⁻ fraction^[24]. Similarly, CD34⁺CD38⁺ fraction of certain AML samples contains all or at least most LSCs^[25]. Furthermore, CD38 is reversibly expressed on CD34⁺ repopulating cells between negative and low levels^[26]. Characteristics that are relevant to therapy may then differ based on the origin of the malignant cell with sensitive AMLs derived from more differentiated stem cells and resistant AMLs derived from earlier stages.

Resistance may in part be provoked by cell adherence to the stromal environment. Inhibition of the CXCR4-SDF-1 axis induces mobilization of cells into circulation and enhances anti-leukemic effects of chemotherapy^[27]. CXCR4 plays a dominant role in cell-trafficking, as confirmed here by up-regulation after CD34⁺ cells egress to the circulation. This was observed for all PB CD34⁺ cells. However, the more mature CD34⁺ cells mobilized earlier than immature CD34⁺ cells, suggesting different degrees of sensitivity. CXCR4 expression favors the enrichment of a non-cycling population of AML cells, which represent dormant leukemia progenitors serving as a reservoir for minimal residual disease. G-CSF results in a decreased

expression of SDF-1 in the BM, resulting in premature release of immature cells^[9]. This was in accordance with our results showing an increased expression of CXCR4 on immature CD34⁺ cells after reaching the PB circulation. The peak number of labeled cells was shown after 72 h preventing an early homing back to the BM^[28]. CXCR4 may contribute to immature cell clearance from the blood observed after T15 by directing cells again to the BM.

Many other proteins, including the VLA-4 integrins, are regarded as essential for AML cell adhesion to stromal cells and their protection from drug-induced apoptosis^[29], but only slight variations in their expression level were noted during and after therapy, suggesting a minor involvement in immature CD34⁺ cell trafficking.

Mobilizing treatments cause CD47 to be transiently up-regulated on progenitors just prior to and during their migratory phase^[30]. This was confirmed by our study, showing an increased expression of CD47 most particularly in the circulating CD34⁺CD38⁻ cell subgroup after chemotherapy \pm G-CSF. Overexpression of CD47 on AML cells is known to increase their pathogenicity by allowing them to evade macrophage phagocytosis^[31,32]. This could explain the strong correlation found between the evolution of CD47 expression and that of CXCR4 on immature CD34⁺ cells.

Circulating CD34⁺ cells tended to be more sensitive to chemotherapy after priming with G-CSF. However, the impact of priming mainly concerned the more mature CD34⁺ cells containing “bulk” leukemic cells, while it was limited on immature CD34⁺ cells susceptible to contain leukemia-initiating cells. All CD34⁺ annexin V⁺ cell populations were involved in the apoptotic/necrotic process, which concerned a larger proportion of PB cells than BM cells. Cell survival seemed to be promoted by G-CSF, as indicated by a decrease in annexin V⁺ cells. At T15, the necrotic process involved preferentially the more mature cell subsets (CD34⁺CD38^{low} and CD34⁺CD38⁺ populations), suggesting a higher protection of CD34⁺CD38⁻ cells from the action of cytotoxic drugs.

Despite disappointing results regarding priming of the most immature CD34⁺ cells, our study represents a first step towards further explorations. Mobilization of LSCs is a concept that is presently being revisited by novel targeted therapies. Inhibition of CXCR4, based on the ability of the CXCR4 antagonist plerixafor to push LSCs out of their BM niches, induces the rapid mobilization of stem cells from BM to PB^[33], and has been shown to sensitize leukemic blasts to chemotherapy^[27,34-36]. A mobilization of up to 80% leukemic cells, including the more primitive CD34⁺CD38⁻ cell subset, was observed when HGF was followed by anti-CXCR4^[37]. Accompanied by chemotherapy, this might lead to a better eradication of LSCs. From that perspective, plerixafor is currently being tested by French centers for the mobilization of dormant LSCs. Consequently, new paradigms must be devised for evaluating the therapeutic agents. For instance, clinical trial design can use intermediate end points such as time

to progression following the administration of an agent that can target LSCs.

COMMENTS

Background

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder that originates from leukemia stem cells (LSCs) with the ability to generate an excessive amount of malignant myeloid blasts. Interactions of the leukemic cells with the bone marrow (BM) microenvironment via specific receptor and adhesion molecules, such as the CXCR4 chemokine receptor 4 (CXCR4)-stromal cell-derived factor-1 (SDF-1) axis, are in part responsible for chemotherapy resistance. AMLs with high CXCR4 cell surface expression and therefore a high tendency of stromal protection have been shown to have a poor prognosis.

Research frontiers

The cell cycle is a critical regulator of the processes of cell proliferation and growth. One strategy to increase the cytotoxicity of cycle-dependent antileukemic agents is to enter more leukemic cells into cell cycle. Timed sequential chemotherapy is based on the findings that the initial cytoreductive drug induces the remaining malignant cell cohort to enter a proliferative state at a predictable time following drug administration. Combination with hematopoietic growth factors (HGFs) has also been developed to enhance the efficacy of cytotoxic agents.

Innovations and breakthroughs

The study represents a first step towards further explorations. Mobilization of LSCs is a concept that is presently being revisited by novel targeted therapies. Inhibition of CXCR4, based on the ability of the CXCR4 antagonist plerixafor to push LSCs out of their BM niches, induces the rapid mobilization of stem cells from BM to PB and has been shown to sensitize leukemic blasts to chemotherapy. A mobilization of up to 80% leukemic cells, including the more primitive CD34⁺CD38⁻ cell subset, was observed when HGF was followed by anti-CXCR4. Accompanied by chemotherapy, this might lead to a better eradication of LSCs. From that perspective, plerixafor is currently being tested by French centers for the mobilization of dormant LSCs. Consequently, new paradigms must be devised for evaluating the therapeutic agents. For instance, clinical trial design can use intermediate end points such as time to progression following the administration of an agent that can target LSCs.

Applications

The study was to evaluate quantitatively and qualitatively the different CD34⁺ cell subsets after priming by chemotherapy and granulocyte colony-stimulating factor (\pm G-CSF) in patients with acute myeloid leukemia.

Peer review

The authors studied the mobilization of CD34⁺ bone marrow stem cells into the circulation in AML patients. The results show that CD34⁺ cells are more sensitive to chemotherapy after priming with GM-CSF associated with an increase in CXCR4 and CD47 expression.

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Human adipose tissue contains erythroid progenitors expressing fetal hemoglobin

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Abstract

AIM: To investigate the origin of hematopoietic progenitors contained in the stromal vascular fraction (SVF) of human adipose tissue.

METHODS: Tissue samples obtained from lipectomies were subjected to enzymatic digestion with collagenase to obtain a single-cell suspension. The centrifuged cell pellet, termed SVF, was separated immunomagnetically into CD45⁺ and CD45⁻ cells and cultured in serum-free medium containing hematopoietic cytokines. The freshly isolated and cultured cells were evaluated to determine their ability to form hematopoietic colony-forming units in clonogenic assays and for the expression of certain hematopoietic transcription factors by reverse

transcription-polymerase chain reaction; the gene expression level was compared to that in CD34⁺ hematopoietic progenitor cells from cord blood (CB) and adult peripheral blood (PB). To characterize erythroid progenitors, burst-forming units-erythroid (BFU-E) were developed in a semisolid medium under different culture conditions, and the hemoglobin composition and globin gene expression in the erythroid colonies were determined.

RESULTS: The transcription factors *SCL/TAL1*, *RUNX1*, *RUNX2* and *GATA2* were expressed in both the CD45⁺ and CD45⁻ SVF populations; however, in contrast to our observations in the CD34⁺ cells from CB and adult PB, *GATA1* was not detected. Nevertheless, *GATA1* could be detected in the SVF cells after seven days in culture, whereas its expression was upregulated in the CB CD34⁺ cells. The analysis of BFU-E-derived colonies revealed that virtually all erythroid cells produced by SVF cells expressed fetal hemoglobin, and the γ -globin mRNA levels ranged between those obtained in the adult- and neonatal-derived erythroid cells. Moreover, the SVF-derived erythroid cells synthesized similar levels of α - and β -globin mRNA, whereas the α -globin transcript levels were consistently higher than those of β -globin in the cells derived from CB or PB CD34⁺ cells. Furthermore, although the cellular distribution of hemoglobin in the erythroid cells derived from the CD34⁺ cells obtained from hematopoietic tissues was dependent on the presence or absence of serum in the culture medium, this did not affect the SVF-derived erythroid cells.

CONCLUSION: Our results demonstrate that hematopoietic progenitors in SVF have molecular and functional features that differ from those exhibited by circulating progenitors, suggesting the possibility of a different origin.

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Key words: Hemoglobin; Adipose tissue; Stromal vascu-

lar fraction; Erythroid cells; Hematopoietic progenitors

Core tip: Stromal vascular fraction (SVF) from human adipose tissue contains mesodermal precursors with the ability to form mixed hematoendothelial colonies and hematopoietic colony-forming units, though this occurs at an extremely low frequency. It is well known that hematopoietic progenitors residing in the bone marrow are released into the circulation and enter peripheral tissues; therefore, the most plausible explanation for this hematopoietic activity is that these cells are actually circulating hematopoietic progenitors. However, it is also possible that they may originate from the adipose tissue itself. To address this hypothesis, we compared the expression levels of the most relevant hematopoietic transcription factors in cells isolated from SVF with their expression levels in CD34⁺ cells isolated from adult peripheral blood and cord blood. Moreover, because the composition of hemoglobin in erythroid cells varies depending on the origin of the hematopoietic progenitors and their ontogenic stage, burst-forming units-erythroid were developed in culture, and the hemoglobin composition and globin gene expression in erythroid colonies were determined. Our results provide evidence that erythroid progenitors contained in SVF exhibit features that differ from those of circulating progenitors. These findings should encourage further research on stem cells and the microenvironment of human adipose tissue.

Navarro A, Carbonell-Uberos F, Marín S, Miñana MD. Human adipose tissue contains erythroid progenitors expressing fetal hemoglobin. *World J Stem Cells* 2013; 5(4): 205-216 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v5/i4/205.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v5.i4.205>

INTRODUCTION

Adipogenesis and angiogenesis are two closely related processes during prenatal and postnatal life^[1,2], and it has been proposed that CD34⁺CD31⁻ cells are a common precursor for both adipocytes and endothelial cells^[3]. In addition, angiogenesis is rapidly induced in growing adipose tissue^[4-6]. Therefore, antiangiogenic factors could be potential targets for regulating fat cell development^[7]. Because bone marrow-derived endothelial progenitor cells do not contribute significantly to neovascularization^[8], endothelial cells must be present in the supportive stroma to enable the vascularization of the tissue. In support of this view, it has been reported that stromal-derived factor-1 produced by CD34⁺CD31⁻ cells induces the chemotaxis of CD34⁺CD31⁻ cells expressing CXCR4 and leads to their differentiation into endothelial cells^[9]. Moreover, a primitive population of CD34⁺CD90⁺ cells in human adipose tissue with a high proliferative capacity has been shown to be capable of differentiating into endothelial cells, even in the absence of angiogenic factors^[10]. We also previously described, for the first time,

the existence of a rare CD45⁻KDR⁺ cell population that exhibits hemangioblastic properties, as they give rise to hematoendothelial colonies^[11]. These findings demonstrate the existence of mesodermal progenitors that can provide adipose tissue with endothelial progenitor cells when required. Additionally, CD45⁻ cells produce hematopoietic colony-forming units (CFUs) when seeded in a methylcellulose-based medium^[11]. Because adipose tissue is not hematopoietic in origin, it is possible that these CFUs are generated by circulating hematopoietic progenitors that target adipose tissue^[12]. However, given that hemangioblasts are bipotent cells that give rise to endothelial and hematopoietic progenitor cells, it is tempting to speculate that these cells could be responsible for the production of these hematopoietic progenitors. To address this hypothesis, we examined the expression of genes directly involved in the process of hematopoiesis and analyzed the hemoglobin levels and globin gene expression in burst-forming units-erythroid (BFU-E)-derived colonies.

MATERIALS AND METHODS

Purification of human adipose tissue and cells

Human adipose tissue was obtained, after informed consent, from female patients between 30 and 40 years of age who were undergoing lipectomy at the University General Hospital of Valencia. The stromal vascular fraction (SVF) was obtained as previously described^[11]. The SVF cells were labeled with anti-CD45 microbeads and separated into CD45⁺ and CD45⁻ cells using a magnetic-activated cell sorting separation system (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany). In some experiments, the CD45⁻ cells were separated on the basis of CD34 expression into CD45⁻CD34⁺ and CD45⁻CD34⁻ subpopulations using anti-CD34 microbeads. To achieve a high degree of purity, the positive and negative cells were passed through a second column. After informed consent, cord blood (CB) samples were obtained from normal full-term deliveries, and peripheral blood (PB) samples were obtained from healthy adults between 20 and 50 years of age. The CD34⁺ cells were purified by positive selection using a CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec). The purity of the isolated cells was determined by flow cytometry.

Short-term liquid culture

CD45⁺ and CD45⁻ cells from the SVF and CB CD34⁺ cells were cultured in a serum-free medium (StemSpan; STEMCELL Technologies, SARL, Grenoble, France) in the presence of stem cell factor and Flt3-L (100 ng/mL each) in addition to thrombopoietin and interleukin-6 (20 ng/mL each) (R and D Systems, Abingdon, United Kingdom). The medium was supplemented with 40 µg/mL lipoproteins (MP Biomedicals LLC, Solon, OH, United States) and 1% bovine serum albumin (BSA). The SVF cells were plated at 5×10^5 cells/mL, and the CB cells were plated at 5×10^4 cells/mL. The cultures were incu-

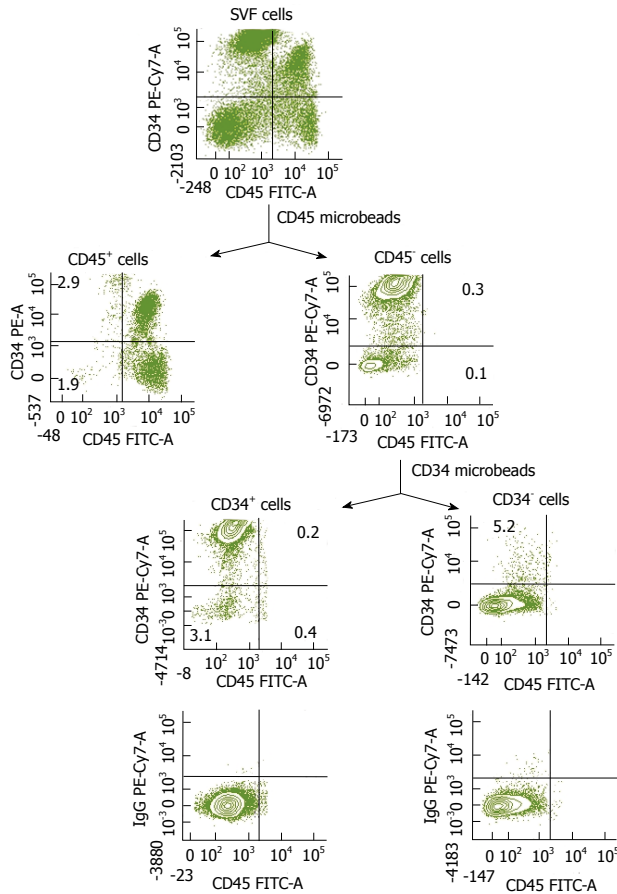


Figure 1 Purity of stromal vascular fraction populations. Selected cell subsets from stromal vascular fraction were separated using specific monoclonal antibodies coupled to magnetic particles following magnetic cell separation technology. Representative dot plots of selected cells are shown. The numbers in the quadrants indicate the percentages of cells expressing the corresponding antigen. SVF: Stromal vascular fraction.

bated at 37 °C in a humidified atmosphere containing 5% CO₂ and either 20% O₂ or 5% O₂. After seven days, the cells were harvested, counted and used for gene expression assays and clonogenic potential determination.

Clonogenic progenitor cell assay

The SVF CD45⁻ and CD45⁺ cells (either freshly isolated or after seven days in liquid culture) and the CB or PB CD34⁺ cells were seeded in methylcellulose-based media with or without fetal bovine serum (Methocult GF H4435 or Methocult SF H4436; STEMCELL Technologies). Isolated or liquid-cultured SVF cells were plated at 5×10^5 cells per dish, and purified CD34⁺ cells were plated at 150 cells per dish. The cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ and either 20% O₂ or 5% O₂. After 14–21 d, CFUs were scored according to their morphology using an inverted microscope.

Hemoglobin analysis

Individual BFU-E-derived colonies were selected from the semisolid cultures, pooled and washed. The cells were divided into two aliquots: one for FACS analysis to deter-

mine hemoglobin expression and the other globin gene expression analysis by real-time reverse transcription-polymerase chain reaction (RT-PCR). For the hemoglobin analysis, erythroid cells were fixed and permeabilized prior to staining with CD45-peridinin-chlorophyll protein complex, glycophorin A (CD235A) conjugated to allophycocyanin, fetal hemoglobin conjugated to FITC (all from BD Biosciences, Erembodegem, Belgium) and β -hemoglobin-PE (Santa Cruz Biotechnologies, Santa Cruz, CA, United States). The cells were analyzed using a FACSCanto II (BD Biosciences) with FACSDiva software (BD Biosciences).

Gene expression analysis

Total RNA was extracted using TRIzol Reagent and was subsequently treated with DNase (Deoxyribonuclease). cDNA was prepared using TaqMan Reverse Transcription Reagents. The real-time PCR experiments were performed using Mastermix and primers from Taqman Gene Expression Assays. All reagents and probes were purchased from Life Technologies (Foster City, CA, United States). The amplification protocol consisted of 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each reaction was performed in duplicate. For each sample, the cycle threshold (Ct) readings were determined using the Sequence Detection Software. All expression data were calculated relative to *GAPDH* controls as $2^{-\Delta Ct}$. The following primers were used: SCL/TAL1 (Hs001097987_m1), RUNX1 (Hs01021971_m1), RUNX2 (Hs01047978_m1), GATA1 (Hs01085823_m1), GATA2 (Hs00231119_m1), α -globin (HS00361191_g1), β -globin (HS00747223_g1) and γ -globin (HS00361131_g1).

Statistical analysis

Significant differences among the samples were tested using the Student *t* test or Mann-Whitney test where applicable. A *P* value less than 0.05 was considered statistically significant. The data were analyzed using GraphPad Prism Software 5.0 (GraphPad Software Inc., La Jolla, CA, United States).

RESULTS

SVF cells have hematopoietic activity in vitro

To demonstrate the presence of hematopoietic progenitor cells in human adipose tissue, SVF cells were separated into CD45⁺ and CD45⁻ populations, and CD45⁻ cells were further separated into CD45⁻CD34⁺ and CD45⁻CD34⁻ populations (Figure 1). Clonogenic assays showed that the colony-forming ability of CD45⁻ cells was restricted to CD34-expressing cells. As shown in Table 1, the CD45⁺ cells, which accounted for approximately 10%–20% of the SVF cells, generated four times more CFUs than their complementary CD45⁻ cells; however, no differences in CFU distribution were found. Notably, this colony-forming ability was not affected by either serum deprivation or a low oxygen concentration (Table 1).

Table 1 Number of CFUs per 10⁵ CD45⁺ or CD45⁻ cells isolated from human adipose tissue stromal vascular fraction

	Freshly isolated cells			Liquid-cultured cells	
	Normoxia		Hypoxia	Normoxia	Hypoxia
	With serum	Without serum	With serum	With serum	
CD45 ⁺ cells					
Total CFUs	1.53 ± 0.26 ^b	1.55 ± 0.35 ^b	1.79 ± 0.42 ^b	10.13 ± 3.19 ^{a,d}	13.73 ± 3.93 ^d
Erythroid colonies	0.59 ± 0.15 ^b	0.80 ± 0.05 ^b	0.72 ± 0.17 ^b	5.93 ± 1.58 ^{a,d}	7.01 ± 1.39 ^d
Myeloid colonies	0.94 ± 0.16 ^b	0.75 ± 0.35 ^a	1.01 ± 0.25 ^a	4.20 ± 1.67 ^{a,d}	6.72 ± 3.17 ^d
CD45 ⁻ cells					
Total CFUs	0.37 ± 0.08	0.42 ± 0.11	0.34 ± 0.19	2.68 ± 0.86 ^d	7.91 ± 2.77 ^d
Erythroid colonies	0.12 ± 0.03	0.12 ± 0.05	0.09 ± 0.06	2.01 ± 0.99 ^d	4.70 ± 1.85 ^d
Myeloid colonies	0.25 ± 0.05	0.30 ± 0.07	0.25 ± 0.21	0.67 ± 0.16 ^d	3.21 ± 1.16 ^d

CD45⁺ and CD45⁻ cells from the stromal vascular fraction that were freshly isolated or cultured in a serum-free liquid medium for 7 d were seeded in a methylcellulose-based medium with or without serum, and colony-forming units (CFUs) were scored under a microscope. The numbers of CFUs per 10⁵ cells are provided. The total CFU values correspond to the sum of erythroid and myeloid colonies. The myeloid colonies include CFU-GM, CFU-M and CFU-GEMM. The data are provided as the mean ± standard error of the mean. Isolated cells: normoxia with serum (*n* = 30-32), normoxia without serum (CD45⁺, *n* = 5; CD45⁻, *n* = 16), hypoxia (*n* = 5); cultured cells (*n* = 5). ^a*P* < 0.05, ^b*P* < 0.01 vs CD45⁻ cells; ^d*P* < 0.01 CD45⁻ or CD45⁺ cultured cells vs their corresponding isolated cells.

To evaluate the potential of hematopoietic progenitors to expand *in vitro*, SVF cells were cultured in a serum-free liquid medium containing early-acting cytokines for seven days and then tested for their colony-forming capacity. Under these culture conditions, the number of CFUs increased by approximately 7-fold in both the CD45⁺ and CD45⁻ cell populations. Moreover, when the cells were cultured under hypoxic conditions (5% O₂), the number of hematopoietic progenitors contained in the CD45⁻ cell subset increased 3-fold over that under the normoxic condition (20% O₂).

Hematopoietic transcription factors are expressed in SVF cells

To characterize the expression of hematopoietic progenitors in SVF, transcription factors involved in hematopoietic differentiation were analyzed by RT-PCR, and the gene expression levels were compared to those in the CD34⁺ cells from CB and from adult PB. As shown in Figure 2A, *SCL/TAL1*, *RUNX1*, *RUNX2* and *GATA2* were expressed at significantly higher levels in the SVF CD45⁺ cells than CD45⁻ cells; however, *GATA1* was not detected in either cell subset. When CD34⁺ hematopoietic cells were analyzed, the results showed that *SCL/TAL1* and *GATA2* were expressed at similar levels in the cells of neonatal and adult origin. However, the *GATA1*, *RUNX1* and *RUNX2* mRNA levels were significantly higher in the CD34⁺ cells from adult PB compared to CB (Figure 2A). We also compared the gene expression profiles of the SVF cells with those of

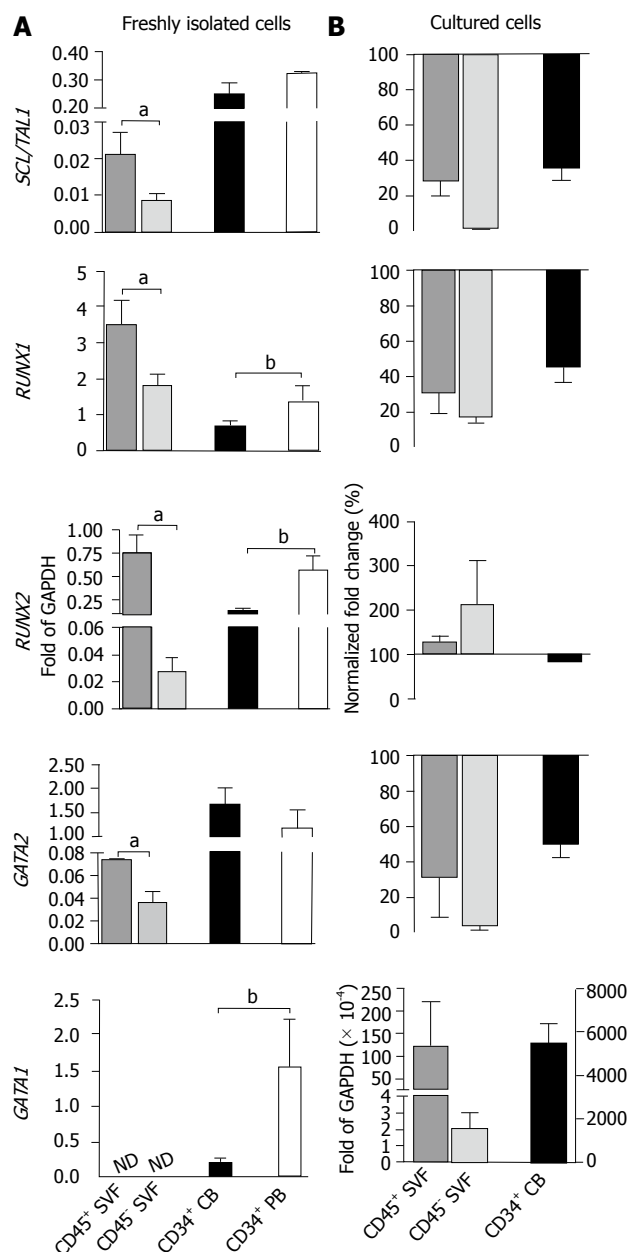


Figure 2 Hematopoietic transcription factors genes are expressed in isolated stromal vascular fraction cell populations. A: Freshly isolated CD45⁺ and CD45⁻ cells from stromal vascular fraction (SVF) and CD34⁺ cells from cord blood (CB) or adult peripheral blood (PB) were isolated via immunomagnetic methods and used for a gene expression analysis by reverse transcription-polymerase chain reaction (RT-PCR). The transcripts were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); B: CB CD34⁺ cells and CD45⁺ and CD45⁻ cells from the SVF were cultured in liquid serum-free medium containing early-acting cytokines for 7 d and then analyzed by RT-PCR to determine changes in the expression of selected genes. The transcripts were normalized to GAPDH, and the ratio between the gene quantity in cultured cells and the gene quantity in isolated cells was determined to yield a normalized fold change. CB CD34⁺ cells, *n* = 6-8; PB CD34⁺ cells, *n* = 3-4; SVF populations - CD45⁺ cells, *n* = 6; CD45⁻ cells, *n* = 6. Levels of statistical significance: ^a*P* < 0.02, ^b*P* < 0.001. ND: Not detected. All samples were assayed in duplicate. SVF: Stromal vascular fraction; CB: Cord blood; PB: Peripheral blood; GAPD: Glyceraldehyde-3-phosphate dehydrogenase.

the CD34⁺ cells from hematopoietic tissues and found that *SCL/TAL1* and *GATA2* were expressed at significantly higher levels in hematopoietic CD34⁺ cells than in

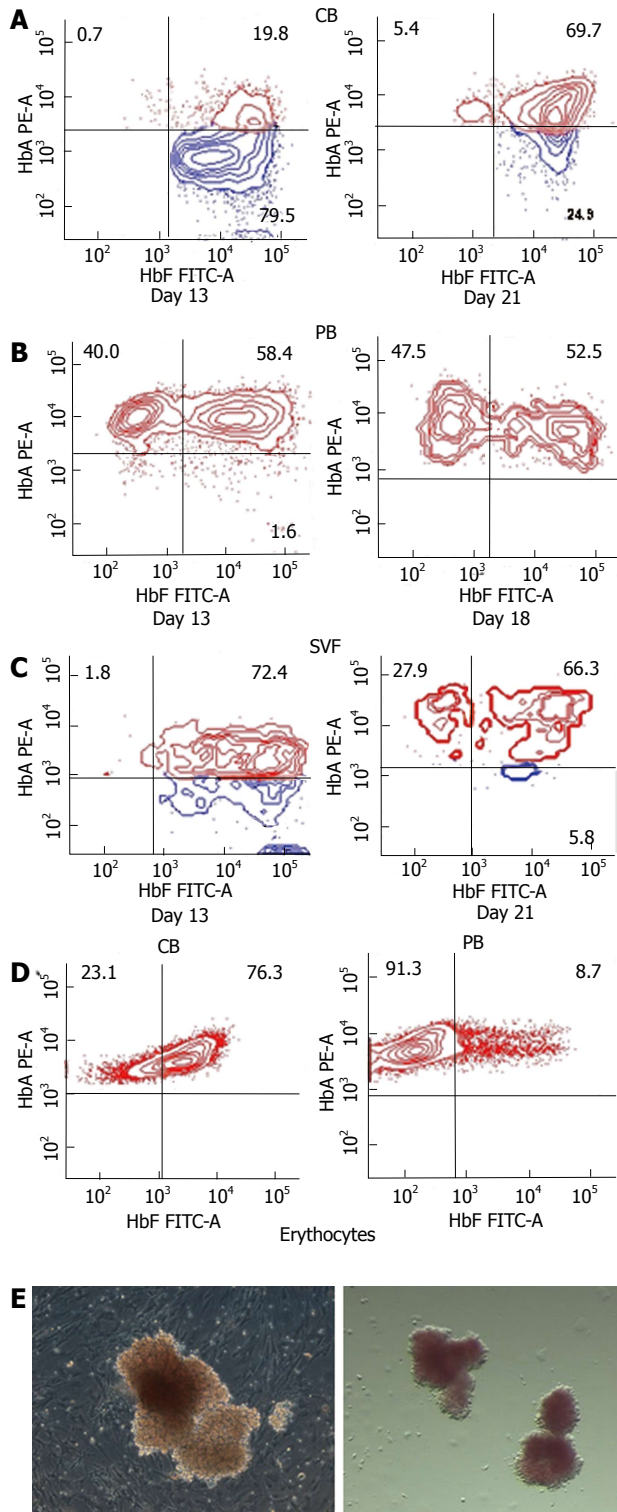


Figure 3 Representative flow cytometric dot plots of hemoglobin expression in erythroid cells. Burst-forming units-erythroid (BFU-E)-derived colonies were analyzed for HbF and HbA expression by flow cytometry after different lengths of time in culture. Erythroid cells generated from cord blood (CB) CD34⁺ cells (A), from peripheral blood (PB) CD34⁺ cells (B), and from stromal vascular fraction (SVF) cells (C). Representative fluorescence-activated cell sorting plots showing hemoglobin expression in circulating erythrocytes from CB and PB (D). Representative phase-contrast photomicrographs showing the morphological aspect of the BFU-Es generated from CD45⁺ (left) and CD45⁺ (right) cells isolated from SVF (E). HbF: Fetal hemoglobin; HbA: Adult hemoglobin; CB: Cord blood; PB: Peripheral blood.

SVF cells. However, the adult PB CD34⁺ cells expressed *RUNX1* and *RUNX2* at levels similar to the CD45⁺ and CD45⁺ cells from SVF, respectively. Lastly, the SVF cells and CB CD34⁺ cells were cultured in liquid for seven days, and changes in their gene expression patterns were compared. The most important finding was that *GATA1* could only be detected in the SVF cells after seven days of culture, whereas *GATA1* expression was upregulated in the CB CD34⁺ cells. The *SCL/TAL1*, *RUNX1* and *GATA2* mRNA levels were decreased in all the cultured cells. Additionally, although *RUNX2* gene expression was unchanged in both the CB cells and SVF CD45⁺ cells, its expression was slightly increased in the SVF CD45⁺ cells (Figure 2B).

Erythroid cells derived from SVF express fetal hemoglobin

BFU-E-derived erythroid cells generated in semisolid culture from selected SVF cells or CB cells or PB CD34⁺ cells were analyzed for hemoglobin expression. As expected, virtually all the neonatal erythroid cells expressed fetal hemoglobin (HbF), whereas adult hemoglobin (HbA) was expressed in all the erythroid cells derived from adult PB CD34⁺ cells (Figure 3A and B). As shown in Figure 3E, the BFU-E-derived colonies generated from the SVF CD45⁺ cells developed on a monolayer of stromal cells, whereas adherent cells were not found in the SVF CD45⁺ cell cultures. It is important to note that when the SVF-derived erythroid cells were analyzed, a high proportion of cells containing HbF together with HbA (HbF⁺HbA⁺) were observed; unexpectedly, some cells expressing only HbF (HbF⁺HbA⁻) and a very small proportion of cells expressing only HbA (HbF⁻HbA⁺) were also detected (Figure 3C).

The mean fluorescence intensity (MFI) of intracellular Hb-associated immunofluorescence was used to estimate the amount of intracellular hemoglobin per cell. However, because the majority of CB- and SVF-derived erythroid cells were growing in clusters containing a wide range of HbF and HbA levels, the samples were separated into two groups based on the MFI of HbF in the HbF⁺HbA⁺ cells. First, a comparison between the neonatal- and adult-derived erythroid cells was established. As shown in Table 2, the neonatal erythroid cells in group A exhibited the highest MFIs for HbF and HbA. The samples in group B exhibited an MFI for HbF similar to that observed in the adult-derived cells, but HbA was significantly lower in both, HbF⁺HbA⁺ and HbF⁻HbA⁺ cells compared to their corresponding adult-derived cells. Next, the MFIs of HbF and HbA in the SVF-derived cells were compared to those observed in the erythroid cells derived from the CD34⁺ hematopoietic cells from CB and adult PB. An analysis of the HbF⁺HbA⁻ cells showed that HbF was expressed at similar and higher levels in the cells derived from the SVF CD45⁺ population than in the neonatal cells corresponding to group A and B, respectively. In contrast, the erythroid cells derived from the SVF CD45⁺ population expressed HbF at a lower

Table 2 Mean fluorescence intensity of fetal hemoglobin and adult hemoglobin in hemoglobin-expressing erythroid cells

		HbF		HbA		HbF/HbF + HbA
		HbF ⁺ HbA ⁺ cells	HbF ⁺ HbA ⁺ cells	HbF ⁺ HbA ⁺ cells	HbF ⁺ HbA ⁺ cells	
CB CD34 ⁺ cells	A	124082 ± 10917	204968 ± 17070 ^b	22139 ± 4457 ^c	25171 ± 3211 ^c	8.49 ± 1.40
	B	16989 ± 1382	21627 ± 1027	6139 ± 182 ^a	5248 ± 398 ^a	5.58 ± 0.38
PB CD34 ⁺ cells			23495 ± 1326	10746 ± 893	9496 ± 548	1.26 ± 0.14
SVF CD45 ⁺ cells	A	90164 ± 23126	78566 ± 2697 ^{b,c,f}	28229 ± 5204 ^c	21022 ± 3720 ^c	3.86 ± 0.71
	B	34612 ± 2701 ^c	29051 ± 1514	9637 ± 1094	5433 ± 325 ^a	5.92 ± 1.10
SVF CD45 ⁺ cells	A	61429 ± 4085 ^c	47907 ± 2011 ^{b,d}	16388 ± 986	16668 ± 587 ^a	3.53 ± 0.18
	B	20971 ± 4696	15731 ± 1875	8006 ± 1653	9219 ± 1533	3.46 ± 1.03

The mean fluorescence intensity values are expressed as the mean ± SE and correspond to the samples analyzed in Figure 2. Statistical significance: ^a*P* < 0.05, ^b*P* < 0.01 *vs* PB CD34⁺ cells; ^c*P* < 0.05, ^d*P* < 0.01 *vs* CB CD34⁺ cells; ^e*P* < 0.05 CD45⁺ cells *vs* CD45⁺ cells. SVF: Stromal vascular fraction; CB: Cord blood; PB: Peripheral blood; HbF: Fetal hemoglobin; HbA: Adult hemoglobin.

and similar intensity than the neonatal cells in group A and B, respectively. The expression level of HbF in the SVF HbF⁺HbA⁺ cells from group A was significantly lower than that observed in the cells of neonatal origin from the same group. However, the HbF expression level in the SVF-derived erythroid cells in group B was similar to that in both the neonatal and adult-derived erythroid cells of the same group.

Although the proportion of HbF⁺HbA⁺ cells in the SVF-derived erythroid colonies was very small, the MFI values showed that the cells from group B expressed HbA at levels similar to the adult-derived cells. However, no significant differences were found between the SVF-derived cells from group B and neonatal-derived cells in the same group. Lastly, an analysis of HbA in the SVF-derived HbF⁺HbA⁺ cells in group B revealed that HbA was expressed at similar levels (*i.e.*, a similar MFI intensity) in the erythroid cells derived from CD45⁺ progenitors and neonatal-derived cells in the same group. However, the MFI of HbA in the cells derived from CD45⁺ progenitors was similar to that of the adult-derived cells, suggesting that the erythroid progenitors contained within the CD45⁺ population must be at a later stage of maturation or differentiation.

MFI values were also used to estimate the contribution of HbF to the total hemoglobin levels. As shown in Table 2, the HbF/HbF + HbA ratio for erythroid cells derived from CB CD34⁺ cells was approximately 6.5- and 4.5-fold higher than that obtained for adult PB-derived cells, providing evidence for the neonatal origin of CB hematopoietic progenitors. The HbF/HbF + HbA ratio for the erythroid cells derived from SVF progenitors was between the levels obtained for the neonatal- and adult-derived cells.

In addition to our analysis at the cellular level, the expression of the globin gene was also examined. Interestingly, when the Ct values of the globin genes were normalized against *GAPDH* expression, the globin transcript levels in the erythroid cells derived from SVF or from CB varied widely. Therefore, three groups were defined on the basis of α -globin transcript levels (designated as I, II and III in Figure 4). We observed that the level of α -globin mRNA was always higher than that of β -globin

in the erythroid cells generated from CD34⁺ cells from hematopoietic tissues (either CB or PB) (Figure 4A and D), whereas similar levels of α - and β -globin mRNA were observed in the SVF-derived erythroid cells (Figure 4B and C). As expected, the highest and lowest levels of γ -globin transcripts corresponded to neonatal- and adult-derived erythroid cells, respectively, whereas the erythroid cells generated from SVFs expressed γ -globin mRNA at levels ranging between those observed in the CB- and PB-derived cells (Figure 4A-D).

The globin chain mRNA ratios were also calculated. As shown in Figure 5A, the $\alpha/(\beta + \gamma)$ globin ratio was 2.55 for the PB-derived cells and 0.55, 1.15 and 2.50 for CB-derived groups I, II and III, respectively. Additionally, the $\gamma/(\beta + \gamma)$ globin ratios were 0.74, 0.57 and 0.56 for groups I, II and III, respectively, though this ratio decreased to 0.04 in the PB-derived cells (Figure 5B).

When erythroid cells derived from the SVF were analyzed, the $\alpha/(\beta + \gamma)$ globin gene mRNA ratios were very similar to those obtained in the CB-derived cells from groups I and II, ranging from 0.54 to 1.11 and from 0.56 to 0.87 in the cells derived from CD45⁺ cells or CD45⁺ cells, respectively (Figure 5A). However, the $\gamma/(\beta + \gamma)$ globin gene ratios were between those obtained for the CB- and PB-derived cells (Figure 5B).

Cellular distribution of hemoglobin in erythroid cells derived from SVF is not affected by serum deprivation

An analysis of the hemoglobin expression profile over the culture period showed that the proportion of HbF⁺HbA⁺ cells gradually decreased and that HbF⁺HbA⁺ and HbF⁺HbA⁺ cells increased in the erythroid colonies generated from CB CD34⁺ progenitor cells (Figure 6A). Indeed, at the end of the culture period, the cellular distribution of hemoglobin was very similar to that exhibited by circulating erythrocytes in CB/placenta (Figure 3D). Therefore, this culture system recapitulates the neonatal pattern of hemoglobin expression *in vivo*. However, the cellular distribution of hemoglobin in the adult PB-derived erythroid colonies changed only slightly during the culture period. In contrast, in the cells derived from SVF, the most notable effect was a gradual decrease in the proportion of HbF⁺HbA⁺ cells, which was accompa-

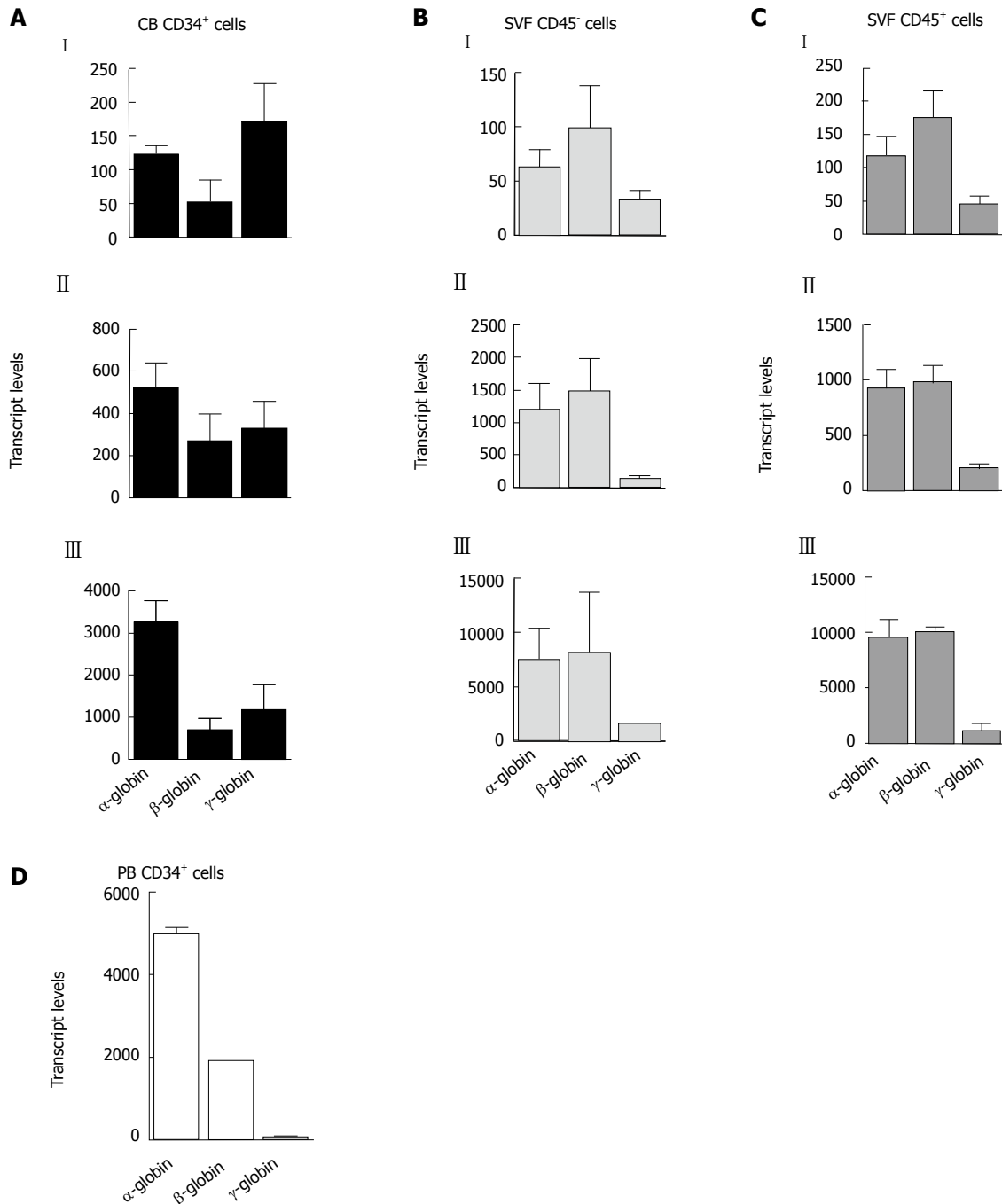


Figure 4 Analysis of globin gene expression in erythroid cells. CD45⁺ and CD45⁻ cells isolated from the stromal vascular fraction (SVF) and CD34⁺ cells from cord blood (CB) or adult peripheral blood (PB) were cultured in a methylcellulose-based medium, and burst-forming units-erythroid -derived erythroid cells were isolated at day 15 of culture to determine globin gene expression by reverse transcription-polymerase chain reaction. The transcripts were normalized to glyceraldehyde-3-phosphate dehydrogenase. Based on the α -globin levels, the values obtained for SVF- and CB-derived cells were placed into three groups (I, II and III). A: CB CD34⁺ cells, $n = 10$; B: SVF CD45⁻ cells, $n = 17$; C: SVF CD45⁺ cells, $n = 17$; D: PB CD34⁺ cells, $n = 4$. All samples were assayed in duplicate.

nied by an increase in the proportion of the HbF⁺HbA⁺ cells. Therefore, at the end of the culture period, a large proportion of HbF⁺HbA⁺ cells continued to be observed in the erythroid colonies derived from both adult PB and SVF hematopoietic progenitors (Figure 6A).

The effect of serum deprivation and hypoxic conditions on hemoglobin expression was also examined. As shown in Figure 6B, when erythroid colonies derived from CB or PB CD34⁺ cells were developed in serum-

free medium, a switch from HbF to HbA production was observed at the cellular level. Thus, the proportion of HbF⁺HbA⁺ cells was significantly decreased, whereas the proportion of HbF⁺HbA⁻ cells increased. However, no change in the proportion of HbF⁺HbA⁻ cells was observed in the CB-derived colonies. Under these conditions, the cellular distribution of hemoglobin in the adult-derived erythroid cells was nearly identical to that observed in the circulating adult erythrocytes (Figure

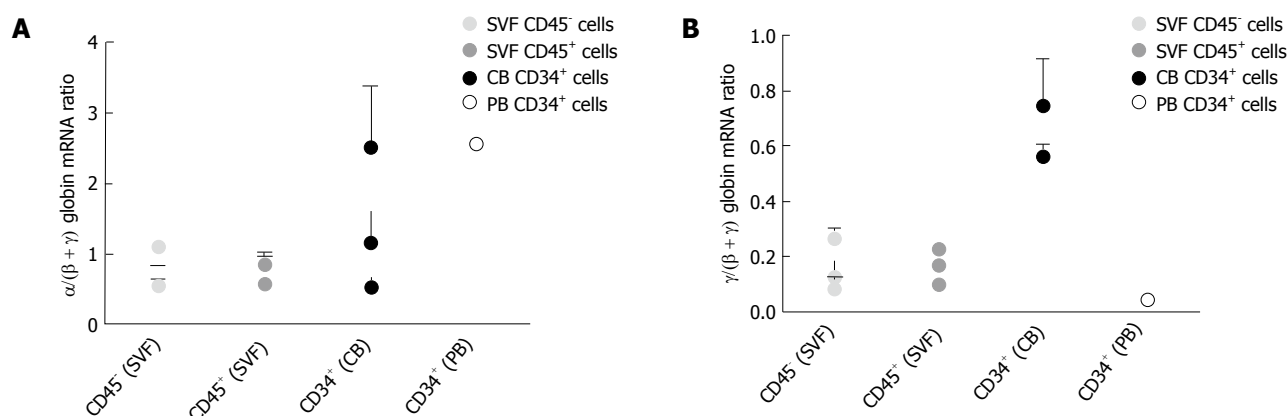


Figure 5 Ratio of α to $(\beta + \gamma)$ and of γ to $(\beta + \gamma)$ globin mRNA in erythroid cells. The values of transcript levels corresponding to the samples analyzed in Figure 4 were used to calculate the $\alpha/(\beta + \gamma)$ globin ratios (A) and $\gamma/(\beta + \gamma)$ globin ratios (B). The results are expressed as the mean \pm SE. SVF: Stromal vascular fraction; CB: Cord blood; PB: Peripheral blood.

3D). In sharp contrast, serum deprivation did not induce significant changes in the cellular distribution of hemoglobin in the erythroid colonies generated from SVF progenitors (Figure 6B). Serum deprivation also induced a dramatic reduction in the mRNA levels of all globins: the α - and β -globin transcript levels were reduced by approximately 90%-95% in erythroid cells derived from all three tissue sources. However, although the γ -globin transcript level was reduced by 95% in the SVF-derived erythroid cells, its level was decreased by 75% and 85% in the CB- and PB-derived cells, respectively.

Lastly, the effect of a low concentration of oxygen (5% O₂) on hemoglobin synthesis was studied. As shown in Figure 6B, the erythroid colonies generated from CB CD34⁺ cells exhibited the same hemoglobin profile in both normoxia (20% O₂) and hypoxia (5% O₂). However, hypoxia induced a significant decrease in the proportion of HbF/HbA⁺ cells in the SVF erythroid cells, though no significant changes in HbF-expressing cell populations were observed.

When globin gene expression was analyzed, the results showed that the β -globin transcript level was significantly reduced (by approximately 55%, $P < 0.05$) in the CB-derived erythroid cells, whereas α - and γ -globin mRNA levels were only slightly decreased (by 20% and 25%, respectively). In contrast, α -globin expression was reduced (by approximately 45%, $P < 0.04$) in the erythroid cells derived from SVF, whereas the expression levels of β - and γ -globins were decreased and increased, respectively, by approximately 25%.

DISCUSSION

It is widely accepted that human hematopoietic stem and progenitor cells reside in the lineage (Lin)⁻ fraction and are enriched in CD34⁺ cells that also express CD45 at moderate levels^[13]. However, Lin⁻CD34⁺CD45⁻ primitive hematopoietic cells with hematopoietic activity *in vivo* (with or without clonogenicity in *in vitro* assays) have recently been identified^[14-18]. Although bone marrow is the

primary site of hematopoiesis in adult humans, our group first described the presence of CD45⁻ cells with hemangioblastic properties in human adipose tissue SVF^[11]. Indeed, the finding that hematopoietic progenitors exist in adipose tissue is very exciting.

The present study shows that CD34-expressing cells are responsible for the ability of SVF-derived CD45⁻ cells to produce CFUs and that the clonogenic efficiency of CD45⁻ cells is significantly lower than that of their counterpart CD45⁺ cells. It has been proposed that adipose tissue in mouse models of obesity and in obese human subjects is in a hypoxic state^[19,20]; thus, unlike circulating hematopoietic progenitors, hematopoietic progenitors in SVF may be exposed to mild hypoxia. Although hematopoietic stem cells are better preserved in culture under low O₂ conditions compared to 20% O₂^[21-23] and hypoxia has been shown to be essential for the proliferation of embryonic hematopoietic progenitors^[24], it remains controversial whether hematopoietic progenitors are better preserved or can be expanded under hypoxic conditions^[23,25]. Herein, we show that hematopoietic progenitors derived from the SVF CD45⁻ cell fraction, but not those derived from the SVF CD45⁺ cell fraction, were expanded under hypoxic conditions. These results could indicate that CD45⁻ progenitors are at an earlier stage of differentiation than CD45⁺ progenitors.

In support of this hypothesis, the CD45⁺ cells expressed much higher levels of *SCL/TAL1*, *GATA2*, *RUNX1* and *RUNX2* compared to CD45⁻ cells, which is consistent with their clonogenic potential. *RUNX1* and *RUNX2* mRNA levels were also significantly increased in the adult CD34⁺ cells compared to neonatal CD34⁺ cells. Because purified CD34⁺ cells include both hematopoietic stem and progenitor cells, these differences in gene expression could be explained by the higher number of proliferating hematopoietic progenitor cells in PB, as previously suggested^[26-28]. However, *GATA1* was unexpectedly not detected in either SVF population, in contrast to what was observed in the CD34⁺ cells from adult PB or CB. This finding was very surprising because *GATA1*,

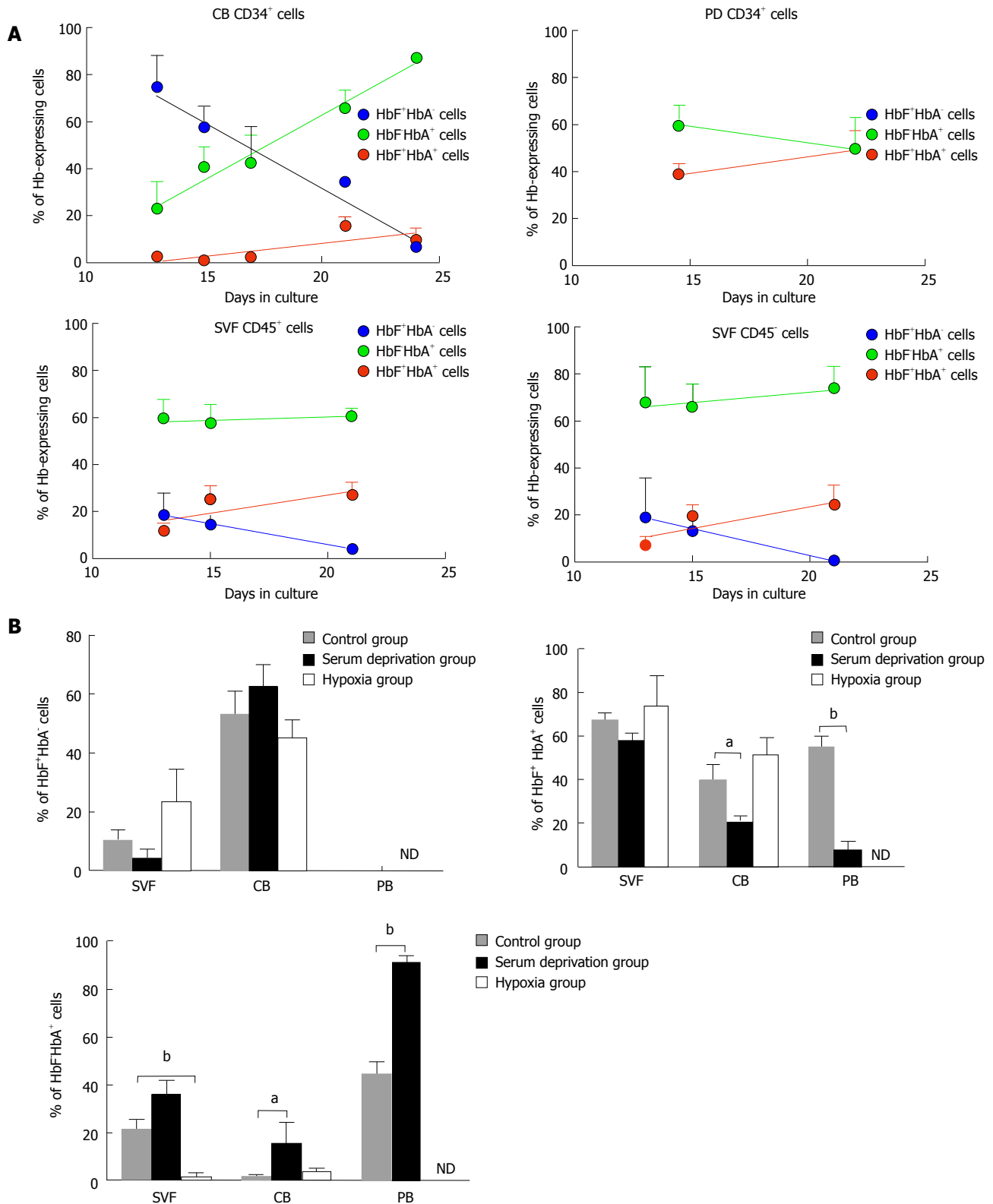


Figure 6 Effects of time and cell culture conditions on the pattern of hemoglobin expression in erythroid cells. Individual burst-forming units-erythroid (BFU-E)-derived colonies generated in a methylcellulose-based medium were selected, pooled and analyzed for hemoglobin expression by flow cytometry. A: The kinetics of HbF⁺HbA⁺ cells, HbF⁺HbA⁺ cells and HbF⁺HbA⁺ cells of erythroid cells in the different cultures over time are given. From top to bottom: erythroid cells derived from cord blood (CB) CD34⁺ cells, from peripheral blood (PB) CD34⁺ cells, from stromal vascular fraction (SVF) CD45⁺ cells and from SVF CD45⁺ cells. The results are expressed as the percentage of total Hb-expressing cells (CB CD34⁺ cells, $n = 7$; PB CD34⁺ cells, $n = 4$; SVF CD45⁺ cells, $n = 10$ and SVF CD45⁺ cells, $n = 10$). The data are provided as the means \pm SE; B: A parallel series of cultures were performed to examine the effect of serum deprivation and hypoxia (5% O₂) on hemoglobin expression. Control cultures were grown under conditions of normoxia (20% O₂) using a serum-containing medium. SVF cells, $n = 10$; CB CD34⁺ cells, $n = 10$; PB CD34⁺ cells, $n = 4$. BFU-E-derived erythroid cells were analyzed by flow cytometry for hemoglobin composition at day 15 of culture. The results are expressed as the percentage of total Hb-expressing cells. The data are provided as the mean \pm SE. ^a $P < 0.02$, ^b $P < 0.0001$. ND: Not detected. CB: Cord blood; PB: Peripheral blood; SVF: Stromal vascular fraction.

a late hematopoietic transcription factor, is directly involved in erythropoiesis^[29] and the SVF cells were able to generate erythroid colonies. Importantly, after seven days in culture under conditions that facilitate the expansion of hematopoietic progenitor cells, *GATA1* was detected in the SVF-derived cells and was overexpressed in the CD34⁺ neonatal cells. Moreover, *GATA1* expression was higher in the PB CD34⁺ cells than in neonatal CD34⁺ cells, which was expected because GATA1 is upregulated in the later stages of commitment and differentiation^[30-32]. Although *GATA1* and *GATA2* are essential in embryonic and adult hematopoiesis and the expression of *GATA2* overlaps that of *GATA1* in hematopoietic lineages^[33], there are substantial differences between the *GATA2* and *GATA1* activities. *GATA2* is highly expressed in quiescent hematopoietic stem cells and is necessary for their maintenance and expansion^[34-36], whereas *GATA1* is required for erythroid differentiation^[29,32,36]. It has also been reported that the hematopoietic GATA factors are not functionally equivalent during adult hematopoiesis^[37]. Therefore, differences in the expression levels of these key hematopoietic transcription factors between CD34⁺ cells from hematopoietic tissues and SVF cells lead us to suggest that these progenitors have a different origin.

To further characterize the hematopoietic function of the SVF-derived cells, we utilized the results from the study of BFU-E-derived colonies because the composition of hemoglobin in erythroid cells varies depending on the origin of the hematopoietic progenitors and their ontogenic stage^[38-40]. HbF⁺HbA⁻ cells were not only the most abundant cells in CB-derived erythroid colonies, but HbA was expressed at significantly lower levels in these cells compared to the adult PB-derived cells, indicating their early ontogenic stage. However, unlike the observations in the PB-derived erythroid cells, HbF was expressed by the majority of the erythroid cells produced by SVF progenitors, and γ -globin mRNA was more highly expressed in these cells than in the PB-derived cells.

Several important findings support the hypothesis that SVF hematopoietic progenitors may not have originated in the bone marrow. The first of these findings indicated that the mRNA level of α -globin was always higher than that of β -globin in the erythroid cells derived from hematopoietic tissues, which may be interpreted as a signature of their hematopoietic origin^[41]. In contrast, α - and β -globins were synthesized at similar levels in SVF-derived cells.

The second of these findings is based on the different patterns of hemoglobin “switching” in response to culture conditions. As expected based on previous studies showing the serum-induced reversal of the hemoglobin switch^[42-46], the erythroid progenitors from both adult and neonatal origin gave rise to a significantly higher proportion of HbF⁺HbA⁺ cells and a lower proportion of HbF⁻HbA⁺ cells when cultured in serum-containing medium. However, the profile of the hemoglobin synthesized by erythroid progenitors from the SVF was nearly identical in the presence or absence of serum.

Additionally, it is largely known that low oxygen concentrations affect erythropoiesis and the synthesis of the HbF^[25,47-49]. Furthermore, in erythroid cells derived from erythroid progenitors from bone marrow or PB, it has been reported that the increase in HbF associated with hypoxia is concomitant with the early induction of γ -globin mRNA, and, in some circumstances, with the reduction of the β -globin mRNA, most likely as a consequence of the presence or absence of serum in the culture medium^[49,50]. However, it has also been proposed that fetal and neonatal cells produce HbF irrespective of oxygen concentration^[49]. Herein, we show that, although the neonatal-derived erythroid cells generated displayed the same hemoglobin profile under 20% O₂ or 5% O₂, importantly, β -globin expression was reduced by 55% in hypoxia, whereas γ -globin expression was not affected. However, in sharp contrast to the reactivation of HbF observed in the erythroid cells derived from adults, hypoxia did not induce an increase in the proportion of cells expressing HbF among the erythroid cells derived from SVF; rather, hypoxia caused a decrease in the number of cells expressing only HbA. A significant reduction in the level of α -globin, but not β -globin, was also observed.

Taken together, these results indicate the presence of early erythroid progenitors within the SVF and show that there are both differences in the regulation of globin gene expression at the transcriptional level and differences in the mechanisms that control the hemoglobin switch in these cells when compared to erythroid cells derived from hematopoietic tissues. Although this finding suggests that the origin of the early erythroid progenitors and therefore the origin of hematopoietic progenitors within the SVF is the adipose tissue rather than the hematopoietic tissue, this result is not conclusive. We cannot rule out that the adipose tissue niche might influence epigenetic patterns and the tissue-specific regulation of globin gene expression and hemoglobin production.

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COMMENTS

Background

The authors' previous observation of the existence of a small subset of CD45⁺ cells in the stromal vascular fraction (SVF) of human adipose tissue with hemangioblastic properties *in vitro* and with the ability to produce hematopoietic colony-forming units led to the determination of the molecular and functional features of the hematopoietic progenitors contained in SVF.

Research frontiers

In this study, the authors demonstrated that the cellular distribution of hemoglobin and globin gene expression in the erythroid cells produced by the early erythroid progenitors in SVF are different from that observed in the erythroid cells derived from CD34⁺ hematopoietic progenitors from cord blood and adult peripheral blood, indicating a different origin of these cells. However, it is also possible that the adipose tissue niche might influence epigenetic patterns. Further studies on the basic biology of adipose-derived primitive mesodermal cells

and the adipose tissue niche may be important for a better understanding of adult stem cells and for cell-based therapies.

Innovations and breakthroughs

Adipose tissue, similar to bone marrow, is derived from the embryonic mesoderm and contains a highly heterogeneous stromal cell population; however, unlike bone marrow, adipose is not a hematopoietic tissue. To our knowledge, this is the first report to demonstrate the existence of hematopoietic progenitors in human adipose tissue and reveal their characteristics at the cellular and molecular levels.

Applications

Evidence is provided that the erythroid progenitors contained in human adipose tissue are more primitive than those in the circulation and that the regulation of globin gene expression is tissue-specific. This study may provide new insights that will be helpful for elucidating the potential of adipose-derived primitive mesodermal cells and for understanding the characteristics and function of the adipose stem cell niche.

Terminology

Hematopoietic stem cells are responsible for the maintenance of all blood cell types and are characterized by their self-renewal capacity. These cells give rise to multipotent, oligopotent and unipotent hematopoietic progenitor cells. Burst-forming units-erythroid is the earliest erythroid progenitor responsible for erythrocyte generation.

Peer review

In the manuscript, the authors demonstrated that stromal vascular fraction of human adipose tissues expressed fetal hemoglobin. This is an interesting study. The methods of this study are reasonable and results are clear.

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Insulin producing cells established using non-integrated lentiviral vector harboring *PDX1* gene

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Abstract

AIM: To investigate reprogramming of human adipose tissue derived stem cells into insulin producing cells using non-integrated lentivirus harboring *PDX1* gene.

METHODS: In this study, human adipose tissue derived stem cells (hADSCs) were obtained from abdominal adipose tissues by liposuction, selected by plastic adhesion, and characterized by flow cytometric analysis. Human ADSCs were differentiated into adipocytes and osteocytes using differentiating medium to confirm their multipotency. Non-integrated lentiviruses harboring *PDX1* (Non-integrated LV-*PDX1*) were constructed using specific plasmids (pLV-HELP, pMD2G, LV-105-*PDX1*-1). Then, hADSCs were transduced with non-integrated LV-*PDX1*. After transduction, ADSCs^{*PDX1*+} were cultured in high glucose DMEM medium supplement by B27, nicotinamide and β FGF for 21 d. Expressions of *PDX1* and

insulin were detected at protein level by immunofluorescence analysis. Expressions of *PDX1*, neurogenin3 (Ngn3), glucagon, glucose transporter2 (Glut2) and somatostatin as specific marker genes were investigated at mRNA level by quantitative RT-PCR. Insulin secretion of hADSCs^{*PDX1*+} in the high-glucose medium was detected by electrochemiluminescence test. Human ADSCs^{*PDX1*+} were implanted into hyperglycemic rats.

RESULTS: Human ADSCs exhibited their fibroblast-like morphology and made colonies after 7-10 d of culture. Determination of hADSCs identified by FACS analysis showed that hADSCs were positive for mesenchymal cell markers and negative for hematopoietic cell markers that guaranteed the lack of hematopoietic contamination. *In vitro* differentiation of hADSCs into osteocytes and adipocytes were detected by Alizarin red and Oil red O staining and confirmed their multilineage differentiation ability. Transduced hADSCs^{*PDX1*+} became round and clusters in the differentiation medium. The appropriate expression of *PDX1* and insulin proteins was confirmed using immunocytochemistry analysis. Significant expressions of *PDX1*, Ngn3, glucagon, Glut2 and somatostatin were detected by quantitative RT-PCR. hADSCs^{*PDX1*+} revealed the glucose sensing ability by expressing Glut2 when they were cultured in the medium containing high glucose concentration. The insulin secretion of hADSCs^{*PDX1*+} in the high glucose medium was 2.32 μ U/mL. hADSCs^{*PDX1*+} implantation into hyperglycemic rats cured it two days after injection by reducing blood glucose levels from 485 mg/dL to the normal level.

CONCLUSION: Human ADSCs can differentiate into IPCs by non-integrated LV-*PDX1* transduction and have the potential to be used as a resource in type 1 diabetes cell therapy.

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Key words: Diabetes mellitus; Human adipose tissue

derived stem cells; Non-integrated lentiviruses; PDX1; Insulin producing cells

Core tip: Common treatments for diabetes mellitus are based on insulin injections and pancreas transplantation, limited by hypoglycemia, shortage of donors, immunosuppression and organ rejection. Cell therapy using human adipose tissue derived stem cells (hADSCs) offers a novel strategy for diabetes treatment without tumor formation and ethical concerns. Different viral vectors have been used for pancreatic differentiation. However, integration of provirus into host chromatin has induced insertional mutagenesis and malignancy. This is the first study to investigate the application of non-integrated Lentiviral vectors harboring PDX1 for differentiation of hADSCs into insulin producing cells and its usage in treatment of diabetic rats.

Boroujeni ZN, Aleyasin A. Insulin producing cells established using non-integrated lentiviral vector harboring *PDX1* gene. *World J Stem Cells* 2013; 5(4): 217-228 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v5/i4/217.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v5.i4.217>

INTRODUCTION

Type 1 diabetes mellitus is a devastating condition that affects millions of people worldwide. It is characterized by autoimmune destruction of pancreatic β -cells, leading to insufficient insulin production and increasing blood glucose level. Treatment of diabetes mellitus has been commonly performed by frequent insulin injections or by pancreas replacement. Some problems, such as immunosuppression and difficult surgery, limit the transplantation of pancreas tissue. Now, application of stem cell therapy has been under intensive investigation as another candidate for pancreatic β -cell replacement.

Stem cells can be converted to obtain required metabolic functions using genetic and epigenetic manipulations. In this way, embryonic stem cells (ESCs) derived from blastocysts^[1] and adult stem cells derived from different adult tissues^[2] have been genetically manipulated to have abilities like β -cell function^[3,4]. ESCs are pluripotent cells but are limited by immune rejection, tumorigenicity and ethical problems^[5]. Adult stem cells are not limited as such and can be obtained from many adult tissues, such as skin^[6], salivary gland^[7], liver^[8], spleen^[9], bone marrow^[10] and adipose tissue^[11-13]. Among them, adipose tissue derived stem cells (ADSCs) are good candidates for possible clinical applications without ethical concerns. They can undergo mesodermal differentiation as well as ectodermal and endodermal differentiation both *in vitro* and *in vivo*^[14,15]. ADSCs may become a useful target for β -cell replacement in diabetic patients because of their potential to adoption of characteristics^[16].

Application of stem cell therapy for diabetes treatment has been under investigation by working on differ-

ent kinds of genes, transfer vectors and differentiating mediums^[17]. Many transcription factors, such as PDX1, neurogenin3 and Nkx2.2, have been used for pancreatic cells differentiation. Variable transforming vectors, such as adenovirus, retrovirus, adeno-associated virus and lentivirus, have been used for gene transfer to cells^[18-21]. The homeodomain transcription factor PDX-1 is an important transcription factor of pancreatic islet development and function^[22]. PDX-1 induces differentiation of ADSCs into insulin-producing cells^[23,24] due to its ability to regulate downstream islet cell-specific gene expression and production of pancreatic hormones^[25-28]. This gene can be packaged in recombinant viral vectors to express PDX1 protein in the nucleus^[29].

Lentiviral vectors (LV) can deliver large cDNA to dividing and nondividing target cells^[30]. These vectors transduce a variety of cell types, including embryonic and adult stem cells for both *in vivo* and *ex vivo* gene therapy applications^[31]. However, integration of a viral genome into a host cell genome may induce insertional mutagenesis that has been highlighted by induction of malignancy in mouse models^[32] and development of leukemia in five patients in two clinical gene therapy trials^[33]. Improving safety and efficiency of LV has been achieved, for example, by modifications of packaging cassette on virus integrase gene or on other regions of virus genome. This virus is called non-integrated LV that cannot integrate into the host genome^[34]. The aim of this study was to transform hADSCs with non-integrated LV harboring PDX1 to obtain functional pancreatic beta-like cells with less genome manipulation.

MATERIALS AND METHODS

Collection and culture of human ADSCs

Human ADSCs were isolated from 80 to 100 mL aspirates from the abdominal fat tissue of normal donors. Informed consent was obtained from all participants and experiments performed according to the guidelines set with the Local Ethics Committee in the National Institute of Genetic Engineering and Biotechnology on Human Research. Lipoaspirate samples were collected into a sterile bottle with serum-free Dulbecco's modified Eagle's medium (DMEM). The samples were washed by phosphate buffered saline (PBS) and digested by collagenase solution (Sigma) at a final concentration of 0.075%. Digested tissues were centrifuged at 400 g for 10 min^[15]. Pellets were resuspended in erythrocyte lysis buffer and centrifuged at 300 g for 10 min. Pellets were cultured at a density of 2×10^6 cells/mL in 25 cm² plastic culture flask in low glucose DMEM containing 50% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin for 24 h. Non adherent cells were washed away with PBS and hADSCs were cultured in DMEM containing 20% FBS and antibiotics. Upon 80% confluency, the cells were harvested using 0.25% Trypsin-0.02% EDTA for 1-2 min at 37 °C and were kept frozen in liquid nitrogen for later use.

Table 1 Sequences of primers used in real-time reverse transcription-polymerase chain reaction and polymerase chain reaction experiments

Gene	Gene ID	Size (bp)	Strand	Sequence (5'–3')	Annealing temperature
<i>Somatostatin</i>	NM_001048.3	114	F	GCACCCGAGAACGCAAAGC	56
			R	TGTGGGGGCGAGGGATCAGAG	
<i>Ngn3</i>	NM_020999.3	241	F	GTAGAAAGGATGACGCCTCAACC	54
			R	TCAGTGCCAACTCGCTCTTAGG	
<i>Glucagon</i>	NM_002054.4	187	F	CAGTGCCTTGGTGCAGAAGT	58
			R	GGAACGTTGCCAGCTGCCTTG	
<i>Glut2</i>	NM_000340	300	F	AGCTTTCAGTTGGTGAAT	52
			R	AATAAGAATGCCGTGACGA	
<i>PDX1</i>	NM_000209.3	201	F	GTCCGGTGCCAGAGTTCAGT	63
			R	CCCAGTCTCGGTTCATTTCG	
β -actin	NM_001101.3	161	F	GAGACCTTCAACACCCAGCC	56
			R	AGACGCAGGATGGCATGGG	

Ngn3: neurogenin3; Glut2: glucose transporter2.

Flow cytometry analysis

Human ADSCs at the third passage were detached with trypsin-EDTA. The cells were centrifuged at 1500 rpm for 6 min and then were resuspended in PBS at the concentration of 1×10^6 /mL. The fluorescent labeled antibodies (10 μ L for each sample) were added and incubated for 30 min at room temperature^[35,36]. The labeled cells were analyzed on a FACS Caliber (Becton-Dickinson, Franklin Lakes, NJ, United States) following labeled antibodies against human CD45-FITC/CD34-PE, CD31-FITC/CD73-PE, CD90-FITC/CD105-PE, CD11b-PE, CD44PE/ CD106-PEcy5, CD16-FITC/CD29-PE, CD14-PE/CD55-PE5 (Scotect, United States).

Multilineage differentiation of hADSCs

Human ADSCs were differentiated into adipocytes and osteocytes using differentiating medium to confirm their multipotency ability. For differentiation into adipocytes, third-passage hADSCs were plated at 30000 cells per cm² in 6-well plates in adipocyte differentiation medium (Gibco) consisting of DMEM, 10% FBS, 0.5 mmol/L 3-isobutyl-1 methylxanthine, 1 mmol/L dexamethasone, 200 mmol/L indomethacin and 10 mmol/L insulin for two weeks. Medium was changed twice a week. Cultured cells were stained with Oil Red O to detect lipid accumulation in differentiated cells^[4,14,37,38].

For osteogenic differentiation, third passage hADSCs were cultured in osteogenic medium containing DMEM, 10% FBS, 0.1 mol/L dexamethasone, 50 mol/L ascorbate-2-phosphate and 10 mmol/L glycerophosphate for two weeks. Cultured cells were stained with Alizarin Red to detect bone matrix in differentiated cells^[3,14,37,38].

Construction of the non-integrated LV harboring PDX1

HEK293 cells were cultured in 75 cm² plastic culture flask in high glucose DMEM and were prepared to a level of confluency. The transfer construct LV-105 harboring PDX1 (GeneCopia) was co-transfected with the enveloped plasmid pMD2G (InvivoGen) and the packaging plasmid pLV-HELP (InvivoGen) into HEK 293T cell culture. The culture medium was changed 14 h after

transfection. One day after medium change, active lentiviral vectors were released in culture medium. Culture medium was removed and viral vectors become concentrated with MILLIPORE falcons and kept in -80 °C for further usage^[36].

Confirmation of virus construction by detection of PDX1 in HEK 293

Total RNA was extracted using RNA X PLUS (CinnaGen Co) and first-strand cDNA was prepared using cDNA RT Kit (Fermentas). RT-PCR was prepared in a 20 μ L reaction volume by appropriate primers for PDX1 gene (Table 1). After amplification, 5 μ L of products were loaded on a 2% agarose gel. DNA bands were stained and visualized by UV transilluminator.

Cytopathic effect of non-integrated LV-PDX1

The MTT assay was performed on transduced hADSCs to determine the cytopathic effect of non-integrated LV-PDX1. The transduced and untransduced hADSCs culture mediums were replaced with a volume of 100 μ L high glucose DMEM medium containing 10% tetrazolium dye 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) solution in the 96-well plates and incubated for 3 hours at 37 °C. The medium was changed with 100 μ L DMSO solution and incubated for 10 minutes at room temperature to develop the purple color. The optical density (OD) of DMSO solution was measured at 580 nm to determine the relative cell viability.

Transduction of ADSCs with non integrated LV-PDX1

Third passage human ADSCs (2×10^5 cells/mL) were cultured in a 25 cm² plastic culture flask in low glucose DMEM and 10% FBS for 24 h. Concentrated non-integrated LV-PDX1 (100 μ L) was added to cultures and incubated for 12 hours. The viral transduction was repeated twice to obtain better PDX1 expression in hADSCs and exposed to 2.5 μ g/mL puromycin for 2 d to obtain stable transduction^[36]. The selection was continued with 2 μ g/mL puromycin for 3 wk until individual colonies expressing PDX1 appeared.

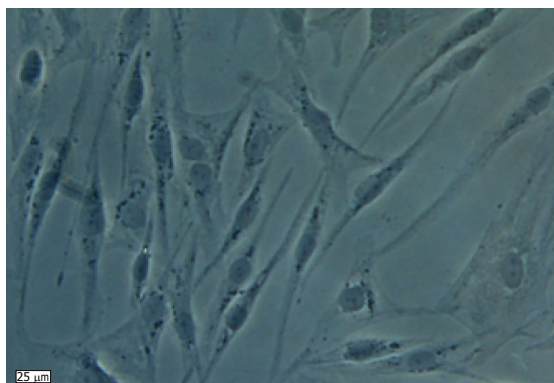


Figure 1 *In vitro* morphology of human adipose derived stem cells after collagenase digestion and plastic adherence selection. In the primary human adipose tissue derived stem cell culture, cells exhibited a spindle-shaped fibroblastic morphology in colonies after 7-10 d.

Induction of differentiation in hADSCs^{PDX1+} into insulin producing cells

Human ADSCs^{PDX1+} were cultured in the medium containing high glucose (25 mmol/L) DMEM, 2% FBS, 10 mmol/L nicotinamide and 2% B27 for 7 d. Culture medium was later supplemented with FGFβ (1 μL/mL) for 14 d^[35]. A parallel negative control of untransduced hADSCs with the same condition in the culture medium was prepared to compare the morphological changes.

Analysis of glucagon, Glut2, PDX1, Ngn3 and somatostatin gene expressions by real time RT-PCR

Expression of beta cell marker genes consisted of PDX1, Ngn3, Glut2, glucagon and somatostatin and were determined in transduced and untransduced hADSCs in three weeks of cell culture in comparison to *β-actin* gene which was used as housekeeping B acting gene using quantitative RT-PCR^[4,36]. Total mRNA was extracted from all triplicate groups cell culture using RNA X PLUS (CinnaGen Co). The first and second cDNA was prepared using cDNA RT Kit (Fermentas). RT-PCR was performed using *AccuPower*® 2X Greenstar qPCR Master Mix Kit (Bioneer) in Rotor-GeneTM 6000 (Corbett) thermal cycler. Primer sequences and their annealing temperature and products length are shown in Table 1. The identity of PCR products was confirmed by electrophoresis and sequencing. Their relative gene expression data were analyzed using 2^{-ΔΔCt} method^[39].

Immunofluorescence cytochemistry for insulin and PDX1 proteins expression

Transduced and untransduced hADSCs (10⁵) were seeded on glass cover slides in triplicate cultures using 6-well culture plates for 3 wk. Cells were washed with PBS containing 0.05% TWEEN 20 and fixed with cold absolute methanol for 5 min and washed with PBS 1% Triton X-100 for permeabilization of the cells. Cells were treated with 1% BSA (bovine serum albumin) for 20 min at room temperature and washed with PBS 1% Tritone X-100. Cells were reacted separately with primary rabbit

anti PDX1 antibody (1:1000, Abcam) and primary guinea pig polyclonal anti insulin (1:200, Abcam) overnight in 37 °C. After washing with PBS 1% Tritone X-100, they were incubated with FITC-conjugated goat anti-rabbit IgG (1:250, Abcam) and FITC-conjugated goat anti-guinea pig IgG for 45 min in a dark room. Cells were reacted with 4,6-diamidino-2-phenylindole in a dark room for 10 minutes and washed with PBS^[21]. The signals were observed under a fluorescence microscope (Zeiss, Axioplan, Germany) and the images were taken with connected digital camera.

Insulin secretion assay

To assay the insulin secretion of transduced cells, triplicate culture groups from transduced and untransduced hADSCs were prepared and washed by PBS buffer before low glucose DMEM (5.5 mmol/L glucose) containing 2% FBS was added in the media and incubated for 24 h. Transduced and untransduced hADSCs were washed again with PBS and incubated in high glucose DMEM (25 mmol/L glucose) containing 2% FBS for 24 h^[35]. Culture mediums were collected and measured insulin contents using electrochemiluminescence test^[36] performed in Bahar Laboratory, Tehran, Iran.

In vivo ADSCs^{PDX1+} transplantation in rats

Hyperglycemia was induced in 20 Sprague-Dawley adult male rats through intraperitoneal injection of 120 mg/kg of alloxan monohydrate (Sigma) that resolved in sodium citrate solution^[40]. All experimental procedures were approved and supervised by the National Institute of Genetic Engineering and Biotechnology Animal Care and Use Committee which conforms to the principles laid down by the National Academy of Science for the care and use of laboratory animals.

Rats were housed individually in special clear sided cages at 22 °C and 12:12 light: dark cycle. After two weeks adaptation period, rats were fasted for 12 h before an intraperitoneal injection of 120 mg/kg of alloxan. Blood glucose level was determined using a blood glucose meter before and after alloxan injection. Rats were diabetic if their blood glucose ranges reached levels between 400 and 600 mg/dL and were maintained stably for one week. Diabetic rats were divided into three groups, each contained 7 diabetic rats. One group of diabetic rats received an intraperitoneal transplant of 4 × 10⁶ semi differentiated hADSCs^{PDX1+} (5 d after transduction) that was harvested with trypsin and resuspended in 500 μL of PBS. Another group of diabetic rats were injected IP with 4 × 10⁶ untransduced hADSCs. The third group of diabetic rats was not injected as control diabetic rats. Blood glucose levels were monitored twice a week after cell injection using the glucose oxidase method by ACCU-CHEK active strips (Roche Diagnostics, Germany).

Statistical analysis

For gene expression and insulin secretion comparison among transduced and untransduced hADSCs, statistical

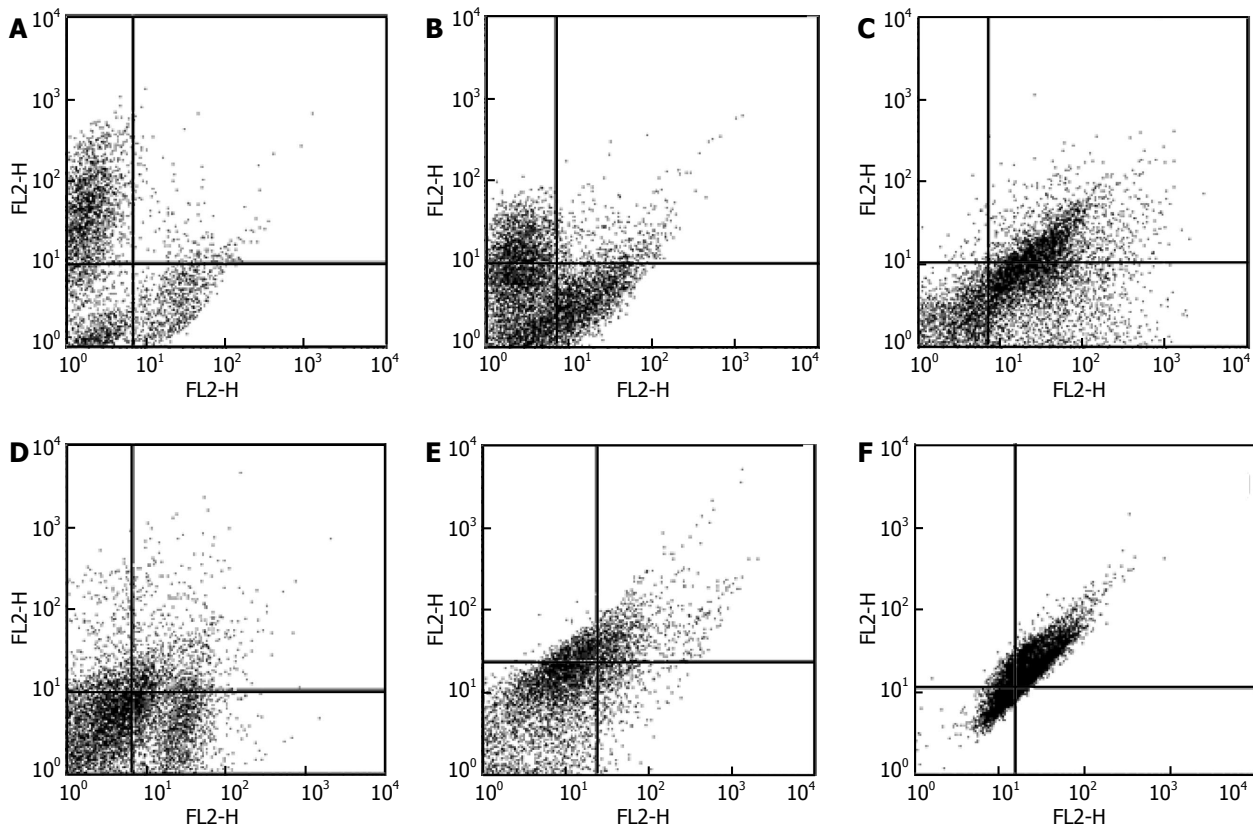


Figure 2 Characterization of human adipose tissue derived stem cells by flow cytometry. Human adipose tissue derived stem cells in third passage were tested against the following antibodies and analyzed by FACS Caliber. A: CD45-FITC/CD34-PE; B: CD31-FITC/CD73-PE; C: CD90-FITC/CD105-PE; D: CD16-FITC/CD29-PE; E: CD44PE/CD106-PEcy5; F: CD14-PE/CD55-PE5. MSCs were negative for CD31, CD34 and CD45 and positive for CD44, CD73, CD90, CD105, CD29, CD106 and CD55.

analysis was performed by one-way analysis of variance using SPSS version 12.0 (SPSS, Chicago, United States). In all statistical analyses, $P < 0.05$ was statistically judged significant and all P values were two-sided.

RESULTS

Isolation and characterization of hADSCs

Human ADSCs were isolated from abdominal adipose tissue through collagenase digestion successfully and expanded in DMEM culture medium at a density of 2×10^4 cells/mL after plastic adherence selection. Hematopoietic cells were removed during subsequent changes of medium and passaging. Human ADSCs exhibited their fibroblast-like morphology and made colonies after 7-10 d of culture (Figure 1). The hADSCs were harvested and labeled with antibodies against CD73, CD90, CD29, CD34, CD31, CD45, CD105, CD16, CD29, CD14, CD55, CD106 and CD44. Determination of hADSCs identity by FACS analysis showed that hADSCs were negative for CD31, CD34 and CD45, which guaranteed the lack of hematopoietic contamination. These cells expressed high levels of CD44, CD73, CD90, CD105, CD29, CD106 and CD55 (Figure 2).

Differentiation capability of hADSCs towards adipocytes and osteocytes

Human ADSCs were differentiated into mesodermal

lineage, such as adipocytes and osteocytes, by a differentiating medium for confirming their multilineage differentiation ability. *In vitro* differentiation of hADSCs into osteocytes and adipocytes was detected by Alizarin red and Oil red O staining. Human ADSCs contained adipose (Figure 3A) and calcium granules (Figure 3B) after 3 wk of treating with adipogenic and osteogenic mediums.

Production of non-integrated LV-PDX1 in HEK 293 cells

The actual non-integrated LV-PDX1 vector was amplified and packaged in HEK 293 cells. The sign of viral production was an early color change in the medium from red to yellow, compared to non transfected HEK 293. As viral production proceeded, some of the cells rounded up and detached from the plate. The culture medium contained active non-integrated LV-PDX1 vector and was collected two days after transfection. The activity and identity of isolated non-integrated LV-PDX1 were confirmed by infecting the new culture of HEK293 cells followed by RNA extraction and cDNA synthesis. PDX1 mRNA expressions were detected by RT-PCR in HEK293.

Isolation of hADSCs^{+PDX1}

Transduced hADSCs culture was screened against puromycin antibiotic for 2 wk for hADSCs^{PDX1+} selection from untransduced hADSCs. Expression of PDX1 was confirmed by amplification of 201 bp fragments from

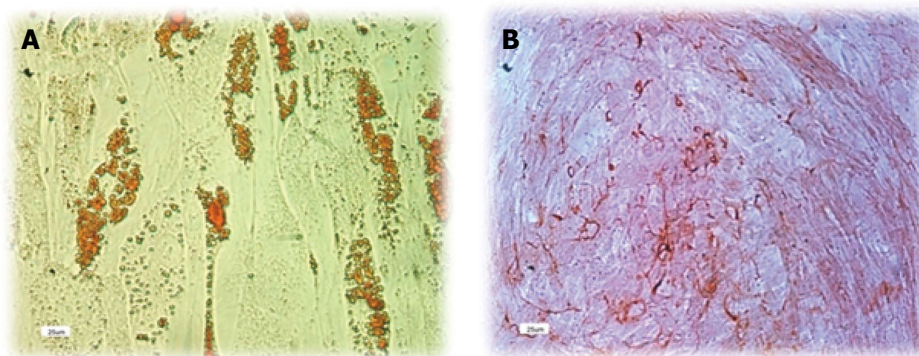


Figure 3 Multi lineage differentiation ability of human adipose tissue derived stem cells. Human adipose tissue derived stem cells of 3rd passage were cultured in specific differentiation mediums for 14-21 d. A: Oil Red O staining shows the presence of fat vacuoles in adipocytes; B: Differentiation into the osteocyte lineage was shown by staining with Alizarin.

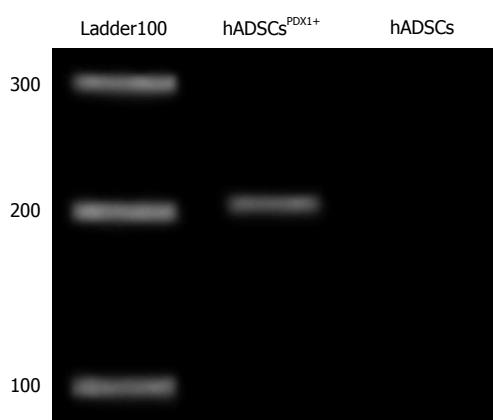


Figure 4 Real-time reverse transcription-polymerase chain reaction of *PDX1* gene from puromycin resistance human adipose tissue derived stem cells^{PDX1+}. Expression of *PDX1* gene in transduced human adipose tissue derived stem cells (hADSCs) was confirmed by amplification of a 201 bp fragment compared to no amplification in control hADSCs.

hADSCs^{PDX1+}, compared to no amplification from control hADSCs (Figure 4).

MTT viability test

The optimum volumes for viral suspension of non-integrated LV-PDX1 that has been used in hADSCs transductions were obtained in MTT test as 0.8 μ L/15000 hADSCs. Increasing the volume of non-integrated LV-PDX1 more than 0.8 μ L/15000 hADSCs resulted in significant decrease in viability and optical density (OD) of cells (Figure 5).

Differentiation and morphological changes of transduced hADSCs^{PDX1+}

Untransduced hADSCs were typically adherent as a spindle shape (Figure 6A). Transduced hADSCs^{+PDX1} became oval-shaped cells (Figure 6B) and clusters appeared using culture medium containing high glucose (25 mmol/L) DMEM, nicotinamide and B27 (Figure 6C). These cells continued to differentiate and eventually resulted in larger clusters under high glucose (25 mmol/L) DMEM, nicotinamide and B27 supplemented with FGF β (Figure 6D).

Analysis of pancreatic marker genes expression in hADSCs^{PDX1+}

Differentiation of hADSCs^{PDX1+} towards insulin pro-

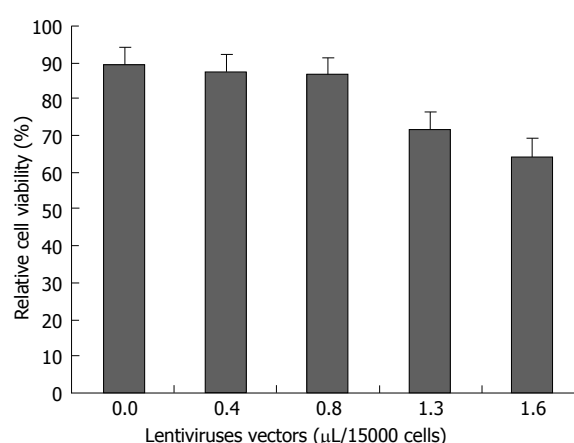


Figure 5 Relative cell viability of transduced human adipose tissue derived stem cells compared to untransduced human adipose tissue derived stem cells determined by MTT assay. In the horizontal axis, the first value 0.0 is related to untransduced human adipose tissue derived stem cells (hADSCs) without any virus transduction. The optimum volume of non-integrated lentiviruses harboring *PDX1* was obtained as 0.8 μ L/15000 hADSCs that has been used for cell transductions.

ducing cells was performed using specific culture media consisting of high glucose DMEM, 2% B27, 2% FBS, 10 mmol/L nicotinamide and β FGF. To investigate endocrine pancreatic cell characteristics, expression of pancreatic marker genes such as *PDX1*, *Ngn3*, *glucagon*, *somatostatin* and *Glut2* were studied by quantitative RT-PCR from hADSCs^{PDX1+}, hADSCs and pancreatic cells cDNAs isolated in the 1st and 3rd week after first viral transduction (Figure 7). Expressions of *PDX1* and *Ngn3* were high after the first week and decreased in the 3rd week in hADSCs^{PDX1+} culture compared to the untransduced one. On the other hand, expression of somatostatin, glucagon and *Glut2* increased in the 3rd week compared to the 1st week (equal to 0.0), which indicates that differentiated hADSCs^{PDX1+} had obtained the glucose-sensing ability after 3 wk (Figure 7). Reduction in *Ngn3* expression in the 3rd week in hADSCs^{PDX1+} is an indication for the phenotypical differentiation toward beta cell characteristics. However, no differentiations happened in untransduced hADSCs since no expressions were detected for *PDX1*, *Ngn3*, *Glut2* and glucagon and somatostatin genes were observed in untransduced hADSCs after the 1st and 3rd week of culture.

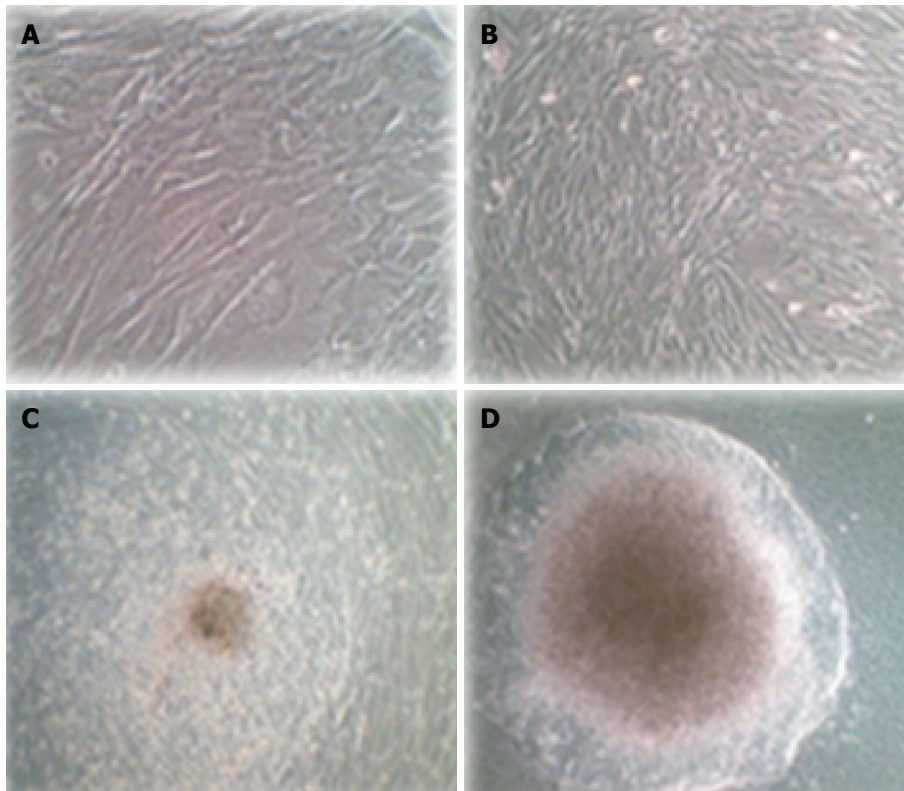


Figure 6 Morphological differentiation of human adipose tissue derived stem cells^{PDX1+} three weeks after second transduction cultured in differentiation medium containing high glucose Dulbecco's modified Eagle's medium supplement by B27, nicotinamide and β FGF. A: Untransduced human adipose tissue derived stem cells (hADSCs) were a typically adherent spindle shape; B: Human ADSCs^{PDX1+} became oval-type cells after second transduction; C: Human ADSCs^{PDX1+} formed clusters in the medium containing high glucose Dulbecco's modified Eagle's medium (DMEM), B27 and nicotinamide 7 d after transduction; D: Human ADSCs^{PDX1+} continued to differentiate and formed larger clusters in 3 wk in the medium containing high glucose DMEM, B27, nicotinamide and β FGF.

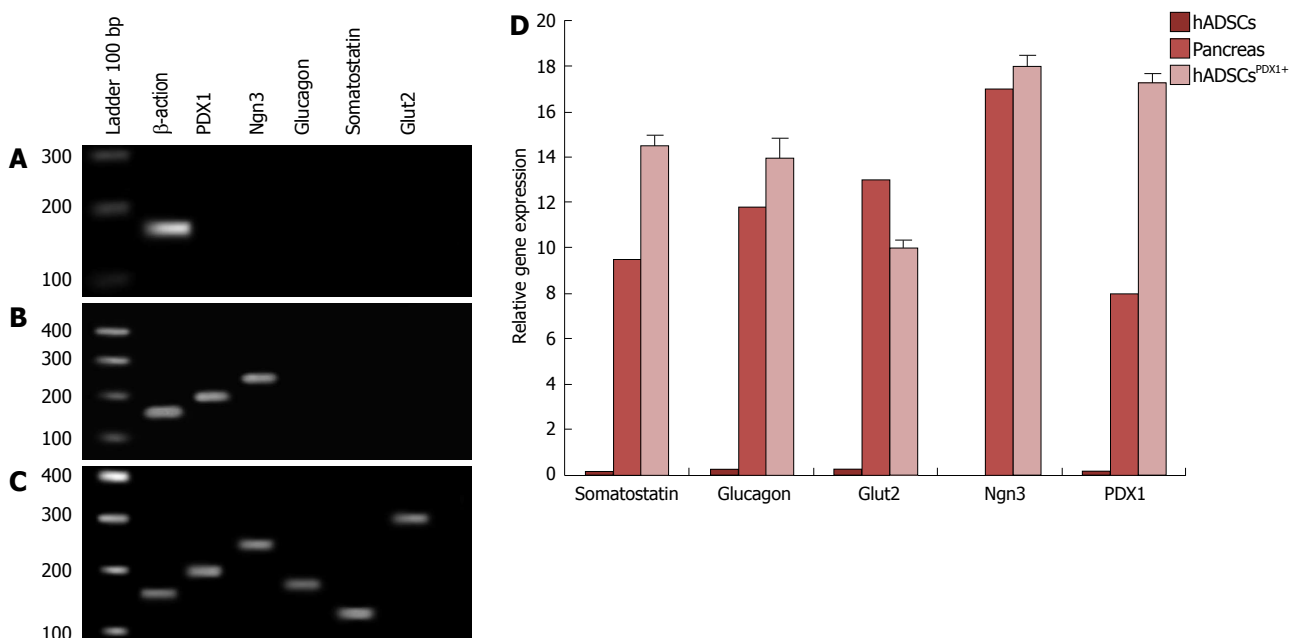


Figure 7 Experiment of pancreatic marker genes consisting of *Glut2*, *PDX-1*, *Ngn3*, *somatostatin* and *glucagon* in human adipose tissue derived stem cells^{PDX1+} compared to human adipose tissue derived stem cells (negative control) and human pancreatic tissue (positive control) by real time PCR. A: In human adipose tissue derived stem cells (hADSCs), no tested pancreatic markers were amplified except B-Actin as internal real-time reverse transcription-polymerase chain reaction positive control; B: Expressions of *PDX-1* and *Ngn3* markers in hADSCs^{PDX1+}, 7 d after second viral transduction; C: Expressions of *PDX1*, *Ngn3*, *glucagon*, *somatostatin* and *Glut2* markers in hADSCs^{PDX1+}, 21 d after second viral transduction indicating for beta like cell differentiation; D: Relative gene expression of *PDX1*, *Ngn3*, *glucagon*, *somatostatin* and *Glut2* was mostly increased in hADSCs^{PDX1+} compared to human pancreatic tissue on the 21st day after second viral transduction. All PCR products were verified by electrophoresis and sequencing and relative gene expression data were analyzed using $2^{-\Delta\Delta Ct}$ method.

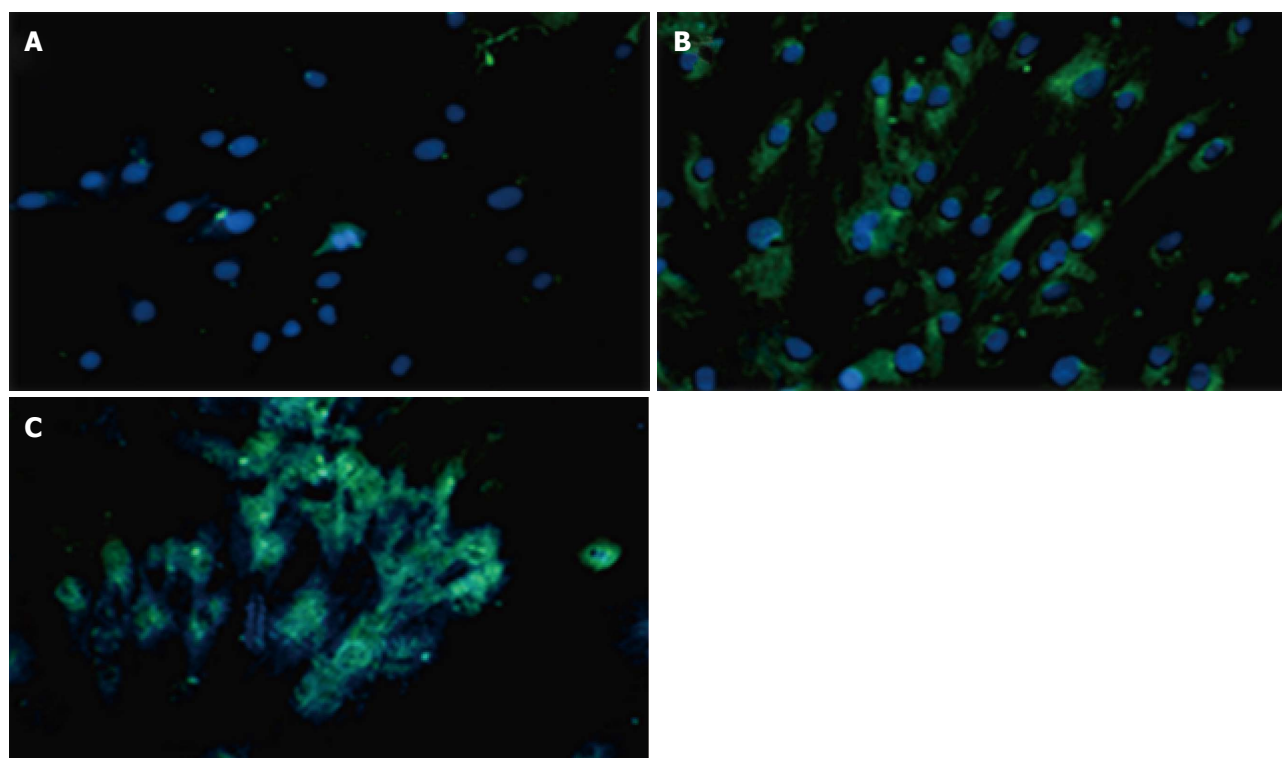


Figure 8 The immunocytochemistry experiments using PDX1 and insulin antibodies 7 d after transduction of human adipose tissue derived stem cells with non-integrated lentiviruses harboring PDX1 and selection of transduced cells with puromycin. FITC conjugated PDX1 antibody (green color) and FITC conjugated insulin antibody (green color) were used for immune staining of PDX1 and insulin proteins and DAPI (blue color) was used for nuclear staining. A: Untransduced human adipose tissue derived stem cells (hADSCs) represented no green signals; B: hADSCs^{PDX1+} represented green signals related to insulin expression in the cytoplasm; C: hADSCs^{PDX1+} represented green signals related to PDX1 expression in the nucleus.

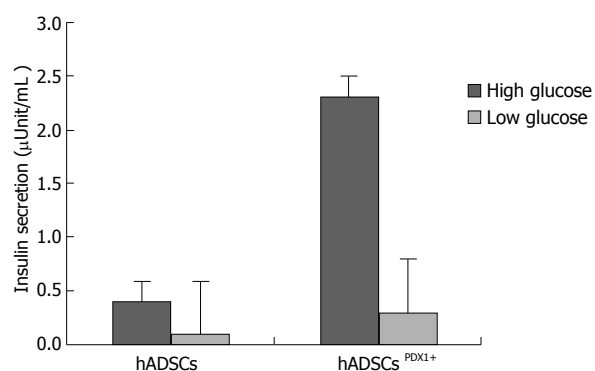


Figure 9 Insulin secretion assay in culture medium of human adipose tissue derived stem cells^{PDX1+} and human adipose tissue derived stem cells after 3 wk. It was performed using electrochemiluminescence test (Bahar lab, Tehran, Iran). Insulin concentration was increased to 2.32 fold in human adipose tissue derived stem cells (hADSCs)^{PDX1+} compared to hADSCs control, when it was exposed to a high glucose concentration.

Immunofluorescence cytochemistry

Expressions of PDX1 and insulin proteins were shown in transduced hADSCs^{+PDX1} using anti-PDX1 and anti-insulin antibodies, which were represented as green dots in the immunofluorescence assay (Figure 8). This result illustrated that hADSCs^{PDX1+} are capable of activating the endogenous insulin promoter in high glucose medium containing 25 mmol/L glucose.

Insulin secretion assay

Induction of insulin excretion from hADSCs^{PDX1+} 2.32 fold was shown using electrochemiluminescence assay, a sensitive method for insulin secretion assay, in high glucose medium compared with untransduced hADSCs (Figure 9).

Treatment of diabetic rats after hADSCs^{PDX1+} transplantation

The potential of differentiated hADSCs^{PDX1+} to diabetes treatment was confirmed by hADSCs^{PDX1+} transplantation into 20 alloxan-induced diabetic rats. Two rats died during the anesthetic procedure and the remaining hyperglycemic rats were divided into three groups, each contained 6 diabetic rats. Fasting blood glucose level of all twenty rats before alloxan injection was 160-200 mg/dL. After 12-14 d of receiving 120 mg/kg alloxan, the blood glucose levels in diabetic rats increased to 400-500 mg/dL with approximately 20% weight loss. After two weeks of stable hyperglycemia, two groups of diabetic rats were injected with $3-4 \times 10^6$ semi differentiated hADSCs^{PDX1+} (5 d after transduction) and untransduced hADSCs respectively by IP injection and the remaining third group was kept as a control for stability of diabetes in rats. The blood glucose level of diabetic rats transplanted with hADSCs^{PDX1+} was gradually decreased and normalized within 3-4 d after transplantation. After transplantation, the blood glucose level of untransduced hADSCs transplanted rats decreased 10 d after transplantation tran-

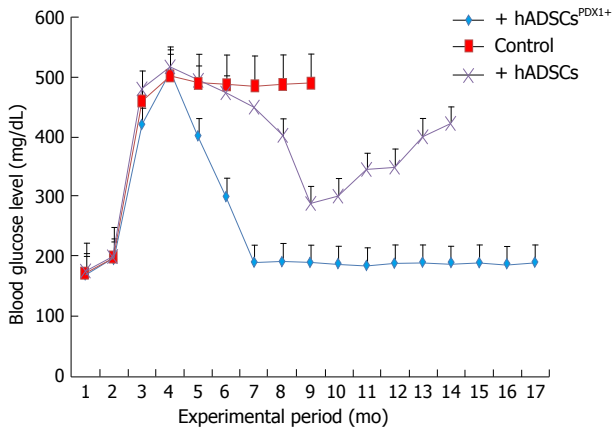


Figure 10 *In vivo* animal study of hyperglycemia. Treatment by transplantation of human adipose tissue derived stem cells^{PDX1+} and human adipose tissue derived stem cells (4×10^6 cells/rat). Before alloxan injection, the blood glucose level was 180 ± 20 and it reached 500 ± 20 mg/mL two weeks after injection. Six alloxan induced diabetic rats were transplanted with human adipose tissue derived stem cells (hADSCs)^{PDX1+} which resulted in decreasing the blood glucose level to 200 ± 20 mg/mL after 3 d. The blood glucose levels of 6 rats transplanted with hADSCs were decreased transiently to 300 ± 20 mg/mL after 10 d and increased again. Control group which consisted of 6 diabetic rats untransplanted were hyperglycemic and died within 6 mo.

siently and increased again. Tumor formation was not observed in any transplanted rats. Control untransplanted diabetic animals remained hyperglycemic and died within 6 months (Figure 10). Our results suggested that the differentiated hADSCs^{PDX1+} may have responded to hyperglycemia in diabetic rats, which represent their possible glucose sensing ability.

DISCUSSION

Mesenchymal stem cells such as hADSCs are attractive therapeutic candidates for insulin dependent diabetes treatment in the clinical setting due to their immunomodulatory properties. hADSCs can modulate immune responses in recipients by releasing co-stimulatory and regulatory cytokines to control autoreactive T cells, B cells proliferation and differentiation, natural killer cells proliferation and dendritic cells maturation, antibody production and chemotactic abilities^[41]. hADSCs express intermediate levels of major histocompatibility complex (MHC) class I molecules and no level of MHC class II molecules on their cell surface that allow their transplantation across MHC barriers^[41]. They stimulate the production of regulatory T cells that inhibit lymphocyte proliferation in allogeneic transplants^[42].

Human ADSCs were isolated from abdominal fat tissues and characterized by flow cytometric analysis. The phenotype of hADSCs was negative for hematopoietic markers, such as CD34 and CD45, and was positive for MSC markers, such as CD44, CD73, CD90, CD105, CD29, CD106 and CD55. Insulin production and secretion of differentiated hADSCs^{PDX1+} were detected using immunofluorescence analysis (Figure 8) and electrochemiluminescence (ECL). Insulin secretion measured by

ECL was equal to $2.32 \mu\text{U/mL}$ in hADSCs^{PDX1+} culture medium but was $0.0 \mu\text{U/mL}$ in control hADSCs culture medium (Figure 9).

Differentiation of mesenchymal stem cells into beta like cells without gene transformation has been reported^[35,43]. However, some animal products such as neuronal conditioned medium have been used for induction of differentiation that may have some complications in clinical usage. Animal products may transmit pathogens, induce antibodies and contaminate with prion particles, nanobacteria, mycoplasma and endotoxins. Proteins exist in animal products that may contaminate stem cells and induce a possible risk of transmitting unknown infectious agents and the risk of initiating xenogeneic immune responses. These antigenic responses may affect the viability, safety and efficacy of transplanted MSCs. In addition to these technical problems, it is inhumane to kill many animals for the collection of animal products^[44].

Lentiviral vectors can deliver transgenes to a wide variety of dividing and nondividing cells and maintain stable long-term transgene expression^[45]. However, integration of a provirus into host chromatin has induced some adverse issues, such as insertional mutagenesis and promoter interference and positional effects have been mentioned by induction of malignancy in some therapeutic trials in human and mouse models^[31,32]. Development of non-integrated lentiviral vectors to deliver transgenes has provided adequate safety and efficiency in clinical applications due to modifications in the packaging cassette that have limited the potential risk for insertional mutagenesis and replication competent of lentiviruses^[34]. This is the first study to investigate the application of non-integrated LV harboring PDX1 in differentiation of hADSCs into islet-like insulin producing cells and in treatment of insulin dependent diabetes in diabetic rat models. Previous attempts used integrated LV harboring PDX1 for differentiation of bone marrow mesenchymal stem cells into islet-like insulin producing cells^[36]. In order to promote insulin secretion, some chemical materials, such as B27, nicotinamide and βFGF , were added into the culture medium of hADSCs^{PDX1+}. Insulin production of hADSCs^{PDX1+} was glucose responsive, which represented efficiency of non-integrated LV vector in strong exogenous gene expression in transplanted cells over other viral vectors^[28]. The nuclear targeting of non-integrated LV vectors enables transduction of both dividing and non-dividing cells, a suitable attribute for a gene therapy vector^[29].

The ectopic PDX1 activated a number of genes, such as Glut2 and Ngn3, which are crucial in cellular insulin production and secretion in respect to sensing environmental glucose level. Ngn3 is not found in mature pancreatic islets^[46] and low expression level of Ngn3 observed in differentiated hADSCs^{PDX1+} after 21 d compared to 7 d, which indicated that these cells may partly resemble mature islet precursor cells that retain its proliferation ability. Detection of alpha and delta cell phenotypes related gene expression, such as glucagon and somatostatin, could be a reflection for multiple islet

genes expression in differentiated hADSCs.

Experimental data for the therapeutic effects of hADSCs^{PDX1+} in the diabetic animal models are necessary tools for safety analyzing before human clinical applications. Alloxan-induced diabetic rats have been used for *in vivo* analysis of allogeneic transplantation of hADSCs^{PDX1+}. Differentiated hADSCs^{PDX1+} could normalize diabetic rat blood glucose levels after three days of cell transplantation without any immune responses. No tumor formation was observed even after 12 mo of follow up of treated rats.

In conclusion, our results demonstrate that hADSCs is a suitable source of cells capable of differentiating into insulin producing and secreting cells, both phenotypically and functionally by non-integrated LV-PDX1 and appropriate microenvironment chemical materials. This hADSCs^{PDX1+} may be used as a source for autologous or allogeneic transplantation for β -cell replacement in the treatment of insulin dependent diabetes.

COMMENTS

Background

Diabetes mellitus is characterized by autoimmune destruction of pancreatic β -cells, leading to insufficient insulin production. Now, application of stem cell therapy has been under intensive investigation as a new candidate for pancreatic β -cells replacement. Adipose tissue derived stem cell is an attractive therapeutic candidate for insulin dependent diabetes treatment in the clinical setting due to its immunomodulatory properties. Application of stem cell therapy for diabetes treatment has been under investigation by working on different kinds of genes, transfer vectors and differentiation medium. Lentiviral vectors (LV) can transduce a variety of cell types both *in vivo* and *ex vivo*. Many transcription factors, such as PDX1, Ngn3 and Nkx2.2, have been used for pancreatic cells differentiation. The homeodomain transcription factor PDX-1 is an important transcription factor of pancreatic islet development and function.

Research frontiers

In the area of treatment of diabetes mellitus using stem cells, the research hotspot is how to differentiate the stem cells by chemicals and gene transduction with different vectors and genes. Many transcription factors, such as PDX1, Ngn3 and Nkx2.2, have been used for pancreatic cells differentiation. Previous attempts used adenoviruses or integrated LV harboring PDX1 for differentiation of mesenchymal stem cells into islet-like insulin producing cells.

Innovations and breakthroughs

In previous diabetes treatment methods, it was found that some animal products such as neuronal conditioned medium used for induction of pancreatic differentiation may have some complications in clinical usage. Animal products may transmit pathogens, induce antibodies and transmit contaminations with prion particles, nanobacteria, mycoplasma and endotoxins. Also, integration of a provirus into host chromatin has induced some adverse issues, such as insertional mutagenesis and malignancy, in some therapeutic trials in human and mouse models. Adenoviral vectors induce an immunological response and transient expression of transgenes. In previous attempts, insulin expression has been reported from partially differentiating embryonic stem cells and mesenchymal stem cells derived from different tissues. Embryonic stem cells are pluripotent cells but are limited by the immune rejection, tumorigenicity and ethical problems. The novelty of this study was to show the application of non-integrated LV harboring PDX1 that is not able to integrate into the host genome and to solve the induced insertional mutagenesis problem that can induce possible malignancies in cell differentiation.

Applications

The study shows that non-integrated LV-PDX1 can be used as a powerful vector in differentiation of hADSCs towards beta like cells. These cells have less potential for malignancy and can be used with less concern in autologous or allogeneic β -cell replacement in the treatment of insulin dependent diabetes.

Terminology

Diabetes mellitus (type 1): Type 1 diabetes mellitus is a devastating condition

that affects millions of people worldwide. Diabetes mellitus is characterized by autoimmune destruction of pancreatic β -cells, leading to insufficient insulin production and increasing blood glucose level; PDX1: The homeodomain transcription factor PDX-1 is an important transcription factor of pancreatic islet development and function. PDX-1 has ability in regulating of downstream islet cell-specific gene expression and production of pancreatic hormones.

Peer review

Overall, a very nice study. It is well written.

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Neural stem cells isolated from amyloid precursor protein-mutated mice for drug discovery

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Abstract

AIM: To develop an *in vitro* model based on neural stem cells derived from transgenic animals, to be used in the study of pathological mechanisms of Alzheimer's disease and for testing new molecules.

METHODS: Neural stem cells (NSCs) were isolated from the subventricular zone of Wild type (Wt) and Tg2576 mice. Primary and secondary neurosphere generation was studied, analysing population doubling and the cell yield per animal. Secondary neurospheres were dissociated and plated on MCM Gel Cultrex 2D and after 6 d *in vitro* (DIVs) in mitogen withdrawal conditions, spontaneous differentiation was studied using specific neural markers (MAP2 and TuJ-1 for neurons, GFAP for

astroglial cells and CNPase for oligodendrocytes). Gene expression pathways were analysed in secondary neurospheres, using the QIAGEN PCR array for neurogenesis, comparing the Tg2576 derived cell expression with the Wt cells. Proteins encoded by the altered genes were clustered using STRING web software.

RESULTS: As revealed by 6E10 positive staining, all Tg2576 derived cells retain the expression of the human transgenic Amyloid Precursor Protein. Tg2576 derived primary neurospheres show a decrease in population doubling. Morphological analysis of differentiated NSCs reveals a decrease in MAP2- and an increase in GFAP-positive cells in Tg2576 derived cells. Analysing the branching of TuJ-1 positive cells, a clear decrease in neurite number and length is observed in Tg2576 cells. The gene expression neurogenesis pathway revealed 11 altered genes in Tg2576 NSCs compared to Wt.

CONCLUSION: Tg2576 NSCs represent an appropriate AD *in vitro* model resembling some cellular alterations observed *in vivo*, both as stem and differentiated cells.

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Key words: Neural stem cells; Alzheimer's disease; Neuron maturation; Drug discovery

Core tip: In this study neural stem cells isolated from Tg2576 mice are characterized as an *in vitro* model for Alzheimer's disease. These cells represent a robust system for studying pathological mechanisms related to Aβ intracellular accumulation, such as stem cell status, or during differentiation processes. This model could provide a new cell platform for developing and screening new molecules.

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INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia, affecting 24.3 million people worldwide^[1] with a 10%-11% and 14%-17% estimated lifetime risk respectively for males and females at age 85^[2]. The cost of caring for afflicted patients is enormous and beyond the capability of most developing countries^[3] and poses a serious problem also for the gross national product (GNP) of western countries^[4]. The prevalence and incidence rates increase exponentially with age^[5] and ageing is actually the only risk factor identified with certainty^[6].

Today the pathophysiology of this disease is still to be elucidated, resulting in a total absence of disease-modifying therapies. However, the finding of amyloid β (A β) peptide deposits in the brains of affected individuals^[7] and the study of the causes of the Familial AD (FAD) indicated A β peptide production as a possible therapeutic target^[8].

The lack of effective therapies leads to the need for new models for drug discovery and screening. In this way stem cells represent a promising tool to create *in vitro* models suitable for studying disease mechanisms, for pharmacological target identification and for drug screening^[9].

In particular, the possibility of deriving primary cultures of neural stem cells (NSCs) from animal models of disease at different ages provides an opportunity to derive cell types carrying human mutations. In this context, the Tg2576 mice, expressing the 695 isoform of the human Amyloid precursor protein (APP) gene carrying the Swedish mutation^[10], represent a well-characterized model for AD. These mice accumulate A β in an age-related manner, particularly in the hippocampus^[11], the cerebral cortex^[12] and the olfactory bulb^[13]. NSCs derived from these animals could be a robust tool for studying the disease mechanisms related to A β intra- and extracellular accumulation^[14]. Cell platforms derived from stem cells can be used at different stages of the differentiation process, thus acting as a useful tool for pharmacological agents active on proliferation, differentiation, functions and pathways of the mature Wild type (Wt) and pathological phenotype.

In this study we have characterized neural stem cells derived from adult Tg2576 AD mice in terms of self-renewal, gene expression profile, multipotency and differentiation capability, compared to neural stem cells derived from Wt age-matched animals.

We derived NSCs from the subventricular zone (SVZ). Neuroblasts generated in this area migrate to the olfactory bulb, renewing inhibitory interneurons in the primary olfactory nucleus and olfactory glomerula, thus contributing to the preservation of the olfactory function. The interest in studying these cells derives from the early

olfactory impairment in AD patients^[15], and from the possibility to use olfaction as a response marker for novel treatments^[16]. Moreover, the neurosphere assay is almost exclusively used in the SVZ, while neuroblast and neural stem cells derived from the subgranular zone of the dentate gyrus of the hippocampus (*e.g.*, the second brain area for constitutive neurogenesis) are predominantly cultured as adherent cells^[17].

The term neural stem cell (NSC) has been used throughout the text to refer to a heterogeneous population of neural stem cells (NSCs), neural precursors and progenitor cells (NPCs)^[18].

MATERIAL AND METHODS

Isolation, generation and expansion of neurospheres

Tg2576 mice and their non-transgenic littermates (001349-W) were purchased from Taconic Europe (Lille Skensved, Denmark). Animal care and treatment were in accordance with the EU Directive 2010/63/EU for animal experiments and in conformity with protocols approved by the Ethical Committee of Animal Experimentation, University of Bologna.

Adult NSCs were isolated following the Ahlenius and Kokaia protocol^[19] with some modifications^[20]. Brains from six month old mice were collected in a 50 mL tube containing ice-cold HBSS (Life Technologies, Milan, Italy).

Using a lancet, the olfactory bulbs were removed. Two 1 mm thick coronal slices were prepared from the rest of the brain and the SVZ was isolated and triturated in cold PBS using scissors. SVZ tissues were transferred to a 15 mL tube and allowed to settle. The PBS was then removed and the tissues incubated with the dissociation buffer consisting of: 1x HBSS; 5.4 mg/mL D-Glucose (SIGMA, St. Louise, MO, United States); 15 mmol/L HEPES (Life Technologies); 1.33 mg/mL Trypsin (SIGMA); 0.7 mg/mL hyaluronidase (SIGMA); 80U/mL DNase (SIGMA). After 15 min incubation at 37 °C tissues were pipetted several times to favour dissociation and incubated again at 37 °C for 10 min. In order to remove the undissociated tissue fragments, the solution was filtered through a 70 μ m filter paper and then centrifuged at 400 \times g for 5 min. The resulting pellet was washed twice, first with a sucrose-HBSS solution (HBSS 0.5 \times ; 0.3 g/mL sucrose), 500 \times g 10 min, then with a solution consisting of BSA (40 mg/mL), HEPES (0.02 mol/L) in EBSS. After 7 min centrifugation at 400 \times g, the cellular pellet was resuspended in serum-free medium (DMEM/F12 GlutaMAX 1 \times ; 8 mmol/L HEPES; 100 U/100 μ g Penicillin/Streptomycin; 0.1 \times B27; 1 \times N-2; 10 ng/mL bFGF; 20 ng/mL EGF) and, after cell count, cells were plated at a density of 50 cells/ μ L in a final volume of 3 mL in low-attachment 6-well plates (NUNC). Medium was changed every three days, centrifuging the cell suspension at 300 \times g for 5 min and gently resuspending the cellular pellet in fresh medium.

To obtain secondary neurospheres, cells were centrifuged at 300 \times g for 5 min and incubated in a 0.5 mg/mL trypsin - 0.2 mg/mL EDTA solution in HBSS at 37 °C

Table 1 Primary and secondary antibodies used in the study

Antibody	Specie	Supplier	Dilution
Primary antibody			
β -III-Tubulin (TuJ-1)	Mouse	R and D	1:1000
GFAP	Rabbit	Eurodiagnostic	1:100
MAP2	Rabbit	S.Cruz	1:250
6E10	Mouse	Covance	1:1000
CNPase	Mouse	Chemicon	1:250
Secondary antibody			
Anti-Mo RRX	Donkey	Alexa	1:600
Anti-Mo Cy2	Donkey	Jackson	1:100
Anti-Rb Cy2	Donkey	Jackson	1:100
Anti-Rb RRX	Donkey	Jackson	1:100

The antibodies used, the species in which they were produced, the manufacturers and the dilutions at which they were used are described in the table. RRX: Rhodamine red-x conjugated.

for 15 min. After inhibiting trypsinization and subsequent centrifugation, the cellular pellet was resuspended in half fresh/half old medium. Cells were counted and re-plated at the same density.

Three different cultures were prepared; all experiments were performed in duplicate.

For population doubling, cell yields and mRNA analysis, undifferentiated neurospheres were used, while for morphology studies, secondary neurosphere derived cells were analysed during spontaneous differentiation.

Cell count and population doubling

Cells were counted from primary and secondary neurospheres 3, 4 and 5 dafter plating. Counting procedure was performed taking images of all neurospheres and statistically calculating the cell number based on single cell and sphere area using Image ProPlus software (Media Cybernetics Inc, Bethesda, MD, United States).

Population doubling was calculated using the following formula^[21]: $PD = \log_{10}(N/N_0) \times 3.33$

Where PD is the Population Doubling, N and N_0 are the final and initial number of cells, respectively.

RNA extraction and PCR array

The RNeasy Micro Kit (QIAGEN) was used for total RNA extraction and 300 ng were retrotranscribed using the RT2 First Strand Kit (QIAGEN) following the manufacturer's instructions.

For the study of NSC gene expression, the 96-well QIAGEN PCR array for neurogenesis was used in combination with the RT2 SYBR Green qPCR Mastermix (QIAGEN), using 10 ng of cDNA per well.

Immunocytochemistry

In brief, 5 d *in vitro* (DIV) secondary neurospheres were dissociated as described above and plated on 0.25 mg/mL MCM Gel 2D Cultrex (TREVIGEN, Helgerman Court, Gaithersburg, MD USA) in 24-well plates at a density of 1×10^4 cells/cm². Cells were grown in the same culture medium without mitogens. After this, 6 DIV cells were

fixed (cold 4% paraformaldehyde, 20 min), washed (two PBS washes, 10 min each) and incubated overnight at 4 °C with primary antibodies diluted in PBS/0.3% Triton x-100. After two washes, incubation with the secondary antibodies was performed at 37°C for 30 min. Cells were then washed and incubated with Hoechst 33258 (1 µg/mL in PBS/0.2% Triton x-100) for 30 min at RT. Finally, cells were washed again and mounted with phenylendiamine solution (0.1% 1,4-phenylendiamine -Sigma-, 50% glycerine -Sigma-, carbonate/bicarbonate buffer pH 8.6). Controls were always performed on secondary antibodies. The primary and secondary antibodies used are described in Table 1, as well as the species in which they were produced, the manufacturers and the working dilutions.

In order to study the development of the filaments net of differentiated neurons, cells positive for the β -tubulin marker (TuJ-1) were analysed using the NIS-Elements Microscope Imaging Software (NIKON). Three random fields from each well were analysed, counting total neurite length and the number of branches per neuron. Hoechst 33258 nuclear staining was used to identify the total number of cells. At least 20 TuJ-1 positive cells per group were analysed.

To quantify the percentage of GFAP, CNPase and MAP2 positive cells, three random fields per well were considered.

In order to identify the expression of the A β /APP transgenic protein, the 6E10 antibody was used. This antibody recognizes the APP whole protein, its cleavage products A β (40 and 42) and the CTF β (C-terminal fragment β), although it is specific for the human protein, identifying in this case only the transgenic protein^[22].

Statistical analysis

Student's *t*-test was used to statistically analyse data obtained from Wt and Tg2576 stem cell cultures. Data were considered significant when $P < 0.05$.

RESULTS

Population doubling and cell morphology

Cells from Tg2576 mice neurospheres express the human transgenic protein APP/A β visualized using the 6E10 antibody (Figure 1A) whereas cells differentiated from Wt neurospheres do not (Figure 1B).

In order to characterize the neural stem cell model, the proliferation and differentiation capability of cultured NSCs derived from Tg2576 compared to Wt cultures were studied. Up to two generations of neurospheres, primary and secondary, were derived from Wt and Tg2576 animals in the presence of EGF and bFGF, and the population doubling calculated. The secondary neurosphere formation is considered the directly related parameter to the self-renewal activity^[23]. When comparing Wt versus Tg2576 derived neurospheres, a statistically significant impairment was observed in population doubling of Tg2576 primary neurospheres (Figure 1C). A decrease in the yield of cells per animal in Tg2576 sec-

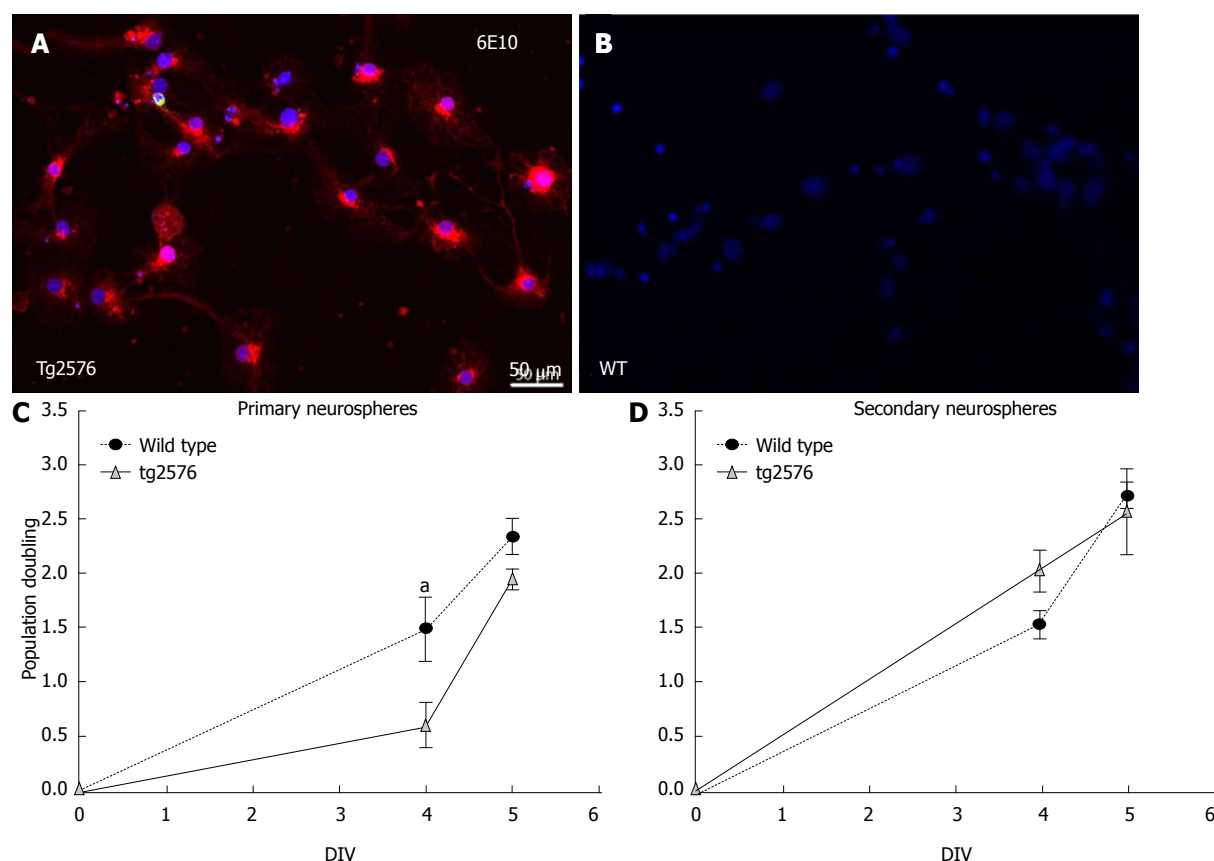


Figure 1 Transgenic protein expression and population doubling. Micrograph shows the positive 6E10 staining of Tg2576 plated cells derived from secondary neurospheres (A) and the related negative staining of wild type derived cells (B). A decrease in population doubling seems to occur in the first few days of primary neurosphere development (C), and is not repeated in secondary neurosphere formation (D). Statistical analysis: Student's *t*-test. Graphs represent mean \pm SE and asterisks represent significant differences between Tg2576 and Wt at the same DIV ($^*P < 0.05$).

ondary neurospheres (Table 2) was also observed, even if the population doubling rate was restored during the generation of secondary neurospheres (Figure 1D).

Specific markers for neurons (Tuj-1 and MAP2), oligodendrocytes (CNPase) and astroglial cells (GFAP) were used to study the differentiation capability of NSCs from primary and secondary neurospheres after mitogen withdrawal (Figure 2). Significant differences were found in the expression of GFAP (glial fibrillary acidic protein) and MAP2 (microtubule associated protein 2) between Wt and Tg2576 differentiated cells, with a lower number of MAP2-positive cells and a higher number of GFAP-positive cells derived from Tg2576 mice (Figure 2E) being observed. Differentiated neurons from these animals also show a lower number (Figure 2F) and length (Figure 2G) of neurites marked using an antibody against the β -III-tubulin (Tuj-1).

Gene expression analysis

In order to detect gene expression alterations between Tg2576 and Wt derived NSCs, a QIAGEN PCR array for neurogenesis was used.

Different genes are affected by the transgenic human mutated APP gene expressed in Tg2576 mice. According

to the manufacturer's instructions, a difference two times greater is considered biologically significant (Table 3). All the genes differently expressed in the two genotypes are overexpressed in Tg2576 cells. These include genes involved in neurotransmission (Acetylcholinesterase and Dopamine receptor D2), growth factors (Glial cell line derived neurotrophic factor, V-erb-b2 and Vascular endothelial growth factor A), cell growth and neuronal differentiation (CDK5, interleukin 3 and Notch gene homolog 2) and an APP-related protein (Amyloid beta precursor protein-binding-1).

Using the web-software STRING [<http://string-db.org/>], proteins derived from the genes of interest were connected in clusters according to their interactions and involvement in biological processes (Figure 3A). The software allows the net of interactions including other proteins closely linked to the one analysed to be extended, in order to obtain a better understanding of the possible pathways affected by the transgene effect in Tg2576 mice (Figure 3B). The analysis shows that almost all genes upregulated in Tg2576 NSCs interact with each other along different pathways, situating VEGF (vascular endothelial growth factor) as a node of the net.

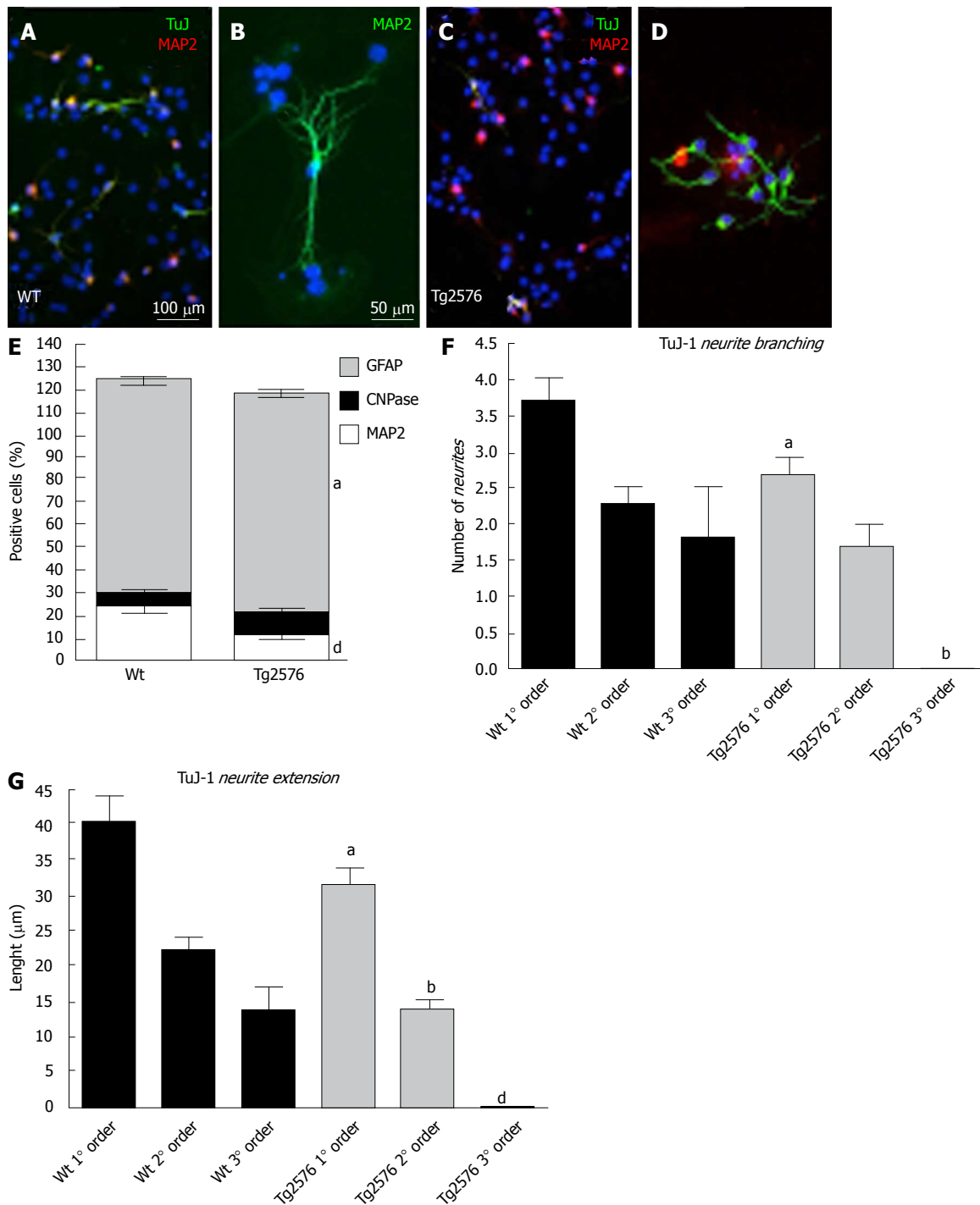


Figure 2 Morphological analysis. A-D: Micrographs showing immunostaining for β -III-tubulin (TuJ-1) and MAP2 in Wt and Tg2576 cells. Hoechst 33258 nuclear staining was used to determine the total cell number; E: The analyses of cell lineage indicate an increase in GFAP- and a decrease in MAP2-positive cells in Tg2576 compared to Wt. The number of CNPase-positive cells does not vary between the two genotypes; F, G: neural maturation analysis shows both a decrease in TuJ-1 positive branching of the third order and in neurite extension in cells derived from Tg2576 animals. Statistical analysis: Student's t-test. Graphs represent mean \pm SE and asterisks represent significant differences between Tg2576 and Wt (^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$). Bars: A, C: 100 μ m; B, D, 50 μ m.

DISCUSSION

At the present time, medical research relies on appropriate model systems to study disease mechanisms and to develop novel therapies. In particular, complex pathologies, such as AD, with no certainly ascertained causes and with no available cure, need new models for *in vitro*

studies and for fast screening new molecules. Even if a number of animal models allow us to recreate diseases *in vivo*, *in vitro* systems are still needed to study pathologies at cellular and molecular level and to generate screening platforms for drug discovery.

The Tg2576 mice, which express the human APP695 transgene carrying the Swedish mutation, display sev-

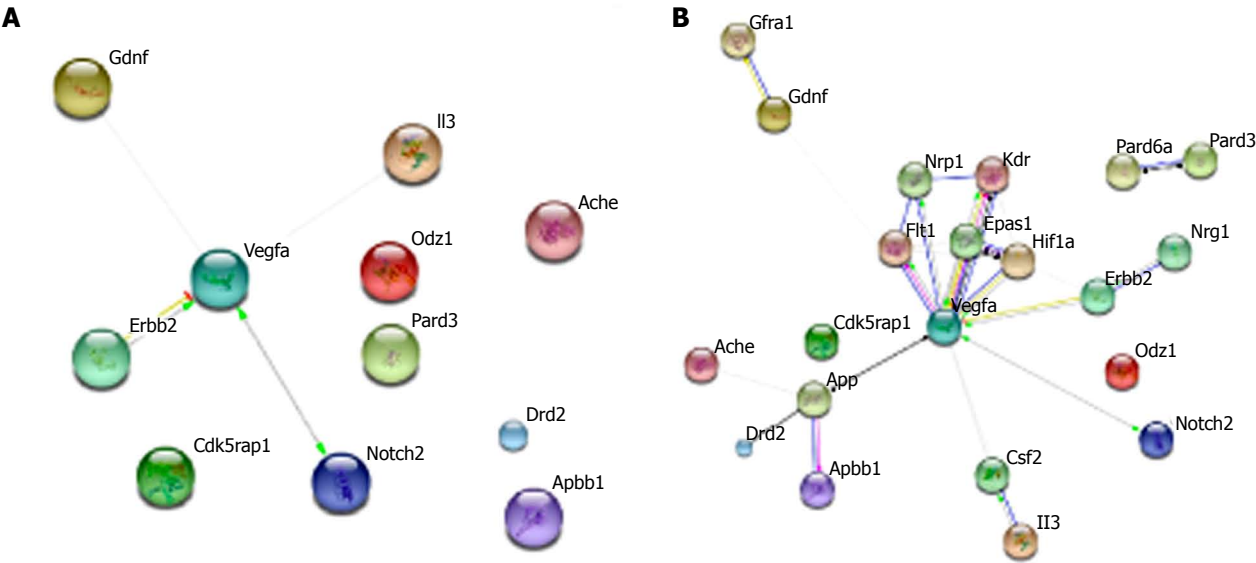


Figure 3 Functional protein net. The list of the neurogenesis-involved genes altered in Tg2576 versus Wt cells is reported in Table 2. The related proteins were clustered using STRING web software. Figure shows the 11 genes (A) and the extended net (B).

Table 2 Genotype-dependent cell yield per animal		
	Wild type	Tg2576
Primary neurospheres	$1.7 \times 10^5 \pm 5 \times 10^4$	$1.7 \times 10^5 \pm 1 \times 10^4$
Secondary neurospheres	$1.7 \times 10^5 \pm 1.4 \times 10^4$	$1.3 \times 10^5 \pm 9.7 \times 10^{3a}$

Cells obtained from 4 animals were counted in primary and secondary neurospheres development. A decrease in the total number of cells per animal in Tg2576-compared to Wt culture was observed, ^a*P* < 0.05. Statistical studies performed by Student’s *t*-test.

eral neuropathological features of AD, including plaque deposition^[8] and early synaptic abnormalities^[24-28]. This transgenic mouse is widely used as an animal model of AD, resembling the inherited form of the pathology related to the APP gene.

In the present study NSCs isolated from Tg2565 mice were characterized as an *in vitro* model for AD due to their ability to preserve transgenic Aβ protein expression. Notably, an altered neurogenesis was showed in AD patients and in different animal models.

The neurospheres generated from the primary culture of adult SVZ mice when cells are grown in the presence of mitogens (EGF and bFGF) are composed of a heterogeneous population of neural stem cells (NSCs), neural precursors and progenitor cells (NPCs). However, this mixed cell population is usually denoted as “neurostem cells”. Neurosphere assay is an accepted model for the study of NSC self-renewal through the formation of different neurosphere generations (primary, secondary neurospheres) and also for the study of their capability to differentiate into neural cell phenotypes (neurons, astrocytes and oligodendrocytes)^[18].

The complex pathology of AD is thought to include an impairment of neurotrophic signaling leading to alterations in the neurogenesis process^[26]. Tg2576 derived

Table 3 Polymerase chain reaction array analysis	
Gene	Regulation
Acetylcholinesterase	3.41
Amyloid beta (A4) precursor protein-binding, family B, member 1	2.33
CDK5 regulatory subunit associated protein 1	2.07
Dopamine receptor D2	2.62
V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	2.17
Glial cell line derived neurotrophic factor	3.13
Interleukin 3	2.69
Notch gene homolog 2 (Drosophila)	2.29
Odd Oz/ten-m homolog 1 (Drosophila)	3.81
Par-3 (partitioning defective 3) homolog (C. elegans)	2.39
Vascular endothelial growth factor A	3.01

Table represent the 11 genes resulted differentially expressed in Tg2576 neural stem cells (NSCs) related to Wt cells. Regulation is indicated as folds of increase. All the included differentially expressed genes resulted overexpressed in Tg2576 cells.

cells show a significant decrease in cell doubling during the first stages of primary neurosphere development and a decreased yield of isolated neurons, thus resembling impairment of neurotrophic alterations in the neurogenesis process in Tg2576 mice^[29] and in AD brains^[30].

The ability of these cells to spontaneously differentiate when plated on a matrix mimicking the extracellular space allows us to study possible lineage and maturation differences between Tg2576- and wild-type-derived cells. A significant decrease was found in MAP2 positive cells in Tg2576 cultures, indicating a defect in neural lineage. Also neuron maturation is altered in Tg2576 compared to wild-type-derived animals. These cultures actually display a decrease in neurite extension of β-III-tubulin positive cells.

The role of APP in neurogenesis and neuron maturation processes has still to be fully elucidated, but a num-

ber of data suggest that the soluble APP (sAPP) alpha and the APP intracellular domain (AICD) affect proliferation, survival and migration of the NSC population^[31], as well as neuronal maturation. The sAPP seems to promotes gliogenesis, whereas AICD negatively modulates proliferation and maturation of the neural precursor^[32]. This observation suggests that an APP overexpression could influence the cell fate of the NSCs, decreasing their number and impairing maturation.

The effect of the human APP transgene expression was thus analysed using neurogenesis-related PCR arrays, resulting in 11 genes overexpressed in the Tg2576 neural stem related to Wt cultures. Among those, APP-binding-protein-1 (APP-BP1) is more than two-fold up-regulated in Tg2576-derived neurospheres. Overexpression of the APP-BP1 gene has already been described in cortices and hippocampus of this animal model^[33] and, in addition, overexpression of this gene in primary neurons is related to apoptosis induction and increase in DNA synthesis^[34]. The expression of the APP-BP1 gene in Tg2576 NSCs seems also to resemble the up-regulated expression found in the lipid rafts in the hippocampi of AD brains^[35].

Genes involved in the neurotransmission and differentiation processes are also affected by the presence of the transgene. Factors involved in driving neurogenesis in AD include the cholinergic system since acetylcholine acts as a growth-regulatory signal in the brain^[36]. The overexpression of the acetylcholinesterase gene found in this *in vitro* model can partially represent an AD environment. Notably, treatments involving acetylcholinesterase inhibitors in clinical use for the symptomatic treatment of memory defects have shown a potential for stimulating neurogenesis^[37]. Overexpression of neurotrophins and growth factors like GDNF^[38] and VEGF^[39] also mimic mechanisms acting to compensate the neurotrophic and differentiation deficits in these cells.

Other factors, such as interleukin-3^[40], dopamine receptor D2^[41], and cyclin-dependent kinase-5 (CDK5)^[42], have already been shown to be involved in neurogenesis and AD models. With regard to CDK5, the A β -induced neurogenesis is coupled with an increase of inhibition of CDK^[43]. Thus, the overexpression of CDK5 in these cells could explain the delay in the population doubling of Tg2576 primary neurospheres, which is restored in secondary, where A β accumulation probably could balance the CDK5 overexpression.

In conclusion, we propose that NSCs derived from animal models carrying human mutations possibly represent a novel and useful tool for drug discovery and drug screening in AD. In particular these cells might be source of mature neurons as a robust model of intraneuronal A β accumulation. Due to the key pathogenetic role of intraneuronal A β in neurodegeneration and AD pathology^[25,44,45], the possibility to derive mature neurons from animal of different age could allow to generate cell systems with different A β overloading, thus providing an ideal system to investigate A β intraneuronal clearance.

COMMENTS

Background

Alzheimer's disease is the most common form of dementia. Genetic studies indicate a possible role of the amyloid precursor protein in the disease mechanisms, thus indicating this protein and derived amyloid- β fragments as potential pharmacological targets. However, the lack of effective therapies leads to the need of new models for drug discovery and for the study of pathological processes.

Research frontiers

Stem and re-programmed cells are looked as robust platforms and promising tools to create *in vitro* models suitable to study disease mechanisms, for pharmacological target identification and drug screening.

Innovations and breakthroughs

Neural stem cells isolated from transgenic animal models of diseases carrying human gene mutations could be used also to mimic *in vitro* neural ageing. Neural stem cells isolated from Tg2576 mice recapitulate aspects of *in vivo* pathology, e.g., APP processing alterations, population doubling and neuronal differentiation process.

Applications

The study results suggest that neural stem cells derived from a mouse model of Alzheimer's disease could be used as a platform for drug screening and to study disease mechanisms.

Terminology

Neural stem cells are multipotent stem cells capable to generate the main central nervous system phenotypes (neurons, astrocytes and oligodendrocytes). Adult neurogenesis: new neurons are formed and integrated in specific brain areas during adulthood. Subventricular zone: this is one of the neurogenic areas of the adult central nervous system.

Peer review

This is a scientific paper that illustrates an *in vitro* model based on neural stem cells derived from transgenic animals of interest in the study of pathological mechanisms of Alzheimer's disease and for testing new molecules for therapeutic purposes.

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