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REVIEW

Effects of living and metabolically inactive mesenchymal stromal cells and their derivatives on monocytes and macrophages

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Abstract

Mesenchymal stromal cells (MSCs) are multipotent and self-renewing stem cells that have great potential as cell therapy for autoimmune and inflammatory disorders, as well as for other clinical conditions, due to their immunoregulatory and regenerative properties. MSCs modulate the inflammatory milieu by releasing soluble factors and acting through cell-to-cell mechanisms. MSCs switch the classical inflammatory status of monocytes and macrophages towards a non-classical and anti-inflammatory phenotype. This is characterized by an increased secretion of anti-inflammatory cytokines, a decreased release of pro-inflammatory cytokines, and changes in the expression of cell membrane molecules and in metabolic pathways. The MSC modulation of monocyte and macrophage phenotypes seems to be critical for therapy effectiveness in several disease models, since when these cells are depleted, no immunoregulatory effects are observed. Here, we review the effects of living MSCs (metabolically active cells) and metabolically inactive MSCs (dead cells that lost metabolic activity by induced inactivation) and their derivatives (extracellular vesicles, soluble factors, extracts, and micro-particles) on the profile of macrophages and monocytes and the implications for immunoregulatory and reparative processes. This review includes mechanisms of action exhibited in these different therapeutic appro-aches, which induce the anti-inflammatory properties of monocytes and macrophages. Finally, we overview several possibilities of therapeutic applications of these cells and their derivatives, with results regarding monocytes and macrophages in animal model studies and some clinical trials.

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Core Tip: Mesenchymal stromal cells (MSCs) and their derivatives possess immunoregulatory and regenerative properties that involve the classical activation of monocytes and macrophages towards an anti-inflammatory profile, marked by the secretion of anti-inflammatory and reparative factors that guide the inflammation resolution and healing processes. This review will comprise the effects of living and metabolically inactive MSCs, MSC extracellular vesicles, subcellular microparticles, and cell extracts on monocytes and macrophages, as well as several possibilities of therapeutic applications.

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INTRODUCTION

Mesenchymal stromal cells (MSCs) are multipotent, self-renewing stem cells with immunoregulatory and regenerative properties. Found in several tissues, these non-hematopoietic progenitor cells have the ability to differentiate into cells of mesenchymal origin, such as chondrocytes, osteoblasts, and adipocytes, as well as muscle, tendon, endothelial, stromal, and neural cells[1,2]. This way, MSCs can be easily isolated from several sources such as the bone marrow, adipose and muscle tissues, the trabecular bone, articular cartilage, deciduous teeth, and the umbilical cord [1,3]. In these tissues, MSCs maintain homeostasis by offering support to other resident cells[4].

MSCs have been targeted by several investigations due to their immunoregulatory and regenerative abilities. These cells secrete soluble factors including cytokines, chemokines, growth factors, and extracellular vesicles (including exosomes and microvesicles) that modulate immune cells such as T cells, B cells, and monocytic cells for orchestrating inflammatory resolution and regenerative processes[2,5-7]. Several published findings have also demonstrated that MSCs support immune suppression through cell-to-cell contact[8-11]. Moreover, MSCs express low levels of the class I major histocompatibility complex (MHC-I) and do not express MHC-II, which makes them cells with a low immunogenicity and hence low rejection risk[12].

Owing to these features, MSCs are great candidates for cell therapy in inflammatory and autoimmune disorders, as well as in other clinical conditions. The therapeutic potential of MSCs and their secreted extracellular vesicles has been demonstrated in several *in vitro* studies, animal models, and clinical trials[5,12]. Successful treatment with MSCs has been observed in experimental models of lupus[13], colitis[14], diabetes[15], graft-versus-host disease (GvHD)[16], cardiovascular malignancies[17], and pulmonary diseases[18].

Currently, there are many MSC clinical trials at different phases registered on US National Institutes of Health database (https://clinicaltrials.gov), demonstrating advances in MSC therapy for GvHD, amyotrophic lateral sclerosis, rheumatoid arthritis, liver cirrhosis, acute respiratory distress syndrome, diabetes, acute myocardial infarction, lupus erythematosus, Crohn's disease, osteoarthritis, fibrosis, Parkinson's disease, cystic fibrosis, multiple sclerosis, ulcerative colitis, organ transplant rejection, and the recent coronavirus disease 2019 (COVID-19) pandemic.

Since previous investigations have shown that MSCs are modulated by the inflammatory milieu and respond specifically to different stimuli, greater therapeutic potential is achieved through MSC priming[19]. MSC activation, for improving their anti-inflammatory capacities, happens through exposure to conditions that are commonly encountered in the inflammatory microenvironment, such as hypoxia, which

enhances the angiogenic properties of MSC extracellular vesicles[20]. Moreover, nutrient deprivation also improves MSCs' immunoregulatory properties[21]. This MSC priming step can be performed *in vitro* before cell administration, with the inflammatory cytokines interferon (IFN)-γ and tumor necrosis factor (TNF)-α or with Toll-like receptor 3 (TLR-3) agonists[19,22,23]. This stimulation enhances the secretion of bioactive factors tumor necrosis factor-inducible gene (TSG)-6, interleukin (IL)-6, and prostaglandin E2 (PGE2) by MSCs[23,24].

MSCs can also be activated by interaction with immune cells. The immunoregulatory potential of MSCs is enhanced in response to the macrophage secretome, which was demonstrated by an attenuation of macrophage pro-inflammatory activity[25,26]. In addition, since macrophages and monocytes are present at the inflammatory milieu and assume either a pro- or anti-inflammatory profile, thereby orchestrating inflammation progression or resolution[27-29], studies investigating the crosstalk between these cells and MSCs are needed to elucidate the mechanisms of action of MSC therapy.

Indeed, macrophages and monocyte-derived macrophages show wide heterogeneity in their responses to the microenvironment. The range of microenvironment stimuli induces different functional states in macrophages, which are usually classified in 2 distinct groups: the classically activated (M1) and alternatively activated (M2) macrophages. M1 macrophages are characterized by a cytotoxic phenotype and by the production of reactive species and pro-inflammatory mediators such as IL-1, IL-6, IL-12, IL-23 and TNF-α[27,30]. Meanwhile, M2 macrophages have a healing profile, pronounced by production of anti-inflammatory and angiogenic molecules, such as transforming growth factor (TGF)-β, IL-10, vascular endothelial growth factor (VEGF) and EGF, which support reparative processes[27,30]. However, new investigations have demonstrated that macrophage activation is more complex than previously thought, and a spectrum of intermediate phenotypes is defined by different transcriptional patterns. In this regard, M2 macrophages can be subdivided in different subsets: M2a, M2b, M2c, and M2d; these activation profiles are induced by distinct stimuli combinations[30]. Nonetheless, such classifications are still being elucidated, and this article will refer to M1 and M2 macrophages for simplification.

Furthermore, monocytes also present broad heterogeneity as recent investigations are uncovering different peripheral blood cell populations. In humans, these are represented by 3 subsets based on the expression of surface markers: classical monocytes are $CD14^{+}CD16^{-}$ and account for almost 90% of the human monocyte population. The remaining cells are subdivided in 2 populations: intermediate ($CD14^{+}CD16^{+}$) and non-classical ($CD14^{low}CD16^{+}$) monocytes[28]. Classical and intermediate monocytes correspond to murine $Ly6C^{+}$ inflammatory monocytes, whilst non-classical monocytes resemble $Ly6C^{-}$ or alternative monocytes[28,31]. The physiological role, as well as the origin and development of monocyte subsets, is still unclear; however, initial evidence in mice indicates that there is a sequential differentiation of classical monocytes into non-classical monocytes, and these might be considered blood-resident macrophages. Therefore, since monocytes can differentiate into macrophages in conditions of altered homeostasis when there is a need for effector cells, monocytes can be recruited to assume either a pro-inflammatory or anti-inflammatory functional phenotype depending on the microenvironment stimuli, which is similar to the concept of macrophage plasticity[31].

The interaction of macrophages and monocytes with MSCs occurs right after intravenous infusion. Németh *et al*[32] demonstrated that MSCs and macrophages colocalize in the lungs after 10 minutes of cell administration. Biodistribution data further showed that MSCs are cleared through phagocytosis by the host's monocytes and macrophages[33,34]. However, the fact that a significant part of infused MSCs get trapped in the lungs raises concerns about the deleterious effects of obstructive events [35-37]. In an attempt to improve therapy efficacy and safety, several studies have thus explored the immunoregulatory features of MSC-derived extracellular vesicles and microparticles, as well as metabolically inactive MSCs, as an alternative to living MSCs [6,38-40]. Their results have demonstrated that these substitutes maintain the immunomodulatory properties that induce a regulatory phenotype in monocytes and macrophages.

Therefore, this review will focus on the modulation of macrophages' and monocytes' immunophenotypes, activation status, and migration by living and metabolically inactive MSCs and their derivatives, as well as the implications on infla-mmation resolution and healing processes in different disease models. Furthermore, this paper will include the mechanisms of action exhibited in these different approaches for inducing anti-inflammatory properties in monocytes and macrophages (Figure 1) and results of therapeutic evidence presented in animal models and some clinical trials.

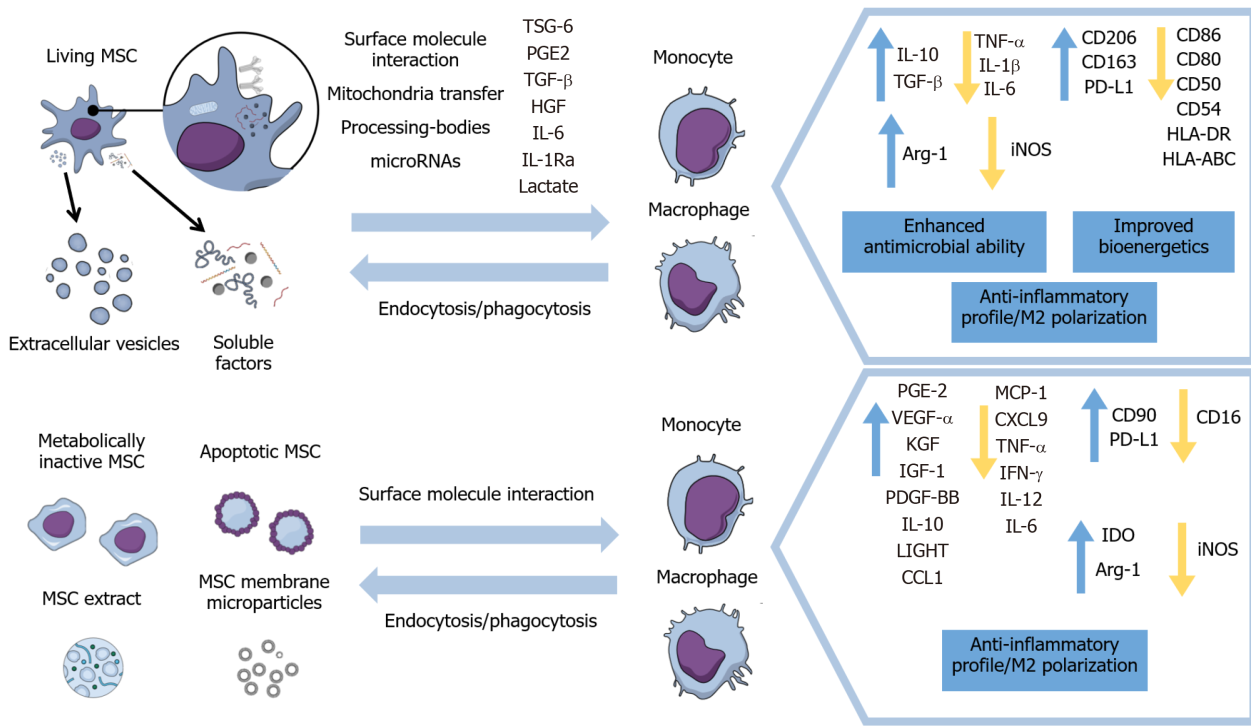

Figure 1 Main effects of viable, inactivated, and apoptotic mesenchymal stromal cells and of mesenchymal stromal cells' secretome and subcellular particles on monocytes and macrophages. MSC: Mesenchymal stromal cell; TSG-6: Tumor necrosis factor-inducible gene 6; PGE2: Prostaglandin E2; TGF-β: Transforming growth factor β; IL: Interleukin; HGF: Hepatocyte growth factor; TNF-α: tumor necrosis factor α; PD-L1: Programmed death-ligand 1; IGF-1: Insulin-like growth factor 1; VEGF: Vascular endothelial growth factor; iNOS: Inducible nitric oxide synthase.

MODULATION OF MACROPHAGES AND MONOCYTES BY LIVING MSCS AND EXTRACELLULAR VESICLES

Cytokine profile in monocytes and macrophages

MSCs and their extracellular vesicles can induce classic inflammatory monocytes towards a non-classic anti-inflammatory profile, as well as classically activated or M1 macrophages into alternatively activated or M2 macrophages[41-44]. This monocytic tolerogenic phenotype is characterized by changes in cytokine expression, represented by an increase in anti-inflammatory IL-10 and TGF-β in monocytes and macrophages [45-47] and a decrease in levels of TNF-α, IL-1β, and IL-6 inflammatory cytokines in macrophages[43,44,47,48].

This shift in the cytokine production pattern of macrophages and monocytes, mainly marked by regulatory IL-10 upregulation, drives inflammation resolution and alleviates injury in experimental models of allergic processes[49], colitis[47], and eye autoimmune and inflammatory disorders[45,50]. Indeed, the IL-10 derived from macrophages preconditioned with MSC exosomes has an inhibitory effect on the proliferation of $CD4^+$ T cells, indicating different ways in which MSCs exert immunosuppressive effects that include macrophage functions[49].

Furthermore, reducing the production of inflammatory mediators like TNF-α and IL-1β has beneficial effects, since these cytokines promote inflammation maintenance. These bioactive factors are involved in the recruitment of inflammatory cells, apoptosis induction, and release of destructive enzymes (such as metalloproteinases) that lead to tissue degeneration. In addition, TNF-α facilitates autoimmunity by inhibiting T regulatory cells[51,52]. Therefore, the immunoregulatory action of MSCs on monocytic cells contributes to the resolution of inflammatory processes and reduction of tissue damage.

Expression of membrane molecules in monocytes and macrophages

The immunoregulatory effect of MSCs on macrophages is also demonstrated by the modulation of membrane protein expression. Murine and human macrophages in co-culture with MSCs were able to reduce the expression of the co-stimulatory molecule CD86 and increase that of mannose receptor CD206, which are well-known markers of M1 and M2 polarization, respectively[53,54]. These changes were also observed in *in*

vivo mouse models of cutaneous wound healing, myocardial infarction, and diabetic cornea, since MSC transplantation decreased the number of CD86$^+$ macrophages while increasing CD163$^+$ and CD206$^+$ anti-inflammatory macrophages[43,55,56]. Similarly, MSC exosomes and extracellular vesicles induce the same marker expression pattern of the M2 phenotype, both *in vitro* and *in vivo*[20,43,47,57].

M1 activated macrophages cultured with MSCs decreased the expression of CD80, CD86, CD50, CD54, HLA-DR, and HLA-ABC cell surface molecules. This indicates that MSC-conditioned macrophages acquire an immunosuppressive profile through the reduction of their antigen presentation functions, since these membrane proteins are involved in this process[58].

Regarding monocytes, de Witte *et al*[33] cultured human monocytes with MSCs and demonstrated that the predominant population of CD14^{++}CD16$^-$ classical monocytes shifted to CD14^{++}CD16$^+$ regulatory intermediate monocytes. Moreover, co-cultured monocytes increased CD163, CD206, and programmed death-ligand 1 (PD-L1) expression.

It is also noteworthy that the co-culture of MSCs with the 3 human monocyte subsets (classical, non-classical, and intermediate) reduced the expression of the class II antigen presentation complex (HLA-DR) while upregulating *MRC1*, *CD163*, *CD163L1*, *CD226*, *CD93*, *LILRB1* and *PTGER2* membrane receptor genes[42]. MRC1 encodes CD206, which, along with CD163 and CD163L1, belongs to the scavenger receptors family, which mediates the remodeling function after tissue damage[42]. CD93 is important to phagocytosis and clearance of apoptotic cells, while CD226 is involved in monocyte migration[59,60]. Further, LILRB1 is an immunoglobulin-like receptor involved in MHC-I mediated immunosuppression[61]. *PTGER2* encodes the EP2 receptor, which is activated through PGE2, one of MSCs' bioactive factors. Meanwhile, researchers observed an upregulation of monocyte cytokines and growth factor genes, such as *IL-10*, *IGF1*, and *VEGF-A*[42]. Not coincidentally, IL-10 production is induced through MSC-derived PGE2, which results in reduced inflammation [32]. This expression profile, along with the CD14 upregulation, shows that MSCs altered the maturation of these monocyte subsets towards an M2 macrophage anti-inflammatory phenotype[42].

Metabolic changes

The MSC-induced M1-M2 phenotype switch is also accompanied by metabolic alterations. MSCs impair monocyte differentiation into antigen-presenting dendritic cells through metabolic reprogramming. Monocytes, instead of assuming an antigen presentation profile, show a transcriptional and phenotypic profile of M2 macrophages that induces a Th2 regulatory cytokine pattern in CD4$^+$ T cells. In addition, these cells acquire higher spare respiratory capacity and more polarized mitochondrial membrane potential, resulting in a better capacity of stimuli response in case of high energy demand[41]. In the same way, monocyte-derived macrophages co-cultured with MSCs had increased mitochondrial function and ATP turnover, which resulted in greater macrophage phagocytosis and antimicrobial ability. These results were demonstrated both *in vitro* and *in vivo*[18].

Importantly, macrophages conditioned with MSCs or MSC exosomes increased their oxygen consumption rate while decreasing proton leak, indicating enhanced bioenergetics and mitochondrial coupling efficiency. In the same work, macrophages challenged with silica particles demonstrated homeostasis alterations highlighted by the mitochondrial production of reactive oxygen species, which was reverted by MSC exosomes[62]. On the other hand, *Salmonella*-infected macrophages co-cultured with MSCs had respiratory burst improvements. This was demonstrated by the enhanced expression of NADPH oxidase subunits, concomitantly with the activation of antioxidant protection mechanisms such as superoxide dismutase 2 (SOD2). These data, along with faster microbial clearance by macrophages promoted in the MSC co-culture, indicate that these metabolic changes enhance the macrophages' ability to respond to pathogens[58].

In addition to improving the antimicrobial ability of macrophages and monocytes, MSC-induced metabolic changes modify macrophage energy generation pathways while promoting their transition towards the M2 phenotype. Since M1 activated macrophages have a high energy demand, they have an augmented expression of glucose transporter 1 (GLUT1), hexokinase 2 (HK2), and mTOR, which are proteins needed in the glycolytic pathway[58]. On the other hand, M2 macrophages exhibit a preference for mitochondrial fatty acid β-oxidation, demonstrated by a higher expression of carnitine palmitoyl trasferase 1α (CPT1α) and phosphorylated AMPKα (p-AMPKα)[58]. This way, the co-culture of M1 macrophages and MSCs reduced GLUT1 and HK2 expression and p-mTOR levels while increasing CPT1α expression

and p-AMPKα levels; this indicated changes in energy metabolism underlying the MSC-induced M2 phenotype[58].

Regarding the amino acid metabolism, macrophages presented an augmented expression of arginase-1 and reduced expression of inducible nitric oxide (NO) synthase (iNOS) in response to MSCs or MSC exosomes[43,56,63,64]. These enzymes are responsible for the L-arginine metabolism, with arginase and iNOS enzymes competing for this substrate to convert it into urea and ornithine or NO, respectively. NO participates in the macrophage microbicidal and effector functions, while ornithine is a polyamine that is necessary for the cell proliferation and tissue remodeling functions of M2 macrophages[27]. The balance between their activities indicates M1 or M2 polarization, and macrophages co-cultured with MSCs exhibit decreased NO production in addition to increased urea levels, which indicates the regenerative and resolutive phenotype typical of M2 polarization[64].

Migration and recruitment

MSCs also modulate the migratory behavior of macrophages and monocytes. *In vitro*, macrophages and monocytes actively migrate towards MSCs[33]; *in vivo*, they are recruited to the lungs where they encounter MSCs after intravenous infusion[65]. In a murine model of myocarditis, MSCs recruited anti-inflammatory $LyC6^{low}$ monocytes to the inflammation site whilst decreasing pro-inflammatory $LyC6^{high}$ and $LyC6^{middle}$ monocyte levels. This regulation occurred through the modulation of local chemokines, reducing levels of MCP-1 (CCL2), MCP-3 (CCL7), and CCL5; abrogating the expression of ICAM-1 and VCAM-1 adhesion molecules; and increasing SDF-1α and CX3CL1 Levels. The migration of the anti-inflammatory monocyte subset helped with tissue repair and led to a reduction in myocarditis severity[66]. Notably, the intravenous infusion of MSC exosomes in experimental mouse models of pulmonary fibrosis also diminished the recruitment of pro-inflammatory Ly6Chigh monocytes whereas it increased the alveolar macrophages and the infiltration of anti-inflammatory monocytes. These changes were accompanied by a reduction in fibrosis measurements, in agreement with the monocyte reparative profile[62,67].

Moreover, the administration of MSCs and MSC-conditioned medium in mice with angiotensin II-induced aortic aneurysm increased $CD206^+$ M2 macrophage infiltration and diminished $iNOS^+$ M1 cells at the injured site, which was concomitant with decreased levels of CCL5, CCL2, CCL3, and CXCL10[48].

On the other hand, previous reports showed that MSC administration in mice increased MCP-1 (CCL2) levels, which recruited monocytic cells to the lungs *via* the CCL2-CCR2 axis. After migration, monocytes and macrophages were consistently modulated by MSCs and assumed an IL-10-producing phenotype[49,50]. Similarly, in a model of skeletal muscle injury, treatment with hypoxia-subjected MSC extracellular vesicles increased the expression of *MCP-1* (CCL2) and the CD206/Ly6c cell ratio when compared to normoxia-derived extracellular vesicles and control groups, indicating M2 polarization[20].

Furthermore, type 2 diabetic mice showed augmented M2 macrophage counts in the liver, adipose tissue, skeletal muscle, pancreatic islands, and spleen after the intravenous infusion of MSCs. Concomitantly with a greater engraftment of administered MSCs in the spleen, this brings up the possibility that MSCs may directly modulate macrophage and monocyte populations in immune organs, which could lead to systemic effects[68]. In fact, mice with myocarditis treated with MSCs retained more pro-inflammatory monocytes in the spleen when compared to the control group, and recruited more anti-inflammatory monocytes to the heart, which improved healing processes and reduced inflammation[66].

In summary, despite the different triggered pathways and chemokine regulation involved in monocyte and macrophage recruitment, several investigations indicate that MSC treatment induces monocyte and macrophage migration to the inflammation site or to immune organs. Once at these sites, MSCs modulate the cell activation status and profile, promoting a monocytic anti-inflammatory phenotype and hence a reparative milieu.

Mechanisms of action

The mechanisms underlying MSC immunoregulatory capacities are still under investigation, but one of the most well-known processes for inducing a suppressive and anti-inflammatory phenotype in monocytes and macrophages is the secretion of soluble factors such as TSG-6, TGF-β, HGF (hepatocyte growth factor), IL-6, and the IL-1 receptor antagonist[45,53,69,70]. Moreover, lactate and PGE2 were also shown to reprogram macrophage metabolism to promote an M2 profile[41,58]. The abrogation of several of these bioactive factors prevented MSC-induced M2 macrophage

polarization and immunoregulatory effects[24,25,41,58].

In addition to their paracrine action, MSCs are phagocytized by monocytic cells in an active process. After *in vitro* phagocytosis, monocytes acquire phenotypic and functional changes of CD14++CD116+ immune regulatory intermediate monocytes, such as upregulated expression of PD-L1 and CD90 surface molecules and IL-1b, IL-6, IL-8, IL-10, and TGF-β cytokines, whilst expression of pro-inflammatory TNF-α decreases[33]. In vivo, monocytes which phagocytized MSCs assume the same anti-inflammatory profile and migrate to other body sites, mainly to the liver, carrying the regulatory properties of MSCs[33]. Further, macrophages also phagocyte MSCs and acquire an anti-inflammatory M2 phenotype, characterized by increased IL-10 and TGF-β expression[33,34].

Organelle transfer is another mechanism triggered by MSCs that enhances macrophage functions. *In vitro* and *in vivo* assays have evidenced that MSCs transfer mitochondria to macrophages through exosomes and cytoplasmic bridges named tunneling nanotubes, which improves the macrophages’ phagocytic ability and bioenergetics[18,62]. Min *et al*[71] reported that monocytes and macrophages engulfed MSCs’ cytoplasmic processing bodies, which are membrane less organelles that store mRNA, miRNA, and proteins. This mechanism was mediated by lipoprotein receptor-related proteins (LPRs) and was critical to the reprogramming of monocytes and macrophages towards a transcriptional profile of reduced antigen presentation, as well as for the inhibition of T cell activation. Moreover, MSC processing bodies were required to prevent the infiltration of CD11b+ inflam-matory monocytes and macrophages in lung tissue in a mouse model of lung inflammation[71].

Furthermore, MSCs can exert immunomodulatory effects through microRNA transfer[62]. The M2 macrophage phenotype promoted by treatment with MSC exosomes is, at least in part, dependent on the post transcriptional control (by miR-182 and miR-181) of TLR-4 and the subsequent downregulation of its downstream nuclear factor-κB (NF-κB) inflammatory pathway[43,72]. The inhibition of TLR-4/NF-κB activation is also triggered by the let-7b miRNA from MSC exosomes while this molecule induces signal transducer and activator of transcription 3 (STAT3) signaling, which in turn participates in M2 conversion[73]. The reduced expression of TLR-4 and enhanced levels of p-STAT3 in the healing wound site demonstrates that the regulation of these signaling pathways in macrophages promotes the M2 phenotype, with reparative properties[74].

miR147, derived from MSC extracellular vesicles, was also found to decrease macrophage activation *via* diminishing HMBG-1 secretion[75]. Moreover, He *et al*[76] reported that M2 macrophage polarization was associated with MSC exosome-derived miR-223 and a consequent decrease in Pknox1 levels, a homeobox protein associated with the regulation of M1 macrophage polarization[77,78]. Interestingly, miR-223 was also shown to reduce NLRP3 Levels; this is a protein of the inflammasome complex whose activation leads to inflammatory cytokine release and to the exacerbation of inflammation in cases of inflammatory bowel disease[79].

Similarly, MSCs induce the association of the yes-associated protein (YAP) and β-catenin in the macrophage nucleus. These are components of protein kinase cascades in the Hippo and Wnt signaling pathways, respectively, and the assembled protein complex operates to negatively control the target gene *XBP1*, which mediates NLRP3 activation. Data demonstrate that MSCs also regulate M2 polarization through Hippo signaling and subsequent repression of inflammasome activation[63]. Finally, MSCs suppress NLRP3 inflammasome-mediated IL-1β production by macrophages through a feedback mechanism where IL-1β may induce COX-2 signaling in MSCs[54].

MODULATION OF MACROPHAGES AND MONOCYTES BY METABOLICALLY INACTIVE, APOPTOTIC MSCS AND SUBCELLULAR PARTICLES

The modulation of macrophages and monocytes by non-viable MSCs or MSC subcellular particles is an emerging issue of interest in research, since investigations can contribute to understanding the immunomodulatory mechanisms of MSCs independently of their soluble secreted factors. In addition, although some studies have shown that MSCs display homing to the injured site[68,80], other experimental models of MSC infusion demonstrate that a great portion of these cells get trapped in the lung capillaries and lose viability after 24 h[35,36,80]. Nevertheless, the immunoregulatory effect of MSCs is maintained, raising questions on how these cells are still able to reduce local and systemic inflammation.

These questions bring up the hypothesis that MSCs transfer their immunomodulatory properties to other host cells, which can then act to decrease inflammatory parameters. In addition, tracking studies have demonstrated that the MSC signal found in the inflammation site, organs, and blood after intravenous administration derived from MSC debris phagocytized by immune cells (such as monocytes) instead of viable MSCs[33,36]. Therefore, inactivated and dead MSCs or even MSC extracts could trigger this immunoregulation without the need for metabolically active cells.

To overcome the low homing efficiency of systemically administered MSCs, studies have demonstrated the therapeutic potential of MSC extract instead of whole cells[81-83]. Song *et al*[81] infused the MSC extract in a chemically induced mouse model of colitis. They found that the extract inhibited inflammatory cytokines, recovered the damaged epithelial barrier, and polarized the macrophages' functional phenotyping from M1 to M2 by reducing the expression of genes encoding for MCP1, CXCL9, and iNOS (M1 markers) and increasing that of genes corresponding to IL-10, LIGHT, CCL1, and Arg-1 (M2 markers).

Studies observed that MSC membrane nanoparticles without any cargo and heat-inactivated MSCs decreased the proportion of pro-inflammatory CD16$^+$ monocytes by inducing apoptosis[40,84]. The MSC membrane nanoparticles were generated from unstimulated and IFN-γ-stimulated MSCs, and this difference seems to be important for the ultimate purpose. For instance, unstimulated and IFN-γ-stimulated nanoparticles were capable of increasing CD90$^+$ monocyte population, (a natural MSC marker), but only IFN-γ-stimulated nanoparticles augmented the PD-L1$^+$ monocyte subset[40]. Moreover, monocytes conditioned with IFN-γ-stimulated nanoparticles, but not with the unstimulated type, had enhanced indoleamine 2,3-dioxygenase (IDO) expression[40]. The possibility of changing stimuli to generate nanoparticles with different features and membrane compositions provides the opportunity of creating specific therapies according to distinct inflammatory disorders[40]. Importantly, PD-L1 is an immune checkpoint protein that inhibits the activation and function of its target PD-1-expressing immune cells, suppressing immune reactivity[85]. In addition, IDO is an enzyme that depletes the essential amino acid tryptophan and generates kynurenine pathway metabolites; these metabolic changes thus contribute to immune regulation[86]. Therefore, IFN-γ-stimulated MSC membrane particles with the ability to induce PD-L1 and IDO expression could be used in the treatment of severe inflammatory conditions that present inflammatory monocytes[40]. These studies also observed that MSC membrane nanoparticles bind and fuse to the monocyte membrane, demonstrating that physical interaction between cell surfaces is important for MSC-induced immunosuppression[40]. Furthermore, MSC membrane nanoparticles maintain ATPase and CD73 enzymatic activities at their surface, converting ATP to ADP and AMP to adenosine, respectively[40]. Adenosine, the last molecule of these reactions, has immunoregulatory functions *via* P1 receptor activation[87]. It is important to note that the activation of monocyte P1 receptors such as A_{2A} and A_{2B} inhibited TNF-α production[87].

Additionally, just as living cells, secretome-deficient heat-inactivated MSCs also disappear after 24 h of infusion in healthy mice and in an experimental model of kidney ischemia/reperfusion injury[39]. Despite their fast clearance, the administration of heat-inactivated MSCs still altered the expression levels of several cytokines and chemokines in the serum and lung tissue and reduced LPS-induced sepsis[39]. *In vitro* assays demonstrated that secretome-deficient heat-inactivated MSCs modulate monocytes through reducing TNF-α production[39,84]. This modulation occurs through phagocytosis of heat-inactivated MSCs, and the recognition of heat-inactivated MSCs by monocytes was even more efficient than that of intact MSCs[84]. Moreover, the supernatant of LPS-stimulated macrophages that phagocytized dead MSCs improved the survival of hypoxic cardiomyocytes[88]. After phagocytosis, macrophages augmented the production of PGE2, VEGF-α, KGF, IGF-1, and PDGF-BB reparative molecules while decreasing that of TNF-α, IFN-γ, IL-12, and IL-6[88]. Together, these data suggest that, at least in some sepsis models, monocytes that had phagocytized inactivated MSCs acquired their immunoregulatory properties and reduced inflammation[39,84].

Another therapeutic approach consists in the administration of apoptotic MSCs. Galleu *et al*[89] demonstrated that mice with GvHD lacking the cytotoxic activity of GvHD effector cells did not respond to MSC therapy due to the need for inducing MSC apoptosis. Therefore, the administration of *in vitro*-produced apoptotic MSCs in GvHD mice eliminated the requirement for promoting MSC apoptosis *in vivo* and induced IDO expression in recipient mice macrophages that had phagocytized the infused cells, which incited immunosuppression[89].

These new MSC-derived alternative therapies bring some advantages. Using non-viable MSCs ensures that the administered product is not altered after infusion, since once inside the target organism, they do not proliferate or secrete any molecules in response to nonspecific host signals. Owing to their small size, MSC membrane nano-particles could pass through the lung capillaries and reach other areas of the body, avoiding problems such as emboli formation induced by the administration of intact MSCs[40,90].

The effects of non-viable and apoptotic MSCs on macrophages and monocytes are still under investigation. The mechanisms of action exhibited by these cells are still not fully understood, but MSC phagocytosis by monocytes and macrophages seems to be essential for the systemic effects of inactivated and apoptotic MSC therapy[89]. The interaction between cell membranes may also have an important role[40]. Future studies will be necessary to reveal the possible interactions between non-viable MSCs and macrophages or monocytes *in vivo*, as well as their implications in treatment results.

THERAPEUTIC APPLICATION POSSIBILITIES — *IN VIVO* STUDIES

The use of MSCs with the proven participation of monocytes and macrophages has been described as having therapeutic potential in several local and systemic disorders studied in animal models. Regarding lung injuries, MSC extracellular vesicles were able to alleviate induced acute lung injury in a murine model: researchers observed alterations in macrophage phenotypes and a decrease in macrophage recruitment[91]. In addition, preconditioned MSC exosomes prevented and reverted experimental pulmonary fibrosis and lung inflammation through the modulation of monocyte phenotypes in adult C57BL/6 mice[67]. Through the modulation of lung macrophage phenotypes, treatment using MSC exosomes alleviated bronchopulmonary dysplasia in a mouse model, resulting in improvement of lung function, decreased fibrosis, remodeling of pulmonary vasculature, and amelioration of pulmonary hypertension [92]. In a mouse model of acute respiratory distress syndrome, an improvement of lung injury was observed when using murine alveolar macrophages previously cultured with MSC extracellular vesicles and through the transference of MSC mitochondria to macrophages, resulting in an enhancement of macrophage phago-cytosis. The enhanced host macrophage phagocytosis could promote a clearance of invading microorganism, which, combined with suppressive pro-inflammatory cytokine secretion, may improve clinical outcomes, since lung injury is associated with high inflammatory response and bacterial burden[18,93]. Moreover, the administration of MSCs increased CCL2 expression and monocyte recruitment in the lungs, suppressing allergic airway inflammation[49].

In relation to cardiac disorders, MSC application in Coxsackievirus B3-induced myocarditis in mice attenuated myocardial inflammation by suppressing the cardiac infiltration of pro-inflammatory monocytes while promoting the cardiac influx of anti-inflammatory monocytes, representing a promising strategy for the resolution of cardiac inflammation and prevention of disease progression[66]. MSC exosomes attenuate myocardial ischemia/reperfusion injury in mice *via* shuttling miR-182, which modifies the macrophages' polarization status[43]. MSCs and their exosomes may also mediate the decrease in pro-inflammatory and increase in anti-inflammatory monocytes/macrophages after acute myocardial infarction[94,95]. Furthermore, a mouse model of dilated cardiomyopathy that received MSC exosomes showed cardiac function improvement, cardiac dilation attenuation, and cardiomyocyte apoptosis reduction due to the decrease in pro-inflammatory macrophages in both the blood and heart[57].

The use of MSCs and their derivatives can also be considered for other organ injuries. The injection of MSCs or their exosomes ameliorated dextran sulfate sodium-induced colitis in mice, and part of the associated mechanism includes a macrophage-dependent phenomenon[47,96]. Previous coculture of MSCs and macrophages induced the M2 phenotype, which combined with host cells, improved liver fibrosis in mice[97]. The internalization of MSC extracellular vesicles by macrophages, with an increasing number of reparative macrophages, was accompanied by a reduction in renal inflammation in a porcine model, suggesting that anti-inflammatory properties underpin the protective effects of MSC extracellular vesicles on the stenotic kidney [98]. In mice secondary lupus disease, MSCs ameliorated lupus nephritis, preventing podocyte injury, possibly through a reduction in macrophage infiltration and polarization into an anti-inflammatory phenotype[13]. MSC exosomes prevented

cerebral injury in rat acute ischemic stroke by inhibiting autophagy-mediated microglial polarization to M1[99]. Therapy with MSC exosomes promoted M2 macrophage polarization and accelerated cutaneous wound healing in skin-defective mice [76]. Mice were protected against a subsequent immune challenge in corneal allotransplantation and experimental autoimmune uveitis after the intravenous infusion of MSC-preconditioned lung monocytes/macrophages[45]. The local administration of MSCs promoted diabetic corneal wound healing by modulating the immune response, inducing alternative activation of infiltrating macrophages towards M2 polarization [56].

In some other conditions, the study of the effects of MSCs on monocytes and macrophages is useful for future therapeutic applications. Regarding sepsis reports, the intravenous infusion of MSCs reduced mortality and bacteremia in gram-negative peritoneal sepsis in mice, partially by enhancing the phagocytic activity of blood monocytes[100]. MSC nanovesicles had protective immunomodulatory effects in a mouse model of sepsis owing to the reduction of pro-inflammatory cytokine production by macrophages and of monocyte infiltration in the peritoneum[101]. Exosomes of pretreated MSCs induced M2 macrophage polarization, increased survival, and effectively ameliorated symptoms in a mouse model of sepsis[102]. Apoptotic MSCs induced immunosuppression in a murine model of GvHD, engulfing recipient phagocytes[89]. MSC treatment prevented and alleviated atherosclerosis in mice, partly by decreasing monocytosis and modulating macrophage activation and differentiation. Plaque size and lipidic deposition in mice that received MSCs in both prevention and treatment groups were significantly smaller than those in the control group[103]. MSC exosomes repaired and regenerated critical osteochondral defects in a rat model of osteoarthritis through coordinated mobilization of multiple cell types and activation of several cellular processes, such as a regenerative immune phenotype characterized by a higher infiltration of $CD163^+$ regenerative M2 macrophages over $CD86^+$ M1 macrophages[104]. M2 macrophage polarization was also the target of the intravenous MSC exosomes studied in rats' spinal cord injury recovery[105,106]. MSC infusion exerted anti-diabetic effects and significantly promoted islet repair in a type 2 diabetes mouse model, and this effect was partially attributed to a suppression of inflammation and induction of M2 macrophage polarization[25]. In diabetes complications, MSC exosomes alleviated neurovascular dysfunction and improved functional recovery in mice with diabetic peripheral neuropathy, including a mechanism of macrophage M1 decrease and M2 increase[107].

In clinical trials, performing some cellular analysis is difficult and may not be possible. Moreover, clinical improvements are the main evaluated outcomes. We will briefly introduce some reports that showed descriptions of clinical improvement using MSCs with a possible involvement of monocytes and macrophages.

MSC infusion in treatment of patients with knee osteoarthritis resulted in overall improvement of pain and symptoms and reduced synovial inflammation. Scores of clinical outcomes showed clinical efficacy and decreased levels of pro-inflammatory monocytes, macrophages, and IL-12 in the synovial fluid after MSC injection. Taken together, the decreases in IL-12 Levels along with pro-inflammatory monocytes/macrophages after MSC injection are supportive of an anti-inflammatory and immunomodulatory mechanism of action of MSCs, which is clinical evidence of the mechanism of these cells in osteoarthritis[108].

Regarding the use of MSCs for immunomodulation after solid organ transplantation, a phase I trial has demonstrated the downregulation of HLA-DR ex-pression by $CD14^+$ monocytes relative to pre liver transplant levels, which can be associated with a decrease in immunological reactivity[109]. MSCs can modulate the maturation and function of monocyte-derived dendritic cells *via* soluble factors, contributing to the improvement of liver allograft histology and suppression of acute rejection in liver transplant recipients[110].

Biopsies of ulcerative colitis showed improved histological results after MSC treatment. Inflammatory cell infiltration at histological evaluation showed that the score of the MSC-treated group was significantly lower when compared to the untreated group[111].

Considering the treatment of infectious diseases, authors have described that the intravenous injection of MSCs significantly improved the inflammation situation in COVID-19; serum levels of pro-inflammatory cytokines and chemokines were dramatically reduced, which attracted less mononuclear cells/macrophages to the fragile lung [112]. Several studies focused on the reduction of the general inflammatory cytokine profile after MSC infusion, and some of them included the specific macrophage inflammatory protein-1 alpha (MIP-1)[113]. Still considering the cytokine profile, the reduction of systemic immune activation after MSC treatment contra-dictorily

improved immune reconstitution in HIV-1-infected immunological nonresponders and decreased specific cytokines such as MCP-1 and MIP-1β[114].

CONCLUSION

The interaction of macrophages and monocytes with either viable or non-viable MSCs seems to be critical for therapy effectiveness, since when these cells are depleted in several models of inflammatory diseases or are prevented from migrating to the inflammation site, no immunoregulatory effects or benefits occur[43,49,50,89,94]. As discussed, these immunoregulatory effects are mainly due to the induced shift towards the anti-inflammatory phenotype of monocytes and macrophages, induced by viable, non-viable, and apoptotic MSCs, as well as their subcellular particles.

This modulation of monocytes and macrophages by MSCs occurs through different complex mechanisms such as secreted soluble factors, mitochondria and micro-RNA transfer, and phagocytosis of MSCs. In addition, the emergence of different therapeutic approaches using non-viable MSCs and MSC membrane particles brings up the need for investigating their immunomodulatory mechanisms. The phagocytosis of MSCs by monocytes and macrophages was also observed, and the interaction between surface molecules of MSC membrane particles and these monocytic cells seems to be important.

Here, we discussed the effects of viable, non-viable, and apoptotic MSCs, as well as their secretome and subcellular particles on monocytes and macrophages (Figure 1). In summary, monocytes and macrophages can acquire the immunomodulatory features of MSCs, and this regulatory action seems to be crucial for therapy success in several clinical conditions.

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REVIEW

Stem cells' centrosomes: How can organelles identified 130 years ago contribute to the future of regenerative medicine?

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Author contributions: Goutas A collected the data; Trachana V collected the data/wrote the paper; All authors have read and approve the final manuscript.

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Abstract

At the core of regenerative medicine lies the expectation of repair or replacement of damaged tissues or whole organs. Donor scarcity and transplant rejection are major obstacles, and exactly the obstacles that stem cell based therapy promises to overcome. These therapies demand a comprehensive understanding of the asymmetric division of stem cells, *i.e.* their ability to produce cells with identical potency or differentiated cells. It is believed that with better understanding, researchers will be able to direct stem cell differentiation. Here, we describe extraordinary advances in manipulating stem cell fate that show that we need to focus on the centrosome and the centrosome-derived primary cilium. This belief comes from the fact that this organelle is the vehicle that coordinates the asymmetric division of stem cells. This is supported by studies that report the significant role of the centrosome/cilium in orchestrating signaling pathways that dictate stem cell fate. We anticipate that there is sufficient evidence to place this organelle at the center of efforts that will shape the future of regenerative medicine.

Key Words: Centrosome; Primary cilium; Asymmetry; Self-renewal; Differentiation; Stemness

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Core Tip: It is believed that the major difficulties that regenerative medicine currently faces are exactly those expected to be resolved by stem cell therapies, which require a comprehensive understanding of the asymmetric division of stem cells, in order to be able to manipulate their fate. Here, we review studies that prove that the centrosome and centrosome-derived primary cilium provide an excellent vehicle for the asymmetric distribution of the determinants of cell fate. We are anticipating that the

quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
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evidence is sufficient to place this organelle at the center of efforts that will shape the future of regenerative medicine.

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INTRODUCTION

Stem cells are undifferentiated cells characterized by two unique properties, the capacity to self-renew and maintain a robust stem cell pool, and the ability to differentiate into all types of specialized cells[1]. The differentiation potential of stem cells divide them into four different cell types: totipotent stem cells, which include the zygote and the cells produced by the first few divisions that give rise to the embryo plus extra-embryonic tissues[2]; pluripotent embryonic stem cells (ESCs) present in the inner cell mass of the developing blastocyst that differentiate to form cells in all three germ layers and gametes[3]; multipotent adult stem cells (ASCs) found in many tissues of the adult body, which can differentiate into several cell types that belong to a particular lineage[4], and unipotent stem cells that can only differentiate into one cell type[5]. In 2006, another category of pluripotent stem cells, occurred from disturbing the stable state of differentiated cells and induced them to revert to the level of pluripotency of ESC, was described by a group of Japanese investigators. These induced pluripotent stem cells (iPSC)[6] have increased the hopes for personalized stem cell therapies, as they are derived from patients, divide indefinitely in vitro, and potentially differentiate into any mature cell type. Moreover, their use is not accompanied by the ethical concerns associated with the use of ESCs[7].

At the heart of stem cell therapies is the hope of repair or replacing damaged tissues or whole organs. Donor scarcity, poor quality donor organs, and transplant rejection are the major difficulties faced by regenerative medicine, and exactly those that stem cell-based therapies promise to eventually overcome[8]. Efforts have been directed toward both repair and replacement, and spectacular advances have been reached in the last 20 years[9]. Repair of damaged tissues or organs mainly depends on injection of isolated stem cells that, either because of their proper differentiation or the secretion of biologically active molecules or both, result in tissue/organ structural regeneration and functional improvement[10]. In those attempts, different types of cells have been used, including ESCs, ASCs, and more recently iPSCs. In addition to the ethical issues accompanying the isolation of human (h)ESCs[11], attempts to use ESCs or iPSCs in the clinic have been problematic because of difficulties in achieving full differentiation and function, risk of tumorigenesis, and significant genomic instability[12,13].

ASCs, on the other hand, have been proven safe, and therefore have high expectations of therapeutic potential[14]. As they are scattered throughout the body in bone marrow, adipose tissue, myocardium, skin, umbilical cord blood, and skeletal muscle, ASCs are relatively easily isolated and expanded *in vitro*[15]. For 50 years, hematopoietic stem cells (HSCs) have been successfully used for the treatment of blood diseases like leukemias and autoimmune disorders[16]. The success of hematopoietic transplantation has raised hopes of the use of other ASCs to treat conditions, such as heart infarction, stroke, spinal cord injury, macular degeneration, diabetes, and skin burns[17]. Despite initial enthusiasm, clinical trials have identified problems such as undesired immune response[18], virus contamination[19], and difficulties with stem cell transport[20]. In addition, the therapeutic use of ASCs requires *in vitro* expansion, which is not free of concerns. Numerous studies demonstrated that ASCs rapidly reach replicative senescence in culture, and that impairs their regenerative potential[21,22].

The only stem cells in routine clinical use are HSCs, as the complications associated with the use of other stem cells have proven greater that originally imagined. It should also be mentioned that the benefits of HSCs and other mesenchymal stem cells (MSCs) depend not only on their *in situ* differentiation to functional tissue cells, but also on their broad repertoire of secreted growth factors, cytokines, chemokines, and other

bioactive components, as well as small circular membrane fragments or extracellular vehicles (EVs), enriched in mRNA, microRNA, bioactive lipids, nucleotides, and proteins[23-25]. Paracrine secretion has regenerative properties and has restore confidence in stem cell therapy. The mechanisms are far from being understood, and additional effort is needed to achieve effective, safe, and powerful regenerative approaches that involve the MSC secretome[26].

In addition to exploiting the self-renewal and differentiation properties of stem cells to repair cell and tissue damage or injury, growing tissues or entire organs in the laboratory is a long-term objective of regenerative medicine. The scientific and clinical community is coming closer to this ultimate goal with advances in our knowledge of the factors essential to directing stem cell differentiation and progress in tissue engineering. In fact, specific tissues and even whole organs generated in the laboratory have been transplanted into patients. These include relatively simple laboratory-grown organs, such as skin, bladder, and windpipe[8]. Encouraging advances have been made in the development of bone, cartilage, heart, nerve, and other tissues[27]. New multidisciplinary advances in organ bioengineering based on advances in cell biology, material science, chemistry, molecular biology, engineering, and medicine, include fabrication of synthetic or natural three-dimensional scaffolds used with stem cells and/or bioactive molecules[28]. However, it is evident that reaching the era of off-the-shelf organs awaits a deeper understanding of organogenesis.

Understanding organogenesis requires a detailed description of the decision-making machinery that controls the ability of stem cells to balance self-renewal and differentiation, while establishing and maintaining cell fate in the right place at the right time. The role of the orientation of stem cell division has emerged as an important mechanism for determining cell fate. A stereotypical asymmetric cell division (ACD) gives rise to one daughter stem cell with the exact same level of potency as the mother cell and another daughter cell that has acquired a more differentiated state. This unique asymmetry allows the stem cell to self-replicate and maintain the stem cell pool, while at the same time produce numerous differentiated progeny. For ACD to occur, cells must previously establish asymmetry/polarity, which is guided by a variable balance of intrinsic *vs* extrinsic cues. Several studies over the last 20 years have revealed the previously unappreciated, multifaceted role of centrosomes in interpreting signals from the extracellular as well as the intracellular environment that govern cellular asymmetry[29-36].

CENTROSOMES

More than a century ago, Theodor Boveri portrayed the centrosome as the dynamic center that governs cell division. He was remarkably accurate in describing its basic organization and function as an organelle that consists of a core structure, the centrioles, and an outer centroplasm, now known as the pericentriolar matrix (PCM), that organizes and anchors the “astral rays” that consist of microtubules (MTs)[37]. These nonmembranous organelles function as the MT organizing center (MTOC) of animal cells and therefore regulate vital processes for cell cycle progression, such as mitotic spindle assembly, chromosome segregation and cytokinesis. We now know that this core structure described by Boveri[38] near the end of the 19th century, *i.e.* the pair of centrioles (mother and daughter) and the surrounding PCM consists of around 200-300 proteins, governs MT nucleation, and also regulates cell cycle checkpoints[39]. The centrosome composition is not fixed, as the PCM materials use the MTs anchored to the centrosome as exchange routes. Cycling cells tightly regulate the centrosome cycle, allowing only one duplication round per cell cycle, so that two centrosomes are present in each mitosis. To ensure that, duplication and segregation of centrosomes is coregulated with the chromosome duplication-segregation cycle[40]. The basis of coregulation is the dependence of both key S phase events on cyclin-dependent kinase 2 (Cdk2) activation[41]. The robustness of the coregulation is ensured by the localization of cyclin E-Cdk2 at the centrosomes during G1/S phase when the initiation of DNA synthesis takes place[42].

Centrosome structure and duplication cycle

In proliferating cells, the centrosome needs to duplicate just before or at the onset of S phase so that it forms two new centrosomes that will orchestrate the assembly and organization of the mitotic spindle. Each centrosome consists of two centrioles, a mature mother centriole, and an immature daughter centriole that was assembled during the previous cell cycle, and is about 80% of the length of the mother centriole.

Except for length, mother and daughter centrioles are structurally distinct, as the distal surface of mother centrioles is associated with two types of outgrowths, the distal and subdistal appendages that are missing from daughter centrioles. The mother and daughter centrioles are in tight orthogonal association with each other[43]. Disorientation or disengagement, with the loss of the tight association, occurs before completion of cytokinesis, and requires the activity of separase, the protease that is also responsible for the separation of sister chromatids before anaphase[44]. Disengagement is necessary for the initiation of centriole duplication, which takes place before, or at the onset of S phase, where the formation of a new centriole (procentriole) starts at the proximal end of each of the already existing centrioles. The next step is elongation of the procentriole that starts during late S phase. The centriole reaches full length during the following cell cycle. Elongation is followed by maturation in G2, with the recruitment of additional PCM material[45]. Complete maturation of a procentriole into a mother centriole extends over one and a half cell cycles, culminating with the acquisition of distal and subdistal appendages[41]. After duplication of centrosome is complete, the fibrous link between parental centrioles is dissolved to allow centrosomes separation and their migration to opposite poles during prophase of mitosis. As the cell exits mitosis, each new cell inherits one centrosome carrying a mother and a daughter centriole, ready to begin the next centrosome-chromosome duplication cycle[46] (Figure 1).

In interphase, centrioles take on another life; the mother centriole matures and docks below the plasma membrane, where it forms the basal body, which serves as a template for the formation of the axoneme that assembles the primary cilium[37]. The appendages that distinguish the mother centriole from the daughter, drive this process, called ciliogenesis. While the subdistal appendages are involved in organizing the interphase MT cytoskeleton, the distal appendages promote membrane docking and are essential for the formation of the primary cilium. In cycling cells, the cilium cycle follows the cell cycle. The cilium is reabsorbed when cells enter mitosis, which allows the formation of centrosomes and the mitotic spindle assembly [47]. The role of the primary cilium, the antenna-like extension present on the majority of nonproliferating or quiescent cells, has been neglected for many years. It has recently become evident that this organelle has both sensory and signaling functions[48,49] that are of key importance for normal development and health. Highlighting this significance, defects in ciliogenesis are characteristic of a set of ciliopathies that affect organs such as the kidneys, eyes, liver and brain[50-52].

Perturbations of centrosome function have also been linked to carcinogenesis, as they compromise the fidelity of chromosome segregation and can result in aneuploidy. That was the basic premise of Theodore Boveri's famous theory of cancer development [53], and is still considered an important hallmark[54]. In addition to compromising chromosome segregation by affecting spindle geometry, it has been established that centrosomes contribute to carcinogenesis *via* several mechanisms that include cellular polarity[55,56], asymmetric centriole inheritance in stem cell lineages[57,58], and ciliary function[59-61]. It is no surprise that centrosome aberrations that might lead to tumorigenesis are related to asymmetries that are intrinsic to their structure and the duplication cycle. The relationship highlights the crucial importance of the asymmetric nature of the centrosome for stem cell physiology. In the following paragraphs we review evidence that supports the maintenance of stem cell renewal and differentiation potential by centrosomes, which direct (1) asymmetric division and distribution of cell fate determinants; and (2) primary cilium-dependent signaling that orchestrates cell fate.

CENTROSOMES AND ASYMMETRIC STEM CELL DIVISION

Stem cells can not only divide symmetrically to expand the stem cell pool, but also asymmetrically. ACD produces one identical stem cell with self-renewal ability and one differentiating cell to produce daughter cells with different fates. This ability of stem cells is the mechanism that balances the need for maintaining the stem cell population with the demand for more differentiated cells, and is vital for tissue homeostasis[62,63]. ACD refers to a polarized/asymmetrical mode of division orchestrated by extrinsic and intrinsic cues that determine the fate of the daughter cells.

ACD depends on cellular polarization

Extrinsic cues consist a molecular signal repertoire that originates in the extracellular environment that stem cells reside in, called the niche[64]. The asymmetry defined by

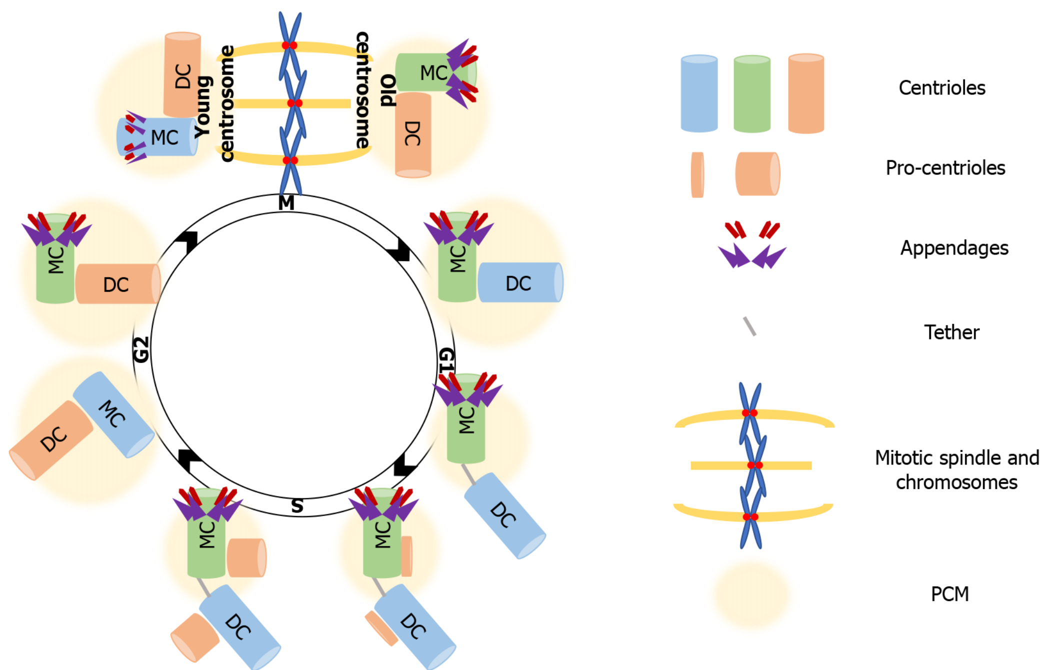

Figure 1 The centrosome cycle. Cycling cells tightly regulate the centrosome cycle, allowing only one duplication round per cell cycle. To ensure this, duplication and segregation of centrosomes is coregulated with the chromosome duplication-segregation cycle. In G1, the centrosome modifies its structure by disorientation or disengagement of the centrioles, with loss of their tight association while remaining connected by the fibrous linker or tether. Two sets of appendages mark the mother centriole. Afterwards, at the onset of S phase, the initiation of the duplication occurs with the formation of a new centriole (procentriole) at the proximal end of each of the already existing centrioles. The next step is the elongation of the procentriole, which starts during late S phase. Elongation is followed by maturation that continues until late G2, with the recruitment of additional pericentriolar matrix material, eventually leading to the formation of two centrosomes, old and young. The old centrosome contains the old mother centriole (green) and the young centrosome contains the new mother centriole (blue). The complete maturation of the new mother centriole (blue) extends over one and a half cell cycles by the acquisition and maturation of full length distal and subdistal appendages. At the end of G2, the tether between mother and daughter centrioles dissolves, freeing the two centrosomes to move to the opposite poles in order to properly orient the mitotic spindle. After mitosis, each daughter cell inherits a single centrosome, either the old or the new one. DC: Daughter centriole; MC: Mother centriole; PCM: Pericentriolar matrix.

the niche depends upon the concept of the delivery of self-renewal signals from the niche to the cells within range. For instance, in *Drosophila melanogaster* testes and ovaries, the niches consist of certain types of postmitotic cells that secrete critical self-renewal ligands to neighboring cells[65]. The cell fate determinants can even reverse the phenotype of partially differentiated cells to become stem cells again[66]. However, that is not a universal phenomenon. HSCs maintain their stemness even after leaving their niche[1], and neural stem cells can also divide symmetrically outside their niche to produce identical self-renewing progeny[67].

The latter evidence highlights the importance of intrinsic cell fate determinants and turns the focus to intracellular cues that are characterized by or provide asymmetry. In other words, the simplest way of producing two different daughter cells, is to distribute fate regulators asymmetrically by polarizing the interphase intracellular environment and ensuring that the determinants will be inherited asymmetrically by properly orienting the mitotic spindle during division. The proper orientation of the spindle to ensure asymmetric division of cell fate determinants, can also been seen as the process by which extrinsic and intrinsic cues merge. Drosophila male germline stem cells (GSCs) are attached to their niche, which provides the signaling ligands necessary for retaining stem cell identity[64]. The asymmetry provided by the niche to the attached progeny is guaranteed by the GSCs orienting their spindle perpendicular to the hub cells, so that one daughter cell maintains the attachment to the cell hub, whereas the sibling cell initiates differentiation because of losing the attachment[68]. The latter emphasizes the fact that ACDs are achieved by polarization of fate determinants coupled with proper spindle orientation[69]. The best described example of polarization of cell fate determinants is also provided by studies in Drosophila. It has been shown that Drosophila neuroblasts produce fate determinants that remain inactive during interphase, are distributed in a polarized manner, and during division the mitotic spindle is oriented in such a way so that these determinants are inherited by only one of the two daughter cells, which will become the ganglion mother cell[70, 71].

Centrosomes serve as means of cellular polarization

Importantly, both cellular polarization as well as orientation of the mitotic spindle depend upon centrosome function. For polarization to occur the most essential requirement is the existence of MTs, and in essentially all non-terminally differentiated somatic cell as well as male germline animal cells, MT organization is governed by centrosomes[72]. For instance, it is well established that centrosome positioning governs the localization of several subcellular compartments, such as the Golgi apparatus, by controling of the nucleation and anchoring of MTs. It has recently been shown that the centrosome might also promote actin filament assembly. As MTs and actin filaments are the two main cytoskeleton networks supporting cell polarity, the latter makes the centrosome the master regulator of intracellular architecture[73].

It is clear that, besides having this fundamental effect on cell geometry, the centrosome determines the position at which the spindle poles will form and how the mitotic spindle will be oriented. Correct orientation of the mitotic spindle ensures not only faithful segregation of chromosomes but also proper segregation of molecules defining cell fate[74]. Various MT subpopulations, *i.e.* kinetochores, interpolar and astral MTs, are involved in controlling the process[75]. For instance, astral MT nucleation, stability, and dynamics as well as anchoring at the cortex are of particular importance in order to achieve proper orientation of the mitotic spindle[74].

More than building polarity/asymmetry, centrosomes provide a mechanism that maintains and transmits differential cell fate information, which also explains how cellular memory is passed on from one cell to the next during division[76]. As mentioned, because of the way centrosomes duplicate, the centrioles within each centrosome can be distinguished by age, one is formed in the preceding cell cycle, and the other is assembled at least one cycle earlier. After duplication, the centrosome that retains the most recently built centriole is the young centrosome and the other one is the old centrosome (see Figure 1). The old centrosome consists of the older mother centriole, which harbors completely mature distal and subdistal appendages. Because the subdistal appendages are the major site for MTs anchoring, the old centrosome typically has higher MT organizing activity (MTOC) than the young centrosome, which contains the recently formed mother centriole. Even more, several proteins such ninein (NIN), Cep164 and outer dense fiber protein 2 (ODF2) were found to be localized to the mother centriole, whereas centrobin (Cnb) localizes only to the daughter centriole[77-80]. This differential protein composition enhances the asymmetry of the old and young centrosomes, which in turn ensures that the daughter cells arising following division are "born differently", as one receives the young centrosome and the other receives the old centrosome. Also, this asymmetry carries the intriguing assumption that it would be also functionally relevant to stem cell ACD.

The asymmetric segregation of the centrosomes that defines the fate of progeny has been best described in GSC, Drosophila neuroblasts, and mouse neural progenitors[81-83]. Those studies provided a narration of the asymmetric centrosome cycle, which depends on differential MTOC activity and in turn on centrosome age, as already mentioned. Specifically, as described for Drosophila neuroblasts, the young centrosome -the one containing the younger mother centriole- maintains its MTOC ability throughout interphase, whereas the old centrosome -containing the older mother centriole- downregulates its ability to nucleate MTs as the neuroblasts enter interphase. That helps orient the mitotic spindle along the neuroblast apical-basal polarity axis, as the young centrosome with active MTOC ability, remains tethered to the apical neuroblast cortex. The inactive old centrosome is displaced form the apical cortex as its centriole downregulates MTOC activity through the "shedding" of its PCM content. In that way, the apical centrosome will always be the young centrosome, and will be inherited by the self-renewed progeny, while the old centrosome segregates into the more differentiated daughter cell. Biased centrosome segregation also takes place in male Drosophila GSCs; but in this cell type is the old centrosome that retains the MTOC activity and therefore maintains its localization near the stem cell niche, ensuring that the self-renewal ability is passed on to the proximal progeny [34,36,82,84-87].

Even though it is true that the above mentioned mechanism of biased centrosome inheritance was originally described for a few cell types, several studies have shown that human cells are probably not an exception. For example, studies have revealed the dependance of MTOC ability on specific centrosome components in human cells. The human daughter centriole-associated ciliopathy protein, Cep120, has been shown to have a critical role in MTOC activity, as its depletion results in accumulation of PCM components. Elevated PCM levels result in increased MTOC activity at the centrosome, which is crucial for centrosome homeostasis, potentially underlies the pathogenesis of ciliopathies, and provides further evidence of the dependence of ACD

on centrosomes[88].

Strong evidence has been provided for involvement of the Wnt pathway in the determination of cell fate in humans. Several important Wnt pathway components, such as disheveled 2, which actually transmits the Wnt signal, was found to localize at the centrosome and to regulate spindle orientation[89]. The latter is critical for determining the plane of cell division and defining whether a cell remains within a particular environment, such as the niche, therefore controlling cell fate. The importance of Wnt signaling in the ACD of human skeletal stem cells (hSSCs) was highlighted in a recent study. It was shown that covalently immobilizing Wnt factors onto synthetic materials can polarize single dividing hSSCs, orient the spindle, and simultaneously generate a Wnt-proximal hSSC and a differentiation-prone Wnt-distal cell[90]. The study emphasizes the importance of deciphering the nature and function of centrosomes for the development of promising approaches for tissue repair.

Moreover, recent advances of centriole biology support the universality of biased centrosome segregation[91-95]. Firstly, the novel concept of PCM as a molecular assembly formed *via* liquid-liquid phase separation[96] is an outstanding paradigm that makes the asymmetric nature of young *vs* old centrosomes even more pragmatic and relevant[97,98]. Even more, targeted cotranslation is another concept that adds to the fascinating idea of centrosome-dependent ACD. In zebrafish and various human cell types, the mRNAs of key centrosome scaffold proteins such as pericentrin (PCNT) and nuclear mitotic apparatus protein 1 (NUMA1), among others were found to be located on the centrosome where they are translated during mitosis[99-101]. The *in situ* translation (1) optimizes centrosome maturation, as its core proteins are manufactured at their destination compartment, and (2) adds a sophisticated layer of regulation of centrosome asymmetry that could prove critical for ACD. *In situ* translation provides insights to the mechanism(s) *via* which mutations in PCNT, for example, cause primary microcephaly phenotypes that are thought to arise from proliferation defects in neural progenitors[102]. Moreover, the above studies[99-101], identified eight mRNAs that localize in the centrosomes of human cells. These mRNAs code for centrosome proteins PCNT, NUMA1, CCDC88C, NIN, BICD2, HMMR, CEP350, and ASPM that regulate centrosome maturation, spindle positioning, and MT dynamics. Given the importance of these proteins in centrosome biology, which is indicated by their *in situ* translation, it would be interesting to elucidate their specific role in centrosome-dependent ACD, which will in turn open new horizons in manipulating the determination of cell fate.

Old vs young centrosome: Asymmetries are functionally relevant

The differential segregation of old and young centrosomes in asymmetrically dividing cells is accompanied by functionally relevant consequences. The old centrosome carries ciliary membrane when it is internalized before mitosis. Because of that, the daughter cell that inherits this centrosome will form a primary cilium before its sibling does[103]. The consequence is that the cell that inherited the old centrosome accumulates primary cilium-associated smoothened (SMO) and experiences higher hedgehog (Hh) signaling, that has been demonstrated to promote stem cell identity. On the contrary, the sibling cell that inherits the young centrosome loses self-renewal ability in response to lower Hh signaling and commits to differentiation[103].

As earlier mentioned, the old and young centrosomes differ not only in age and their ability to organize MTs, but also in their molecular composition, *i.e.* proteins and mRNAs that could serve as fate determinants[104,105]. A well-designed study by Lambert and Nagy[104] showed that fate-determining mRNAs are attached to one of the centrosomes during cell division in mollusk embryos. Those mRNAs are inherited by only one daughter cell *via* the asymmetric segregation of the centrosomes, and define the embryonic patterning during mollusk development. A recent study demonstrated that Mindbomb1, a Notch ligand activity regulator, was found to localize onto the daughter centriole in chick neural progenitors, and that the daughter cells that receive this centriole after ACD differentiate into neurons[106].

Another interesting asymmetry that is associated with centrosomes has been reported to accompany cytokinesis. At the end of cytokinesis, the midbody ring is inherited by one of the daughter cells, which studies in HeLa cells have shown, is the cell that inherits the old centrosome[107]. Interestingly, studies with stem cells revealed a correlation between midbody inheritance and self-renewal ability[107-109]. Even though that is an interesting notion, the exact role that the midbody or midbody-associated molecules have in regulating self-renewal is missing. Similarly, the aggresome, a large structure that accumulates damaged or misfolded proteins, was also observed to be associated with centrosomes and to be inherited, together with the young centrosome, during ESC division by the differentiated progeny[110]. Again, it

was implied that the aggresome acts as a cell fate determinant without providing a mechanistic insight that would explain such a function.

Interestingly, a recent study provided even more direct evidence linking the old centrosome's composition to pluripotency maintenance. It was shown that NANOG, the protein that, together with SOX2 and OCT4, has a fundamental role in defining stemness, localizes in the cytoplasm on the appendages of the mother centriole in human tumor cell lines, fibroblasts and hESCs[111]. Even though this important study clearly demonstrated the association of NANOG with the old centrosome, the assumption of its role in centrosome maturation is lacking direct evidence. Nevertheless, these observations gave birth and provide support of the immortal centrosome hypothesis, which proposes that the daughter cell that retains the older centrosome has self-renewal properties[112]. The hypothesis is elegant, but not globally supported. In Drosophila neuroblasts and female GSCs, the cell that inherits the young centrosome maintains self-renewing[109,113]. A similar pattern of asymmetric inheritance of the centrosome was found in human cancer stem cells. It was shown that in human neuroblastoma cell lines, the young centrosome is inherited by the NUMA-retaining cell, which also possesses greater proliferation ability[114]. In any case, the fact that either the young or the old centrosome is associated with self-renewal progeny means that it is the asymmetric nature of the centrosome that contributes to determination of cell fate. Recent studies of the molecular composition of centrosomes provide additional evidence supporting asymmetry. Several proteins reported to be associated with stem cell centrosomes could prove to be functionally relevant to asymmetry and have crucial roles in determining cell fate. For example, NIN is a protein reported to be specific to the mother centriole[79,115] and to accompany inheritance of the old centrosome in radial glial progenitor cells that retain pluripotency in mice[83]. It was also demonstrated that Klp10A, an MT-depolymerizing kinesin, is located on the centrosomes of stem cells but not the centrosomes of differentiating germ cells in Drosophila male germline. Moreover, depletion of Klp10A resulted in an abnormally elongated old centrosome compared with the younger one, which in turn gave rise to asymmetric daughter cell size, with a bigger GSC and a smaller differentiating gonial blast. Even though these results did not reveal the exact significance of centrosome asymmetry, they imply that the old centrosome (or older mother centriole) possesses an ability that is missing from the young centrosome[116]. A recent study identified another mouse neural stem cell centrosome protein, AKNA, that is found only on the subdistal appendages of the mother centriole, and has a vital role in promoting MT nucleation and growth[117]. *Alms1a*, the Drosophila homologue of the gene responsible for the Alstrom syndrome, a characteristic human ciliopathy, is another mother centriole-specific protein that was recently identified and found to interact with both Klp10A and Sak[118], the Drosophila homologue of Polo-like kinase 4 (Plk4), the master regulator of centriole duplication[119]. In addition to centriole duplication, Plk4 was recently reported to support the establishment of centrosome asymmetry. In Drosophila neural stem cells, Plk4 phosphorylates Spd2 on the mother centriole, which is important not only for centrosome asymmetry but also for proper mitotic spindle positioning[120]. These findings add further knowledge of the unique characteristics of old and young centrosomes that will help to elucidate how centrosomal asymmetry drives asymmetric stem cell division, by identifying the centrosome molecules and their functions that drive self-renewal *vs* differentiation. And by deciphering how cell fate determination is controlled, new research directions will open, aiming at producing cells specific for use in tissue repair and regeneration.

PRIMARY CILIA AND STEM CELL DIFFERENTIATION

Among the centrosome asymmetries, no difference is more remarkable than the unique ability of the mother centriole to dictate the formation of the primary cilium. The primary or nonmotile cilium is an organelle consisting of MTs surrounded by a specialized membrane that carries signal receptors. It extends from the apical surface of nearly all vertebrate cells, and forms when the basal body docks on the membrane [121].

Mother centriole and ciliogenesis

Ciliogenesis, is known to be entirely dependent on the mother centriole appendages, with the distal appendages promoting mother centriole to basal body maturation and membrane docking. The subdistal appendages direct cilium positioning (Figure 2)[37].

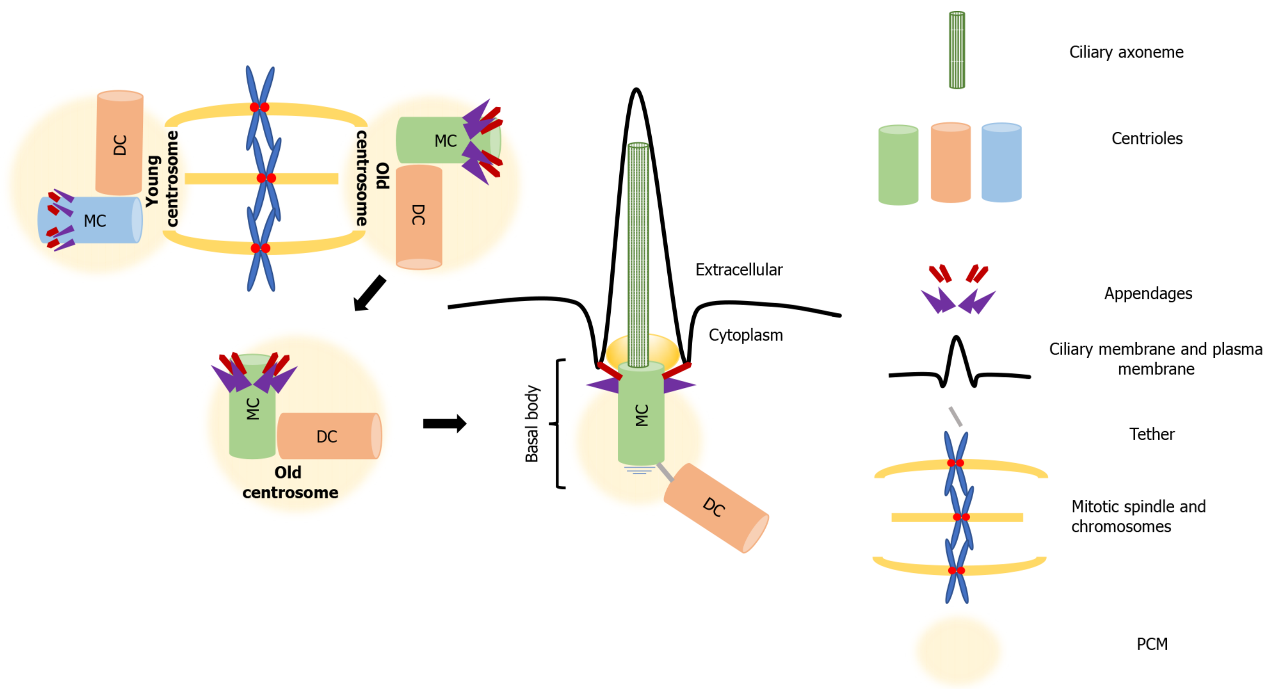

Figure 2 The primary cilium. After mitosis, the mother centriole of the old centrosome undergoes maturation that results into the formation of the basal body and ultimately the development of the primary cilium. The primary cilium is an organelle that contains the microtubule-consisting axoneme surrounded by a specialized membrane that extends from the apical surface of nearly all vertebrate cells. It is formed by ciliogenesis, which is entirely dependent on mother centriole's appendages. The distal appendages (red) promote the maturation of the mother centriole to a basal body and its docking to the membrane. The subdistal appendages (purple) direct the positioning of the primary cilium. DC: Daughter centriole; MC: Mother centriole; PCM: Pericentriolar matrix.

The process is rather complex and involves multiple steps that include (1) transport of preciliary vesicles to the basal body, associating with the distal appendages, and fusing to form a larger vesicle; (2) enrichment of the ciliary vesicle with membrane proteins that promote ciliary membrane expansion and selective trafficking of proteins to the cilium; (3) extension of the centriole/basal body MTs to form the axoneme of the cilium; and (4) the forming of a transition zone that partitions the cilium from the cell body[47,122]. Two distinct ciliogenic pathways have been described. The intracytoplasmic and plasma membrane associated pathways, differ in the position of the formation of the preciliary vesicles, *i.e.* the cytoplasm or plasma membrane, and not in the basic steps[61]. Ciliogenesis results in the construction of a distinctive microenvironment within the primary cilium that facilitates the transduction of extracellular initiated signals. Many components of the cilium, both regulatory and structural, participate in receiving and interpreting a variety of different extracellular cues[123]. As already mentioned, aberrations in ciliogenesis or dysfunction of primary cilia-associated signaling is linked to several human pathologies, These ciliopathies highlight the functional significance of the mother centriole that was neglected for a long time and has recently experienced a renaissance. This renaissance is believed to be attributed to accumulating evidence that support its role as the cell's "antenna", which receives and integrates signals from the extracellular environment that regulate development, cell polarity, and importantly, cell identity[124].

The primary cilium inherits asymmetries

Many studies have reported the presence of primary cilia in a variety of stem and progenitor cells. Aberrations or alterations in their structure/expression, length, and/or protein composition highlight their significance for stem cell function[125-129]. It is becoming evident that the function of the primary cilium in signaling is of crucial importance when it comes to the determination of stem cell fate. As already mentioned, in dividing mouse radial glial progenitor cells, the primary cilium is not completely disassembled prior to cell division. Fragments stay attached to the old centrosome, which includes the older mother centriole (see Figure 1). The latter is believed to result in more rapid formation of a primary cilium in that daughter cell than in its sibling, as its old centrosome had controlled the formation of a primary cilium in a previous cycle. Even more, it was also demonstrated that the old centriole responds to signaling that promotes stemness[103]. A recent study, using live-cell

imaging analysis, demonstrated that cilia grow faster from older centrosomes associated with a ciliary remnant than from "naked" centrosomes that lack a remnant [130]. Additionally, this study clearly demonstrated that the remnants were associated with the distal appendages of the mother centriole and that overexpression of active Nek2A kinase prematurely displaced distal appendages from interphase centrosomes. As previously noted, inheritance of the ciliary remnants seems to give to the cells a temporal advantage in reforming the cilium and therefore becoming responsive to Hh ligands[103]; the latter could prove essential in paving ways to manipulate cell fate determination.

Given the above, a recent study by Vestergaard and colleagues [131] that showed that transcription factors known to be associated with pluripotency, such as SOX2 and NANOG, are located on the primary cilium of hESC did not come as a surprise. Specifically, the study describes the technique used to examine the spatiotemporal regulation and localization of those transcription factors and revealed that in addition to the expected nuclear location, SOX2 and NANOG were associated with a subset of hESC primary cilia. Even though the study is important for indicating a functional relationship between primary cilia and differentiation and/or self-renewal processes, it lacks an explanation of why SOX2 and NANOG were found in some hESC primary cilia and not in all of them. It remains to be shown whether that was a technical limitation of the study or if it is functionally relevant.

Primary cilium-associated signaling pathways determine cell fate

A variety of signaling pathways that are crucial for cell fate determination and differentiation have been reported to be associated with or mediated by the primary cilia of human stem cells[132-135]. The most relevant are the Hh and Wnt signaling pathways, which have already been mentioned[136]. Hh signaling has been shown to be of critical importance in organogenesis, as it promotes stem cell proliferation and migration[139-142]. Two of the most important proteins for Hh signaling, Patched (PTCH) and SMO, were found to be located in the primary cilium. Briefly, when Sonic Hh is present it binds PTCH, thus allowing SMO to move into the ciliary axoneme and activate the glioma-associated oncogene transcription factor (GLI). For a detailed description see Kopinke *et al*[143]. Briefly, the cilium is believed to act as a mediator of the trafficking and accumulation of SMO and GLI proteins in the context of Hh signaling during development and regeneration.

Like the Hh signaling pathway, Wnt signaling is considered extremely crucial for cell fate determination[144]. Wnt signaling includes both canonical and noncanonical Wnt pathways. Canonical Wnt signaling controls cell proliferation and cell fate, and defects have been associated with cancer development. Noncanonical Wnt signaling is thought to give shape to tissues by control of cell migration and orientation driven by cell polarization and ACD. In noncanonical Wnt signaling the receptor of Wnt signals, Frizzled protein, was found to be located on the membrane of the primary cilium. The downstream activity of the Wnt proteins inversin and disheveled is also located at the base of the cilium[144,145]. Recent evidence suggests that components of noncanonical Wnt signaling interact or are associated with the primary cilium[146].

Recent findings also report the dependance of the platelet-derived growth factor (PDGF)[137] and transforming growth factor beta (TGF-β) signaling pathways on the primary cilium[133,138]. The PDGF signaling pathway is considered to be of major importance for wound healing and cancer development, and has been implicated in cell migration and differentiation[147]. PDGF signaling depends upon the interaction between PDGF-AA ligand and its receptor PDGFRα, which was found to occur on the primary cilium membrane. This interaction may be the best described example of the function of the primary cilium as a chemical antenna, as its orientation depended on the concentration of the PDGFRα receptor[148]. TGF-β signaling is also linked to cell proliferation and differentiation. It is particularly important in epithelial-mesenchymal transition (EMT), a procedure that is mediated by shear stress activating TGF-β that is located on the primary cilium[149,150]. The downstream proteins of TGF-β signaling, SMAD 2/3 and extracellular signal-regulated kinase 1/2 (Erk1/2), have also been found at the base of the cilium[133], further supporting its importance in this pathway.

The significance of cilia-mediated signal transduction was further emphasized by a study that investigated hESC mesendoderm and neuroectoderm (NE) fate decisions. It was demonstrated that a specific ciliation pattern occurred within the first 24 h that, coupled with G1 phase lengthening, induced NE lineage specification before any other neural markers were expressed. Notably, it was further shown that cilia formation in NE precursors was accompanied by increased autophagy that resulted in NRF2-mediated transcription inactivation and repression of the expression of pluripotency genes OCT4 and NANOG that allow lineage commitment toward NE[151].

The critical significance of the above signaling molecules being present at the primary cilium was further supported by reports of its fundamental role in defining the offspring of different progenitors. When muscle-resident fibro/adipocyte progenitors (FAP) are injured or aged, proliferation is shifted towards the production of adipocytes, which causes muscle to be replaced by fat. A recent study demonstrated that the process was directed by ciliary Hh signaling[152]. The same study also demonstrated that preventing ciliation in FAP resulted in inhibition of intramuscular adipogenesis and enhanced myofiber regeneration after injury in a Duchenne muscular dystrophy mouse model. A study of electrical field stimulation (EFS)-enhanced osteogenesis of human adipose-derived stem cells (hASC) demonstrated that if the molecular composition of the primary cilium was disrupted, the ability of hASC to detect electrical field signals was compromised. The same study also reported evidence of the primary cilium as a key calcium-signaling module during EFS-osteogenesis[153]. Another recent study added to the above by demonstrating that calcium induction triggered ciliogenesis and adipogenic differentiation of human MSCs by negatively regulating Wnt5a/β-catenin signaling[154].

Another study of hASCs showed the potential implication of ciliary signaling in the pathogenesis of obesity. It was reported that obese hASCs had shortened cilia, and were unable to respond properly to stimuli[155]. Interestingly, another study in obese patients showed that treatment with inhibitors of Aurora A kinase or Erk1/2 rescued both the length and functionality of primary cilia and increased the expression of genes related to self-renewal/stemness. The findings have clinical importance for autologous MSC-based therapies[156]. Further studies revealed that the above cilia aberrations were associated with a deficiency in Hh signaling that affected hASC differentiation capacity. The data support the potential of novel therapies for obesity and associated pathologies[155]. The impact of ciliary Hh signaling in tissue regeneration and tumorigenesis was described in a recent study of the importance of epithelial-EMT programming in stemness. The stemness of both mammary stem cells and their neoplastic counterparts, mammary tumor initiating cells, in the mammary epithelium seem to depend on the EMT program, which in turn relies on primary cilia formation and Hh signaling[157].

Stemness dependance on the primary cilium was also investigated in a recent study in which the authors silenced the expression of two of its components, the ciliary proteins IFT172 and KIF3A, in MSCs. The outcome of siRNA-based knockdowns was the production of fewer and shorter cilia, increased proliferation ability of MSC and reduction of the expression of the stem cell markers OCT4, NANOG, and SOX2[158]. The results suggest the dependance of stemness maintenance on proper cilia function and signaling. Similarly, a recent study reported the dependance on cilia-specific genes of hematopoietic stem and progenitor cell (HSPC) function in the hemogenic endothelium (HE) of zebrafish embryos. The authors described the role of cilia-mediated Notch signaling in HSPC asymmetric division in the production of mature blood cells as well as self-renewing progeny[159].

Not only structure and signaling but also proper disassembly of the primary cilium was reported to be an important factor in stem cell function. It is known that a mutation in the centrosomal-P4.1-associated protein (CPAP) is linked to Seckel syndrome microcephaly[160] and possibly to neural progenitor cell (NPC) dysfunction. It was demonstrated recently that CPAP serves as a scaffold protein that promotes timely cilium disassembly, and mutation results in retarded cilium disassembly as well as delayed cell cycle re-entry and therefore premature differentiation of NPC[161]. The latter further emphasizes the important role that the primary cilium has in ACD that maintains tissue homeostasis.

A recent study investigated the function of cilia-dependent signaling in regeneration and repair of fractured bone. Interestingly, the authors showed that delayed fracture healing in smokers might be attributed to dysfunctional ciliary-mediated TGF-β signaling in MSC[162]. Besides TGF-β, Hh signaling and intraflagellar transport (IFT) were reported to be essential for bone development. IFT moves non-membrane-bound particles from the cytoplasm to the tip of the cilium and is considered crucial for cilium assembly and maintenance[163,164]. A recent study showed that IFT proteins regulated Hh signaling in osteoblasts (OBs), and their silencing resulted in impaired OB differentiation and subsequent craniofacial and skeletal abnormalities[165].

As previously mentioned, MSCs have been accepted as vital for tissue homeostasis and regenerative medicine, as they are present in almost all tissues, are easily isolated, can differentiate into almost any cell lineage, and can be cultured on specific scaffolds used for tissue reconstruction[4,136,166]. Even though initial studies of MSC-based regenerative approaches focused on the musculoskeletal system, studies have recently

been expanded to include other tissues, like the nervous system, heart, liver, cornea, and trachea[136]. Many studies have used this type of adult stem cells to explore the role of the primary cilium in directing regeneration and repair. For example, in one of the first studies, Corbit *et al*.[167] demonstrated that knocking down the cilia protein Kif3a resulted in disruption of the proper structure of the cilium and enhanced canonical Wnt signaling. Similarly, siRNA knockdown of IFT88, another primary cilium-associated protein, was also demonstrated to compromise the osteogenic, chondrogenic and adipogenic differentiation potential of MSCs[126]. Knockdown of another cilia-associated protein, polycystin-1 in human adipose tissue-derived MSC (hASC) resulted in a downregulation of osteocalcin gene, diminished calcium accretion, and reduced alkaline phosphatase activity that abrogated hASC-dependent bone regeneration and repair abilities[168]. MSC cilium structure and the activity of its associated proteins in the control of cell differentiation were investigated in a study analyzing changes in ciliary length. It was reported that MSCs cultured in adipogenic differentiation medium exhibited an elongation of their primary cilia with subsequent upregulation of nuclear PPARγ levels and recruitment of IGF-1Rβ to the cilium, thus contributing to expanding our knowledge of ciliary protein function[169].

Even more essential, the role that MSCs could have in tissue engineering and regenerative medicine was highlighted in a study that investigated the effect of substrate environment architecture on MSC phenotype determination[170]. It was shown that substrate architecture can induce changes in cytoskeletal tension that in turn influence primary cilium signaling. Specifically, it was demonstrated that MSCs cultured on grooved surfaces had more elongated and aligned cilia. It was concluded that the specific architecture enhanced ciliogenesis and suppressed MSC proliferation *via* inhibition of canonical Wnt signaling[171]. Another recent study described the dynamic sensory abilities of hASC primary cilia and the importance of manipulating those abilities. The authors found that hASC cilia length and cilia conformation varied in response to culture conditions (*e.g.*, complete growth, osteogenic differentiation, or adipogenic differentiation culture medium) with the longest cilia expressed in cells differentiating into adipocytes. Importantly, they showed that cyclic tensile strain enhanced hASC osteogenic differentiation while suppressing adipogenic differentiation[172]. The study highlights the importance of the primary cilium in lineage specification and therefore its role as a novel target in attempts to manipulate hASC for tissue engineering applications.

CONCLUSION

Based on all the above, it is safe to say that cellular asymmetry and asymmetric distribution of cell fate determinants as well as ACD define stemness. It is becoming more than evident that the centrosome and the centrosome-derived primary cilium provide an excellent vehicle to serve this asymmetry. The centrosome and centrosome-derived primary cilium illustrates the extraordinary ability of stem cells to maintain the crucial balance between self-renewal and differentiation. As studies regarding stem cell centrosomes and cilia accumulate, we are reaching a better understanding of the requirement of the presence of these structures for orchestrating receiving, interpreting and transducing signals. Essentially, centrosome-dependent signaling -by directing changes in stem cell morphology, gene expression, and cytoskeletal organization- ultimately determine stem cell differentiation. Hence, it is tempting to envision procedures aiming to manipulate and change centrosome composition and/or cilium architecture and trafficking, as means of controlling the direction of differentiation in the context of tissue engineering and regenerative medicine. Already various methods that aim to guide cell phenotype, including chemical or mechanical stimulation as well as modulation of the architecture, composition and/or dimensionality of the substrate microenvironment, have been reported[136]. From what was presented here, it seems that those manipulations, intentionally or unintentionally, directly or indirectly, aimed at exploiting the functions of the centrosome/ cilium. For this reason, we dare to predict that this 130-year-old organelle, originally called the centrosome, in order to acknowledge its location near the geometrical center of the interphase cell, will be at the center of efforts that will shape the future of regenerative medicine.

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REVIEW

Effects of storage media, supplements and cryopreservation methods on quality of stem cells

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Abstract

Despite a vast amount of different methods, protocols and cryoprotective agents (CPA), stem cells are often frozen using standard protocols that have been optimized for use with cell lines, rather than with stem cells. Relatively few comparative studies have been performed to assess the effects of cryopreservation methods on these stem cells. Dimethyl sulfoxide (DMSO) has been a key agent for the development of cryobiology and has been used universally for cryopreservation. However, the use of DMSO has been associated with *in vitro* and *in vivo* toxicity and has been shown to affect many cellular processes due to changes in DNA methylation and dysregulation of gene expression. Despite studies showing that DMSO may affect cell characteristics, DMSO remains the CPA of choice, both in a research setting and in the clinics. However, numerous alternatives to DMSO have been shown to hold promise for use as a CPA and include albumin, trehalose, sucrose, ethylene glycol, polyethylene glycol and many more. Here, we will discuss the use, advantages and disadvantages of these CPAs for cryopreservation of different types of stem cells, including hematopoietic stem cells, mesenchymal stromal/stem cells and induced pluripotent stem cells.

Key Words: Cryoprotective agents; Dimethyl sulfoxide; Hematopoietic stem cells; Mesenchymal stromal/stem cells; Induced pluripotent stem cells

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cold storage of hematopoietic stem cells, mesenchymal stem cells and induced pluripotent stem cells. Although dimethyl sulfoxide (DMSO) is commonly used in cryopreservation of cell lines, primary cells and stem cells, the use of DMSO has been associated with certain toxicity, both directly on the cells, as well as upon infusion with the stem cell product. As a result of this many groups have undertaken efforts to find suitable replacements for DMSO that are equally potent but less toxic. In this review, we summarize the current *status quo* of stem cell freezing protocols and we describe the most commonly used cryoprotective agents and their effects on stem cells and stem cell function.

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INTRODUCTION

Although optimization of stem cell culture, expansion and differentiation methods has been the main focus of stem cell research, an equally important and largely ignored topic in stem cell research is long term storage and cryopreservation. No matter the quality of the stem cell cultures, without optimization and careful control of cryopreservation, reproducibility and clinical (side) effects may be difficult to interpret. Furthermore, effects may be unexpected and suboptimal if cells are not stored, frozen and thawed under the most favorable conditions. Cryopreservation of cells, tissues and embryos has been common practice since the 1950s and took flight with the development of *in vitro* fertilization practices and hematopoietic stem cell (HSC) transplantation.

Storage under low temperature conditions reduces the rates of intracellular enzymatic and chemical reactions that may be harmful and allows the cells to be stored long-term without damage. The basic principle underlying successful cell cryopreservation is prevention of the formation of intra- and extracellular ice crystals during freezing, since this is the primary cause of cell damage[1]. Cryopreservation methods can be classified into slow freezing and fast freezing (vitrification) procedures. Both methods are based on the freezing or solidification of the cells or tissues and may cause cell injury in the process. However, the mechanisms that cause cell damage are quite distinct. Whereas rapid cooling results in the formation of intracellular ice crystals causing physical stress to the cells and mechanical breakdown, slow cooling causes osmotic changes in the cells and mechanical stress due to the formation of extracellular ice[2]. During vitrification a liquid is transformed into a glass-like non-crystalline solid state due to overcooling without freezing. Its most important feature is the prevention of ice formation[3,4]. During vitrification, cells kept in cryoprotectant solutions are briefly exposed to nitrogen vapor and subsequently immersed in liquid nitrogen[5] and usually a permeable cryoprotectant [dimethyl sulfoxide (DMSO) or glycerol] and an impermeable cryoprotectant [hydroxyethyl starch (HES), polyvinyl alcohol, trehalose] are used together[6,7]. During slow freezing, extracellular ice crystals may cause an increase in cellular osmolality and dehydration, and therefore the cooling rate during freezing should be sufficiently slow to allow a suitable amount of water to leave the cell[8,9]. The optimal cooling rate depends on cell size, sample size, water permeability and the presence of nucleating agents, which initiate and catalyze the freezing process. In addition, the cryoprotectant used, the temperature and surface/volume ratio should also be taken into consideration to determine the optimal cooling rate[10]. A cooling rate of 1-3 ℃/min during the initial freezing phase (+4 ℃ to -40 ℃) is optimal for most mammalian cells when frozen in the presence of cryoprotective agents, such as glycerol or DMSO[11]. Automated freezing devices, such as KRYO 10 series III (Planer Products, Sunbury-on-Thames, United Kingdom)[12], CryoMed 1010 (Forma Scientific, Marjetta, OH, United States)[13] and Cryomed (New Baltimore, MD, United States)[14] provide a temperature decrease at a controlled rate. Differences between vitrification and cryopreservation are depicted schematically in Figure 1.

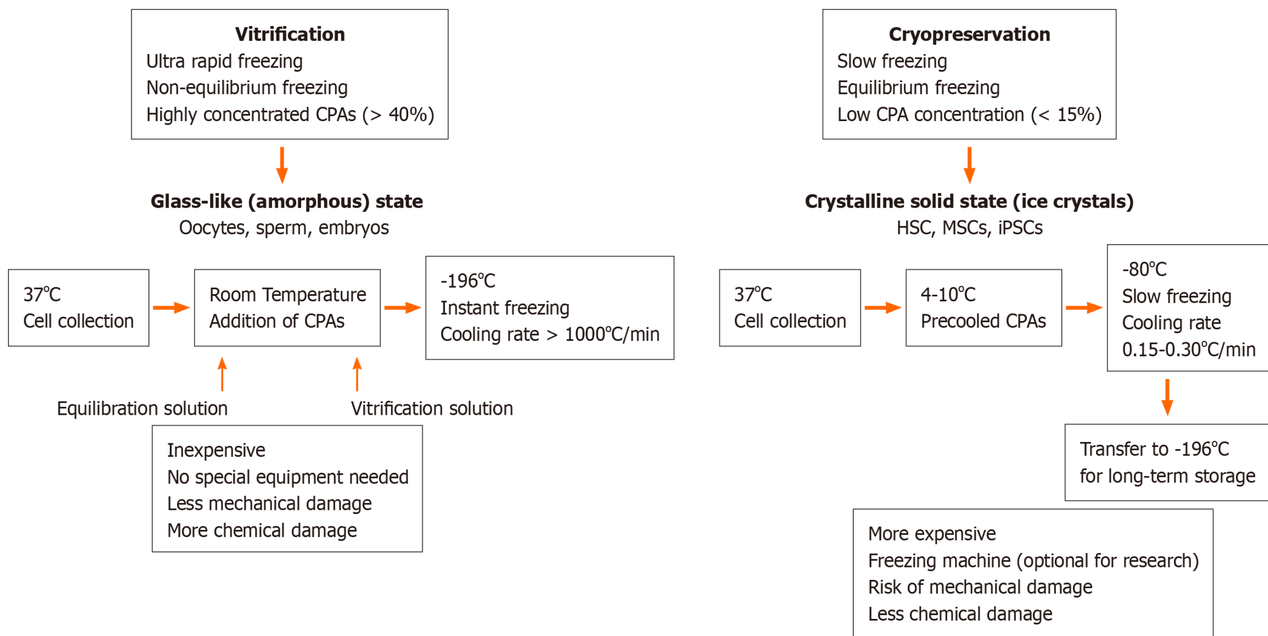

Figure 1 Comparison of vitrification and cryopreservation procedures. CPAs: Cryoprotective agents; HSCs: Hematopoietic stem cells; MSCs: Mesenchymal stem cells; iPSCs: İnduced pluripotent stem cells.

Despite a vast amount of different methods, protocols and cryoprotectants, stem cells are often frozen using protocols optimized for cell lines and relatively few comparative studies have been performed to assess the effects of cryopreservation methods and supplements on stem cell quality and viability. A list of commercially available cryopreservation media is provided as Supplement 1. Here, we summarize the use, advantages and disadvantages of cryopreservation methods used for different types of stem cells, including HSCs, mesenchymal stem cells (MSC) and induced pluripotent stem cells (iPSC).

CRYOPROTECTIVE AGENTS, ADDITIVES AND SOLUTIONS

In order to serve as an effective cellular cryoprotective agent (CPA), the compound should have certain properties, including (1) High water solubility, even at low temperatures; (2) Free penetration of cell membranes; and (3) Low toxicity. Although many compounds may have these properties, including the most commonly used agents DMSO and glycerol, the choice of the compound may differ depending on the type of cell. CPAs are often used in combination with a carrier solution, which may provide different concentrations of (nutritional) salts, a variety of buffers, osmogens and/or apoptosis inhibitors. The contents of this carrier solution further help the cells maintain an isotonic concentration (300 milliosmoles) to prevent swelling or shrinking during the freezing process[15].

DMSO [Me_2SO, $(CH3)_2SO$]

DMSO has been a key agent for the development of cryobiology. For cryopreservation of HSCs, use of DMSO, in combination with a temperature-controlled freezing technique followed by a rapid thawing procedure of 1-2 °C/min, is considered the clinical standard[16]. The use of DMSO as a CPA to prevent freezing-related cell damage was first proposed by Lovelock and Bishop[17], who used it during slow cooling of bull sperm. Due to its low hydrophilicity and molecular weight, DMSO freely penetrates cell membranes. It can disrupt ice crystal nucleation by forming hydrogen bonds with intracellular water molecules and prevents dehydration by reducing the amount of water absorbed into ice crystals[18]. However, prolonged exposure to DMSO negatively affects cellular function and growth by interfering with metabolism, enzymatic activity, cell cycle and apoptosis[19]. DMSO is also thought to modulate intracellular calcium concentrations[19,20] and may induce or inhibit cell apoptosis and differentiation, depending on the cell type, the stage of cell growth and differentiation, the concentration of DMSO (typically 5%-10%), duration of exposure

and temperature[21,22]. Whereas high concentrations of DMSO may cause instant hemolysis, white cell stacking and fibrinogen precipitation, intravenous administration of DMSO has been associated with local irritation and necrosis[23]. Infusion of cell products that contain DMSO is associated with a wide range of gastrointestinal side effects (nausea, vomiting, abdominal pain, diarrhea)[24-26]; cardiovascular effects (hypertension, bradycardia, tachycardia)[25-27]; respiratory (dyspnea) and dermatological effects (urticaria, itching, and redness)[28,29]. Furthermore, even very low concentrations of DMSO can affect cellular processes by causing differential expression of thousands of genes, changing DNA methylation profiles and tissue-specific deregulation of miRNAs[30,31], and may affect stem cell fate by inducing unwanted differentiation[32].

Glycerol ($C_3H_8O_3$)

Glycerol is a simple polyol compound. Its cryoprotective effects have been known since the early 1950s, when glycerol was first tested on fowl spermatozoa, rabbit red blood cells and water amoeba[33,34]. Glycerol is a colligative CPA that prevents dehydration damage by increasing the total solute concentration, including sodium ions, thus preventing ice formation and reducing the amount of water absorbed by ice crystals[7,35]. Although glycerol at low concentrations (< 20%) is not sufficient to prevent crystallization completely, it does protect different cells from cell death. High concentrations (70%) of glycerol were used without significant toxicity and were shown to provide substantial protection[36].

Hydroxyethyl starch

Hydroxyethyl starch was synthesized by Ziese W in 1934. The hydroxyethyl starch molecule is a high molecular weight synthetic polymer and can be purified from corn or potatoes[37]. Since high molecular weight CPAs are generally unable to enter cells, HES accumulates in the extracellular space. Here, it regulates water flow during cooling and heating and provides cryoprotection by absorbing the water molecules and keeping them thermally inert. Although HES remains extracellulary, it can minimize intracellular ice crystal formation and provides membrane stabilization[38]. By increasing the extracellular viscosity it further prevents osmotic stress and damage, reducing the rate at which water is withdrawn from the cells during cooling[39,40].

Trehalose

Trehalose is a non-toxic disaccharide and helps maintaining the structural integrity of cells during freezing and thawing[41,42]. Trehalose has high water retaining properties and is found in a large number of organisms, such as nematodes and yeasts that can survive freezing and drying[43] and can be isolated from yeasts, plants and fungi[42,44]. However, trehalose does not display any significant cryoprotective potential by itself and should therefore be used in combination with other CPAs[45].

Albumin

The albumin protein consists of three homologous domains, each with specific structural and functional properties[46]. Human serum albumin (HSA) is present in serum at high quantities and serves as a buffer or depot for hormones, growth factors, fatty acids and metals. Due to its stabilizing function, albumin is an important component of common preservation and cell culture media. During freezing, albumin is used for its ability to coat surfaces, buffer function and binding capacity[47], but, similar to trehalose, albumin is only used as a supplementary cryoprotective agent during freezing of cells and tissues[48].

Dextran

Dextran is a branched polysaccharide with α-1.6 glycosidic links between glucose molecules[49]. Dextran can interact with lipoproteins, enzymes and cells, and has the ability to stabilize proteins[50]. Dextran is non-toxic, only weakly antigenic and usually used at a concentration of 10%[51,52]. Dextran has been used as a cryoprotect during freezing of HSCs and sperm[53,54]. Similar to albumin and trehalose, dextran is only used in combination with other CPAs, such as DMSO or glycerol.

CRYOPRESERVATION OF HEMATOPOIETIC STEM CELLS

Hematopoietic stem cell transplantation (HSCT) is used for the treatment of various

malignant and non-malignant diseases affecting the hematopoietic and immune system as well as for the treatment of a variety of inborn errors of metabolism[55]. HSC products derived from bone marrow (BM), peripheral blood or umbilical cord blood (UCB) are usually stored for a brief period that may range from a few days to months but may increase up to several years, depending on the disease state of the patient and treatment schedule[56]. Banking of HSC transplants is becoming increasingly important because of the possibility to use previously stored material even years after collection. In addition, storage of UCB for personal (private banking) or transplantation purposes (biobanks) is becoming increasingly popular and may require banking for up to several decades. For this reason, it is critically important that HSCs retain their potential during the freezing, banking and thawing[57]. HSCs can be stored unprocessed at +4 °C or room temperature for approximately 72 h after collection without massive apoptosis, cell death or loss of stem cell function. Within this time period, they can be transported and engrafted without any problems, but additional protocols may be required for longer storage[22,58,59]. Freezing the cells extends their shelf life greatly and increases the safety of HSC therapy by providing time to perform quality controls (microbiologically) and product testing (HSC content, colony assay, CD34+ enumeration). Despite these benefits, cryopreservation of HSCs poses several challenges, most notably a decrease in cell viability after thawing and side effects in patients due to the CPAs used[60]. An overview of current protocols used for cryopreservation of HSCs has been provided in Table 1.

Throughout the years, DMSO has been the CPA of choice in most studies. It has been tested at different concentrations, ranging from 2.5% to 10% with variable results. Since DMSO is highly hyperosmotic, rapid infusion of the cryopreserved cells into the isosmotic blood system may cause osmotic damage, excessive cell expansion and decreased cell viability. This in turn may cause immediate side effects but can also affect engraftment in the long term[14,22]. Generally, lower doses of DMSO provided less toxicity, but in some cases, this was accompanied by a decrease in cell viability. Nevertheless, observed effects and side-effects of DMSO may differ widely between the protocols used due to the addition of other supplements (HES, HSA, Trehalose), cell dose (ranging from 15 x 10^6 cells/mL-4000 x 10^6 cells/mL), cell source (peripheral blood/BM/UCB), use of controlled rate or uncontrolled rate freezing, duration of storage (< 1 wk to > 1 decade) and the temperature used for long-term storage (-80 °C to -196 °C). To reduce the toxic effects of DMSO-cryopreserved HSCs during transplantation, it has been opted to divide the infusions into multiple portions, given at intervals of several hours or days, or alternatively to concentrate further HSC grafts to reduce cryopreservation volume and DMSO content[61]. In addition, alternatives such as different CPAs to reduce or replace DMSO for cryopreservation[14,62] or complete removal of DMSO prior to infusion[63,64] are being investigated. Even though a concentration of 10% DMSO in HSC cryopreservation is widely accepted as the cryopreservation medium of choice[65,66], similar or even more successful results have been obtained using percentages of DMSO as low as 2.5%-5%, with or without the addition of HES. Using these protocols similar engraftment was observed but with less toxicity[14,67,68]. Use of trehalose in combination with DMSO in UCB-derived HSC freezing has been shown to increase survival and cell differentiation capacity of HSCs in comparison to HSCs frozen without trehalose[53]. Direct comparison of trehalose and DMSO for cryopreservation of BM-HSCs showed no differences on viability between both groups[45]. Similarly, in NOD-SCID mice, the use of low amounts of DMSO (5%) and trehalose (5%) to reduce the toxic effects of DMSO showed a positive effect on HSC survival and engraftment after transplantation[69]. When BM-derived HSCs were frozen using a combination of 7.5% DMSO and 4% HSA, cells displayed high viability and sustained engraftment[70]. Studies using combinations of DMSO with dextran-40 showed increased HSC viability and functionality in comparison to the DMSO only group[71]. In conclusion, a lower concentration of DMSO and addition of a non-toxic second CPA or supplement, such as HSA and trehalose, decreases toxicity related to DMSO, while maintaining high HSC viability and sustaining engraftment.

CRYOPRESERVATION OF MESENCHYMAL STEM/STROMAL CELLS

Multipotent mesenchymal stem/stromal cells (MSCs) can be isolated from many tissues, including the bone marrow (BM-MSC), adipose tissue (adipose tissue derived stem cell), umbilical cord Wharton Jelly (Wharton Jelly-MSC), placenta (placenta-MSC), tooth germ (tooth germ MSC) or dental pulp (dental pulp stem cell) and many

Table 1 Comparison of different protocols used during cryopreservation of hematopoietic stem cells

HSC source	Storage period and temperature	Cryopreservation	Viability post freezing	Engraftment in days	Results	Ref.
< 600 x 10^6 cells/mL autologous PBSC	5-15 yr, -150 °C	10% DMSO and 23.3% Plasma Lyte A	66.4%	12	Viable CD34+ cells or CFU-GM is a reliable predictor of rapid engraftment	[13]
< 300 x 10^6 cells/mL autologous PBSC	< 6 mo, -80 °C	3.5% DMSO, 1% HSA and 2.5% HES	72%	14	Low DMSO conc allows successful engraftment and reduces toxicity (8%); Similar engraftment after combination of DMSO with or without HES and HSA	[115]
< 100 x 10^6 cells/ mL autologous PBSC	< 6 mo, -80 °C	5% or 10% DMSO, autologous plasma, 5% ACD	85%	14	19.1% infusion-related toxicity in the 10% DMSO group *vs* 6.8% in the 5% DMSO group, lowering DMSO results in reduction in infusion toxicity and lower costs with a similar hematopoietic reconstitution	[116]
Autologous PBSC	< 11 yr, -80 °C	3.5% DMSO + 1% HSA and 2.5% HES *vs* 6% DMSO + 6% HES	no significant change	11-12	Uncontrolled-rate freezing and cryopreservation with 5% DMSO/HES at −80 °C supports hematopoietic reconstitution comparable to that of controlled-rate freezing and liquid nitrogen storage	[117]
< 4000 x 10^6 cells/mL autologous PBSC	1-98 wk, -80 °C	3.5% DMSO, 2.5% HES and 1% HSA	60.8%	11-20	Reduction in DMSO concentration decreases transfusion-related adverse events. PBPCs cryopreserved in low DMSO/HES/HSA at -80°C allow successful engraftment	[24]
50 x 10^6 cells/mL autologous PBSC and BM	PB: 35 mo (26-78); BM 16 mo (27-71), -90 C	5% DMSO, 6% HES and 4% HSA in RPMI1640	93%		DMSO-associated toxicity during infusion, storage of HSCs at -90°C in DMSO/HES/HSA did not cause loss of cell numbers, viability, and clonogenic activity	[118]
Autologous PBSC	Controlled rate freezing at -186 °C	5% or 10% DMSO and 6% HES		10-20	Two patients who received components cryopreserved with DMSO alone experienced serious neurological toxicity, none of the recipients who received components frozen in DMSO/HES experienced serious infusion-related toxicity, better hematopoietic recovery in presence of HES independent of DMSO concentration	[14]
100 x 10^6 cells/mL – 200 x 10^6 cells/mL autologous PBSC	5-6 yr, controlled rate freezing at -160 °C	2%-10% DMSO, 10% ACD	73% with 5% DMSO	10-14	Cryopreservation using 5% instead of 10% DMSO improves CD34 + cell and leukocyte viability, but has only minor effects on supernatant levels of leukocyte- and platelet-derived soluble mediators	[61]
75 x 10^6 cells/mL - 250 x 10^6 cells/mLautologous PBSC	32-180 d, controlled rate freezing, -196 °C	5% or 10% DMSO	84%-95%	10-14	The use of 5% instead of 10% DMSO was associated with a decrease in side effects, cryopreservation with 5% DMSO followed by storage in nitrogen is a simple, highly standardized, and safe procedure for cryopreservation of autologous stem cell graft	[119]
UCB	1-2 mo, uncontrolled *vs* controlled rate freezing at -90 °C	5% or 10% DMSO	Uncontrolled 84.2%; controlled 92.5%		Best recovery of UCB cells when controlled-rate freezing and 5% DMSO were combined	[120]
15 x 10^6 cells/mL UCB	> 2 wk, controlled rate freezing at -170 C	5%, 10% or 20% DMSO and 2% HSA or autologous plasma	89%		Optimal conditions for cryopreservation were 10% DMSO and 2% HSA with fast addition and removal of DMSO	[121]
800 x 10^6 cells/mL UCB	10 yr, controlled rate freezing at -196 °C	10% DMSO and 5% Dextran	83.7%		Long term storage of UCB units does not affect the quality of the HSCs	[122]
Autologous BM	4 mo, -80 °C	5% DMSO and 6% HES	82.2%	21	BM cells can be rapidly and inexpensively cryopreserved in DMSO/ HES, without need for rate-controlled freezing or storage	[123]

				in liquid nitrogen	
20 x 10^6 cells/mL BM or 17 x 10^6 cells/mL PBSC	Controlled rate freezing at -196 °C	10% DMSO or 0.25-1 mol/L TH with or without 0.25 IU/mL insulin (I)	DMSO: 33%TH: 32%; TH/I: 30%	DMSO-cryopreserved cells exhibited the best median viability-rate after thawing. Comparable results could be achieved with trehalose 0.5 mol/L with/without insulin	[45]
200 x 10^6 cells/mL autologous BM or PBSC	BM: 11.8 yr *vs* PB: 33 d controlled rate freezing at -196 °C	10% Medium 199 , 80% autologous plasma and 10% DMSO	BM: 81.5%; PBSC: 68.0%	BM can be cryopreserved for more than a decade without apparent loss of progenitor activity in comparison to short-term cryopreserved PBSC	[124]

HSC: Hematopoietic stem cell; DMSO: Dimethyl sulfoxide; CFU-GM: Colony Forming Unit-Granulocyte/Macrophage; ACD: Acid citrate dextrose; RPMI: Roswell Park Memorial Institute Medium; HES: Hydroxyethyl starch; HSA: Human serum albumin; BM: Bone marrow; UCB: Umbilical cord blood; TH: Trehalose.

other connective tissues[72,73]. MSCs can differentiate into cells from several mesenchymal lineages, including but not limited to osteoblasts, adipocytes and chondrocytes[74,75]. MSCs are highly positive for cell surface molecules like CD29, CD44, CD73, CD90 and CD105[76]. They hold great potential for clinical application due to their capacity for regeneration of damaged or injured tissues, migration to sites of injury and regulation (usually suppression) of local and generalized immune responses. In order to obtain a sufficient amount of MSCs for clinical application, cells are often profoundly expanded in culture. Since MSCs themselves do not express HLA-DR, the cells are considered immunologically inert and expanded MSCs from unrelated, third-party donors can be used for treatment of a variety of diseases, ranging from graft *vs* host disease to severe acute respiratory distress syndromes[77, 78]. These characteristics make MSCs ideal for ready, off-the-shelf treatments but require significant expansion and long-term cryopreservation[79-81]. Similar to the protocols developed for freezing of HSCs, a variety of freezing solutions and protocols has been tested for cryopreservation of MSCs (Table 2). Similar to freezing protocols used for HSCs, MSC freezing media generally consists of a basic medium [alpha-modified minimal essential medium, Dulbecco's Modified Eagle's Medium (DMEM) or advanced DMEM], supplemented with 3%-10% DMSO. In most studies expression of MSC surface markers (CD29, CD44, CD73, CD90, CD105 and/or CD166) was assessed before and after cryopreservation, and in almost all cases, MSC phenotype was not affected by cryopreservation, with overall expression levels > 90%. Cell viability ranged from 60% to 95% when fetal bovine serum (FBS) was used in addition to DMSO. In the presence of 10% DMSO, viability was typically very high (80% to 100%) after thawing, regardless of the duration of the freezing period[81-84].

While there was no significant difference between 2% and 10% DMSO in terms of viability after a 1 mo freezing period, a significant portion of the cells frozen in presence of 2% DMSO died after long-term cryopreservation[81]. Therefore, in order to reduce the toxicity related to DMSO, either the percentage of DMSO was reduced or secondary CPAs (trehalose, sucrose, boron) were added to the freezing media[83-85]. Alternatively, high molecular weight macromolecules, such as FBS, polyethylene glycol (PEG) or polyvinylpyrrolidone were added as secondary CPAs to the freezing media[83,84,86]. However, since FBS contains animal components, cell products may contain remnants of FBS despite post-thaw washing that may trigger adverse (immune) reactions when used in a clinical setting[87]. Therefore, animal component free media, such as Cryostor, have been developed as an alternative to standard freezing medium formulations[81]. Studies using adipose tissue-derived MSCs frozen with 10% DMSO, 0.9% NaCl and human serum, HSA or knockout serum replacement (KSR)[88] revealed that all FBS replacements supported a similar multilineage differentiation potential, expression of cell surface markers and gene expression of stem cell markers, indicating that these may be good alternatives for clinical use. Carnevale *et al* [89] used 5% DMSO and human serum instead of FBS for cryopreservation of BM-MSCs and found no differences in terms of differentiation or phenotype. Cryopreservation of BM-MSCs using 7.5% DMSO, supplemented with 2.5% PEG and 2% BSA or even 5% DMSO, supplemented with 5% PEG and 2% BSA were shown to be almost as good as 10% DMSO in terms of viability and similar in terms of differentiation[84]. Comparison of mixed osmolyte solutions, consisting of sucrose/glycerol/creatine and sucrose/glycerol/isoleucine with standard DMSO containing freezing media further showed the potential of these type of cryopreservation solutions by improving post-thawing function of MSCs[31].

Table 2 Comparison of different protocols used during cryopreservation of mesenchymal stem/stromal cells

MSC source, passage	Culture medium	Storage period and temperature	Cryopreservation	Viability	Phenotype	Results	Ref.
BM-MSC/P3	MEM, 15% FBS, 1% P/S, 1% L-glutamin	7 wk at -196 °C	90% FBS and 10% DMSO		Osteogenic and adipogenic differentiation, high expression of CD44, CD73, CD90 and CD105	No effects of freezing on function, differentiation and phenotype of the cells	[125]
1 x 10^6BM-MSC/P3, P4, P8, P13, P18	MEM, 10% FBS, 1% P/S, 1% L-glutamin	12 mo, controlled rate freezing at -80 °C	30% FBS, 60% MEM and 10% DMSO	85%-100%	Chondrogenic, adipogenic, neurogenic differentiation, no difference in expression of cell surface markers between passages	No differences in phenotype or differentiation between different cryopreserved MSCs from different passages	[82]
0.5 x 10^6 /mL; BM-MSC	MSC growth medium, 10% FBS	1-5 mo, controlled rate freezing at -196 °C *vs* 4 d at 4 °C	Freezing medium (FM): 10% DMSO, 10% FBS, MSC growth medium, 30% BSA *vs* CryoStor (CS) animal component free freezing medium with 2%, 5% or 10% DMSO *vs* storage in HypoThermosol-FRS medium (HTS-FRS) at 4°C	FM 10% DMSO: 102.8%; CS 2% DMSO: 91.7%; CS 5% DMSO: 95.6%; CS 10% DMSO: 95.4%; HTS-FRS: 85.0% (rapid loss of viability after > 6 d)	Osteogenic differentiation, high expression of CD44, CD90, CD105, CD166, loss of expression of CD9 after hypothermic storage	No difference in differentiation or phenotype before and after freezing; HTS-FRS preserved MSC marker expression, proliferation and osteogenic differentiation after storage for at least 4 d	[81]
1 x 10^6 /mL; BM-MSC	MEM, 10% FBS, 1% P/S	7 wk at -196 °C	10% DMSO ± 10% or 90% FBS, 7.5% DMSO, 2.5% PEG ± 2% BSA, 5% DMSO, 5% PEG, 5% DMSO, 2% PEG, 3% Trehalose ± 2% BSA, 2.5% DMSO, 7.5% PEG ± 2% BSA, 10% Propanediol, 2%BSA, 7.5% Propanediol 2%BSA, 2.5% PEG	Highest viability with 7.5% DMSO, 2.5% PEG and 2% BSA: 82.9% ± 4.3% *vs* 10% DMSO: 82.7% ± 3.7%	Adipogenic, osteogenic and chondrogenic differentiation	In comparison to 10% DMSO, best results with 7.5% DMSO, 2.5% PEG and 2% BSA. In presence of and 2% BSA also good results with 5% DMSO, 5% PEG or 7.5% propanediol with 2.5% PEG	[84]
BM-MSC/P1-6	MEM, 10% Human Serum, 1% L-glutamine, 1% P/S	1 yr at -196 °C	MEM, 40% Human Serum, 5% DMSO		Osteogenic, adipogenic and myogenic differentiation, before and after thawing high expression of CD73, CD90 and CD105, no expression of CD16, CD34, CD45 and HLA-DR	Cryopreserved MSCs show slightly lower proliferation rate, no differences in differentiation, senescence markers, CFU-F or karyotype between frozen and fresh cells	[89]
5 x 10^5 /mL; BM-MSC/P1	MEM, 15% FBS, 1% P/S	< 6 mo *vs* 33-37 mo	CELLBANKER cryopreservation medium (contains serum and DMSO)	90%	Osteogenic differentiation, both fresh and cryopreserved MSCs were negative for CD14, CD34, CD45 and HLA-DR and positive for CD29 and CD105	No difference in osteogenic potential between fresh and cryopreserved cells. Long-term cryopreserved MSCs retained high osteogenic potential, no difference in phenotype	[86]
1 x 10^6 /mL; WJ-MSC	ADMEM, 10% FBS, 1% P/S, 1% L-glutamine	3 mo, controlled rate freezing at -196 °C	A: ADMEM, 10% PVP ± 10% FBS, B: ADMEM, 10% FBS, 0.05 mol/L glucose, 0.05 mol/L sucrose, 1.5 mol/L ethylene glycol ± 10% FBS, C: ADMEM, 10% DMSO ± 10% FBS	A: 62.9% ± 0.4%; A without FBS: 6.8% ± 0.2%; B: 72.2% ± 0.23%; C: 81.2% ± 0.6%	Adipogenic and osteogenic differentiation, both fresh and cryopreserved MSCs were negative for CD34 and CD45 and positive for CD73, CD90 and CD105	Complete elimination of FBS in cryoprotectants resulted in drastic reduction in cell viability. Cryopreservation did not alter basic stem cell characteristics, plasticity and multipotency, except for proliferation rate	[83]
1 x 10^6 /mL; tgMSC	DMEM, 10% FBS, 1% P/S/A	1 d or 6 mo, freezing at -196 °C	20 µg/mL NaB, 20% FBS, 1% P/S/A , 10%, 7%, 5%, 3% or 0% DMSO	First cycle: > 90%; Second cycle: > 70%; Third cycle: > 80%; Fourth cycle: > 80%	Osteogenic, chondrogenic, and adipogenic differentiation, high expression of CD29 and CD73, medium expression of CD90, CD105 and CD166, no	< 5% DMSO in freezing medium resulted in increased cell death, NaB improved cellular viability after freeze-thaw cycles, addition of NaB to the freezing medium did not affect	[85]

					expression of CD14, CD45, CD34	differentiation capacity of MSCs	
5 x 10^5/mL ADSC/P2	DMEM-LG, 10% FBS	2 wk, freezing at -196 °C	0.9% NaCl containing 10% DMSO HSA, HS, KSR or 90% FBS	DMSO + 9%; HSA: 78.0%; DMSO + 90%; HS: 72.4%; DMSO + 90%; KSR: 77.0%; DMSO + 90%; FBS: 78.5%; DMSO alone: 19.6%	No differences in adipogenic, osteogenic, and chondrogenic differentiation, gene expression of *CD73, CD90, CD105, CD106, CD166, SCF, REX1* and *NANOG.* All ADSCs were positive for surface expression of CD44, CD73, CD90, CD105, CD166 and HLA-ABC and negative for CD31, CD34 and HLA-DR	ADSCs frozen with HSA, HS, or KSR showed similar growth kinetics as cells frozen with FBS. Multilineage differentiation of ADSCs did not differ between groups	[88]
1 x 10^6/mL DPSC/P5-7	MEM, 15% FBS, 1% P/S/A, 100 uM L-ascorbic acid 2-phosphate	1 wk, freezing with Mr. Frosty (NMF) *vs* magnetic freezing (MF)	Serum-free cryopreservation medium (SFM) containing 3% DMSO, SFM + 10% DMSO, FBS + 3% DMSO, FBS + 10% DMSO	SFM + 3%; DMSO: 75%; SFM + 10%; DMSO: 78%; FBS + 3%; DMSO: 70%; FBS + 10%; DMSO: 73%	CD29, CD44 and STRO-1 expression did not differ between the NMF and the MF groups, whereas levels of CD73, CD90, CD146 and CD166 in the MF group increased compared to the NMF group.	DPSC viability using MF was significantly superior to that of the NMF using 2%–10% DMSO; Post-thaw MF-DPSCs expressed MSC markers and showed osteogenic and adipogenic differentiation similar to fresh DPSCs	[90]
ESC-derived MSC	MEM, 10% FBS, 1% NEAA	Controlled rate freezing at 196 C	Sucrose, glycerol, creatine (SGC) and sucrose/ glycerol/isoleucine (SGI) solutions were incubated for 1h before freezing, Sucrose, mannitol, creatine (SMC) solutions were incubated for 2 h before freezing	SGI>SGC>SMC	Osteogenic and chondrogenic differentiation, all groups were positive for CD73, CD90 and CD105, and negative for CD45	Osmolyte-based cryopreservation formulations retain MSC post-thaw viability, cell surface markers expression, proliferation, and osteochondral differentiation potential	[31]

MSC: Mesenchymal stem/stromal cell; FBS: Fetal bovine serum; DMSO: Dimethyl sulfoxide; ESC: Embryonic stem cell; NEAA: Non essential aminoacids; MEM: minimal essential medium; KSR: Knockout serum replacement; BSA: Bovine serum albumin; P/S: Penicillin/Streptomycin; DPSC: Dental pulpa stem cells; ADSCs: Adipose derived stem cells.

For research purposes often non-controlled, simple isopropanol-jacketed freezing containers (such as the Mr. Frosty from NALGENE) are used. Using this system, temperature in cryovials decreases approximately 1 C/min[89,90]. In contrast, for clinical use, temperature controlled freezing devices are often preferred. Lee *et al*[90] used a programmed freezer with a magnetic field to freeze human dental pulp MSCs. Using the magnetic freezing procedure, the researchers were able to decrease the level of DMSO to 3% without a significant difference in cell viability. Using the magnetic field freezer "Cells Alive System" (CAS) rat BM-MSCs were frozen in serum-free freezing medium (10% DMSO, 5% Albumin, 0.2% D-Glucose, 0.6% NaCl, 0.03% glutamine, 0.2%$NaHCO_3$)[91]. After 3 years, viability and *in vivo* bone formation in the CAS group was significantly higher than that in cells stored in a non-programmed or non-magnetic freezer (87.7% and 48.5%, respectively). These data show the potential for use of alternative freezing systems for cryopreservation of MSCs as well as the use of secondary CPAs that decrease the need for DMSO. Most clinical trials use MSCs from related donors rather than off-the-shelf products. These MSCs are often directly after expansion infused into the patients. However, considering the increasing requirement for readily available MSC products, MSC culture and cryopreservation protocols under good manufacturing practice conditions will need to be revisited and low DMSO protocols that are optimized for clinical use and support MSC function in the absence of animal components remain to be developed.

CRYOPRESERVATION OF INDUCED PLURIPOTENT STEM CELLS

Whereas studies on HSCs have been the focus of stem cell research since the 1960s-70s, studies assessing the role and function of MSCs have intensified since the 1990s. Since 2006, a substantial portion of the focus within the stem cell field has moved steadily towards the use of the new kid on the block, *i.e.* induced pluripotent stem cells (iPSC).

iPSCs are stem cells with embryonic stem cell (ESC)-like properties, but lack the ethical issues involved with the use of ESCs. This is related to the fact that iPSCs are artificially generated from somatic cells by forced overexpression of the pluripotency transcription factors OCT4, SOX2, KLF4 and c-Myc[92,93]. New protocols using different combinations of transcription factors, including NANOG and LIN28[94] and others, devoid of oncogenic potential, as well as different methods for transfer (*e.g.*, integrating lentiviral vectors, non-integrating sendai based vectors, episomal vectors, direct mRNA transfer, *etc.*)[95] have not affected the characteristics of the derived iPSCs: iPSCs have unlimited self-renewal capacity and the ability to differentiate into cells from all three germ layers (endoderm, mesoderm, ectoderm). iPSCs thus provide the tools to study early developmental biology *in vitro* and can be used for disease modeling and drug discovery. In addition, patient-derived iPSCs offer the opportunity to study the pathophysiology of diseases that could not be studied previously and can be used for the development of personalized medicine. All these features further stimulated iPSCs to become an important source of stem cells, and biobanks for storage of healthy and patient-derived iPSCs have now been established in many countries. However, efficient banking requires cell production facilities where cells can be expanded, maintained and cryopreserved under optimal conditions to ensure protection of iPSC characteristics and properties for weeks to years. In contrast to the cryopreservation protocols developed for HSCs and MSCs, current protocols for cryopreservation of iPSCs have focused on different issues, including freezing of cells in small aggregates *vs* single cell freezing in the presence of absence of DMSO[96-99], cell freezing using vitrification or different combinations of CPAs[100-102], cell recovery after cryopreservation using small molecules, such as the Rho kinase (ROCK) inhibitor Y-27632[103-105] and development of animal-component free formulations of culture and cryopreservation media using KSR instead of serum[106-108] (Table 3).

Using Raman spectroscopy to assess intracellular ice formation in iPSCs during cooling, Li *et al*[96] showed that iPSC aggregates are more sensitive to supercooling than single iPSCs in suspension due to the decreased water permeability of iPSCs in aggregates *vs* single cells. They also showed a greater variation in DMSO concentration across the aggregates than in single cells, suggesting that the size of the aggregates may hinder equal diffusion of the cryoprotectant to the cells. They also found that iPSC aggregates frozen in an optimized solution consisting of non-essential amino acids, sucrose, glycerol, isoleucine and albumin dissolved in a buffer made of poloxamer 188 (P188) in Hank's Balanced Saline Solution, did not exhibit the same sensitivity to undercooling as those frozen in non-optimized solutions or those containing 7.5% DMSO[97]. In addition, cryopreservation of iPSCs in aggregates requires a significantly modified freezing technique, where iPSC aggregates are first incubated at room temperature for 30 min to 1 h before freezing to allow sufficient internalization of the CPAs[97], in contrast to freezing with DMSO, which usually requires working at low temperatures (4 °C) and rapid mixing of cells.

Miyamoto *et al*[100] compared the efficacy of a variety of different cryopreservation media on an established murine iPSC line. These media consisted of control 10% DMSO formulations to reduced DMSO solutions, glycerol-containing solutions, combinations of DMSO and glycerol and commercially available cryopreservation media (CELLBANKER 1, 1+, 2 and STEM-CELLBANKER) and were used to freeze mouse iPSCs in suspension. Comparison of viability, proliferation and multipotency after long-term freezing of iPSCs in these media showed optimal results with the serum-free formulations of CELLBANKER (CELLBANKER 2 and STEM-CELLBANKER)[100]. However, the precise formulations of these freezing media is proprietary, Hank's Balanced Saline Solution and the researchers did not mention whether the STEM-CELLBANKER formulation used contained DMSO. Katkov *et al* [98] compared freezing of iPSCs in aggregates and as single cells using different CPAs including DMSO, ethylene glycol (EG), propylene glycol and glycerol. After extensive comparison, they found that freezing in aggregates resulted in favorable iPSC recovery after thawing. In addition, toxicity tests revealed that EG was not only less toxic than DMSO, it also supported better maintenance of pluripotency than propylene glycol or glycerol[98].

The use of KSR as a serum replacement has shown promising results and is another step in the development of animal component-free cryopreservation solutions. In combination with 10% DMSO, KSR has been used at concentrations of 25%-90% to freeze effectively iPSCs, ESCs and iPSC-derived cells with high post-thaw viability [105,106,108,109]. Inhibition of Rho kinase activity with ROCK inhibitors has shown favorable outcomes after freezing of both ESCs and iPSCs, and although not added during cryopreservation itself, it promotes both plating and cloning efficiency[104,105, 108,110,111] by preventing apoptosis of detached cells[112]. Since addition of ROCK

Table 3 Comparison of different protocols used during cryopreservation of induced pluripotent stem cells

Source of cell	Storage periode and temperature	Cryopreservation	Viability	Parameters	Results	Ref.
1.5 x 10^6-2 x 10^6 hiPSC line UMN PCBC16iPS	Controlled rate; -196 °C	NEAA, sucrose, glycerol, isoleucine and albumin in a P188 in HBSS *vs* 7.5% DMSO; Aggregates *vs* single cells		Viability, adherence and intracellular ice formation	P188 was found here to not only inhibit ice formation significantly but also soften the solid-liquid interface of ice and increase the distance between adjacent ice crystals; The cryoprotective effects of the DMSO- free CPA cocktail could be capitalized only with the optimized composition. Deviation from the optimum may result in less desirable outcomes	[96,97]
H9 hESC and hiPSC	3-6 d,controlled rate; -80 °C	10% DMSO, 10% EG, 10% PG, 10% glycerol, clumps *vs* single cells; ROCK inhibitor after thawing	EG-DMSO> PG|||<***glycerol	Toxicity of CPAs, expression of NANOG by hiPSCs	Freezing single cell iPSCs in the presence of a ROCK inhibitor and EG and programmable freezing drastically improved the yield of iPSCs in comparison to standard freezing in clumps without ROCK inhibitor	[98]
1-2x10^6 hiPSC	-196 °C	A: 10% DMSO/90% FBS; B: 10% DMSO/90% KSR; C: 10% DMSO/ESC medium + 20%KSR + ROCK inhibitor; Single cells	A: 90%; B: 70%; C: 70%	Viability, karyotype, expression of pluripotency markers TRA-1-60, TRA-1-81, Oct4, SSEA-3, and SSEA-4, embryoid body formation, neuronal differentiation, colony formation	Addition of ROCK inhibitor to pre- and post-thaw culture media increased survival rate, hiPSCs retained typical morphology, stable karyotype, expression of pluripotency markers and the potential to differentiate into derivatives of all three germ layers after long-term culture	[103,105,108]
hiPSC	-196 °C	10% DMSO in KO DMEM, 20% KSR, 1% NEAA, 1% L-glutamine, 0.2% b-mercaptoethanol, 1% antibiotic/ antimycotic and 8 ng/mL bFGF; ROCK inhibitor after thawing; Single cells		Colony number and size	ROCK inhibitor Y-27632 significantly improves the recovery of cryopreserved human iPS cells and their growth upon subculture	[104]
hiPSC line 253G4 and 201B2	7 d, Vitrification in; -196 °C	VS2E vitrification solution (40% EG, 10% PEG in Euro-Collins medium), DAP213 vitrification solution (1.2% DMSO, 22% PG, 5.9% acetamide); Single cells	VS2E>DAP213	Proliferation, expression of pluripotency markers Oct3/4, SSEA4, ALP, pluripotency in teratoma assay	Higher recovery rate of hiPSCs with DMSO and serum-free VS2E vitrification medium, cells after vitrification expressed Oct-3/4 and SSEA-4 and alkaline phosphatase and retained their pluripotency	[114]

iPSC: Induced pluripotent stem cells; NEAA: Non-essential amino acids; DMSO: Dimethyl sulfoxide; CPA: Cryoprotective agents; ESC: Embryonic stem cell; bFGF: basic Fibroblast Growth Factor; ROCK: Rho Kinase; ALP: Alkaline phosphatase; KSR: Knockout Replacement; FBS: Fetal Bovine Serum; HBSS: Hank's Balanced Salt Solution.

inhibitors up to 5 d after thawing still promotes colony formation, and since the effects of ROCK inhibition appear to be reversible, it has been also been suggested that ROCK inhibitors may relieve cellular stress[104].

Similar to studies in MSCs, the effects of magnetic fields on iPSC recovery after freezing have been assessed. Using the CAS researchers showed improved survival after thawing of iPSCs, but no effect on proliferation, gene expression and multilineage differentiation[113]. Reubinoff *et al*[101] previously showed that vitrification of both ESCs and iPSCs is feasible, using precooled freezing medium consisting of 90% FBS and 10% DMSO and a cooling rate of 1 C/min. ESC aggregates were preincubated in 80% DMEM, 10% DMSO and 10% EG and then placed into small 1-2 mL droplets containing 60% DMEM, 20% DMSO, 20% EG and 0.5 mol/L sucrose. All vitrified ESC aggregates recovered upon thawing and gave rise to colonies after plating. However, vitrified colonies were significantly smaller and showed increased differentiation compared with control colonies. Nevertheless, colonies generally recovered within 1-2 d of cell culture. Using a similar method for iPSCs, but using a DMSO and serum-free medium based on 40% EG and 10% PEG, Nishigaki *et al*[114] obtained a higher recovery rate of iPSCs than with a vitrification solution containing DMSO and serum.

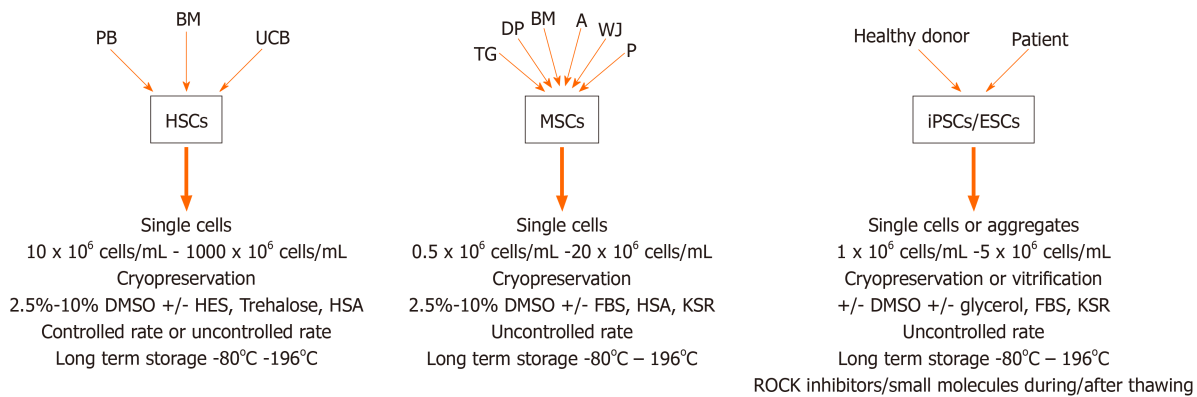

Figure 2 Preferred cryopreservation protocols for different types of stem cells. PB: Peripheral blood; BM: Bone marrow; UCB: Umbilical cord blood; HSCs: Hematopoietic stem cells; DMSO: Dimethyl sulfoxide; HES: Hydroxyethyl starch; HSA: Human serum albumin; TG: Tooth germ; DP: Dental pulp; A: Adipose tissue; WJ: Wharton Jelly; P: Placenta; MSCs: Mesenchymal stem/stromal cells; FBS: Fetal bovine serum; KSR: Knockout serum replacement; iPSCs: Induced pluripotent stem cells; ESCs: Embryonic stem cells; ROCK: Rho-associated protein kinase.

CONCLUSION

The universally used cryoprotectant DMSO has been associated with *in vitro* and *in vivo* toxicity and has been shown to affect many cellular processes through dysregulation of gene expression and changes in DNA methylation. Despite studies showing that DMSO affects cell characteristics including differentiation potential, DMSO remains to be the CPA of choice both in a research setting and in the clinics. Many different protocols have been developed for different types of stem cells and a broad range of alternatives to DMSO have been shown to hold promise for use as a CPA (Figure 2). These alternatives include such molecules as trehalose, sucrose, EG, PEG and many more. It is obvious that a single protocol that can be used for all types of stem cells is not feasible, but the enormous amount of available alternatives should make it possible to adapt and optimize DMSO-free and animal component and serum-free cryopreservation solutions adapted for different types of stem cells in the foreseeable future.

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REVIEW

Recent advances in stem cell therapy for neurodegenerative disease: Three dimensional tracing and its emerging use

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Abstract

Neurodegenerative disease is a brain disorder caused by the loss of structure and function of neurons that lowers the quality of human life. Apart from the limited potential for endogenous regeneration, stem cell-based therapies hold considerable promise for maintaining homeostatic tissue regeneration and enhancing plasticity. Despite many studies, there remains insufficient evidence for stem cell tracing and its correlation with endogenous neural cells in brain tissue with three-dimensional structures. Recent advancements in tissue optical clearing techniques have been developed to overcome the existing shortcomings of cross-sectional tissue analysis in thick and complex tissues. This review focuses on recent progress of stem cell treatments to improve neurodegenerative disease, and introduces tissue optical clearing techniques that can implement a three-dimensional image as a proof of concept. This review provides a more comprehensive understanding of stem cell tracing that will play an important role in evaluating therapeutic efficacy and cellular interrelationship for regeneration in neurodegenerative diseases.

Key Words: Cell tracing; Neurodegenerative disease; Stem cells; Three-dimensional imaging; Tissue clearing

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Core Tip: Although the use of stem cells in neurodegenerative disease has become widespread, a proof of concept (PoC) for three-dimensional analysis of the interrela-

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tionships in brain structure has not been performed *in vivo*. This review will introduce recent stem cell research for therapies and PoC for a three-dimensional analysis based on tissue optical clearing.

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INTRODUCTION

Most brain disorders lead to irreversible consequences in intra- and inter-cellular responses depending on their severity, which commonly causes deterioration of physical or intellectual function. In general, it is known that the adult central nervous system is not capable of neurogenesis, but recent research on stem cells has negated this precept[1,2]. To improve neural regeneration to replace damaged neural cells and/or re-establish dendritic connections, two basic strategies have been established over the past two decades[3-5]. First, endogenous neural stem cells (NSCs) participate in the self-repair process in the subventricular zone (SVZ) lining the lateral ventricles and the subgranular zone within the dentate gyrus of the hippocampus, despite limitations in cell number and regenerative ability[6]. Even if symptomatic treatment is performed when the boundaries of endogenous regeneration in the brain are crossed, they have limited implications, including sustainability and efficiency in the repair of neurodegenerative diseases. Second, exogenous transplantation of stem cells is expected to become a source of neurogenesis. Stem cells derived from pluripotent 'embryonic' stem cells (ESCs), which are more lineage-committed reprogrammed 'embryonic-like' pluripotent stem cells (PSCs) have been used as a therapeutic source in neurodegenerative diseases[7,8]. The fundamental mechanism underlying all therapies is a positive regulation of progressive loss of brain structure, function, or neuronal survival. Although stem cell-derived NSCs or neural progenitors can affect cell replacement therapy, direct transplantation of stem cells or stem cell-free therapy is mostly known to be exerted through paracrine effects, including cytokines, chemokines, and neurotrophic factors at the molecular level[9-11]. Unfortunately, the therapy currently available does not fully account for the mechanism of stem cell function *in vivo*, and it does not prove the relationship between exogenous stem cells and existing brain cells from the site of neurodegenerative disease[12]. To analyze an interconnected network with a molecular biological approach, an integrative description of the microenvironment needs to comprehend the three-dimensional structure of the brain containing exogenous stem cells.

This review will focus on recent improvements of stem cell therapy for neurodegenerative disease, the methodological approach of cell tracking for the definition of stem cell proof-of-concept (PoC), and on the advanced technique of cell tracking for a three-dimensional structure description after stem cell treatment. This clarification will influence future studies by providing insights into the three-dimensional structure approach of stem cell tracing for many therapies of neurodegenerative diseases.

STEM CELL THERAPY IN NEURODEGENERATIVE DISEASE

Neurodegenerative diseases are mainly classified by clinical characteristics which are based on major symptoms and the site of involvement, including Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD). AD and HD are caused by neuronal loss in the brain, and PD is known to involve a specific local loss of dopaminergic neurons in the substantia nigra of the brain[13]. In fact, AD is the most common neurodegenerative disease, usually chronic and progressive, showing a decline in intellectual function, such as memory, judgment, and language skills, and impairments in daily life ability, personality, and behavior. HD is also accompanied by abnormal behavioral movements and cognitive impairment. PD is a degenerative neurological disease with the second highest incidence after Alzheimer's disease,

which is chronically manifested, as well as movement disorders such as tremors or paralysis of the arms, legs, and face, stiffness, stiffness, and postural instability, as well as pain, depression, and dementia. Although there are limited treatment options, the viability of cell therapy treatment has been the focus of recent research.

Stem cells were discovered in the early 1960s and are generally capable of continuous self-renewal and have the ability to differentiate into several types of cell lineages[14,15]. Stem cells include ESCs, progenitor cells, mesenchymal stem cells (MSCs), and PSCs, and are classified as totipotent, pluripotent, or multipotent according to their differentiation ability. Totipotent stem cells such as ESCs and PSCs can be isolated from the four cell stages of the embryo and can differentiate into all types of cells in the body, including tissues outside the embryo. Multipotent stem cells such as MSCs and progenitor cells can be isolated from various tissues in the adult human body and can differentiate into various cells, but only those of a closely related family type of cells. In recent years, the development of stem cell technology has expanded to many human body tissues, including treatment for degenerative neurological diseases using stem cells. The application fields of stem cells used in the treatment of neurodegenerative diseases are shown in Figure 1[16-23]. Because organizations with ineffective recovery systems cannot easily return after injury or extensive degenerative events, it is important to understand the characteristics of the available stem cell type and the specific mechanisms of neurodegenerative diseases, including AD, PD, and HD[12].

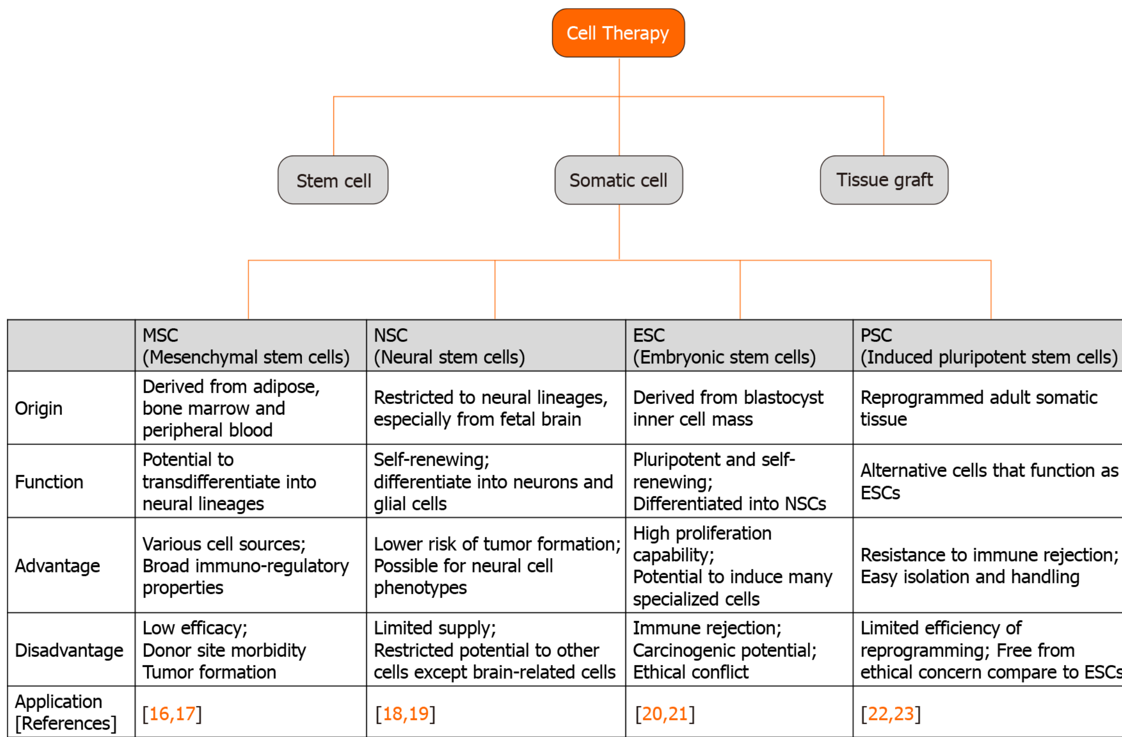
Stem cells and AD

The 2018 Global Alzheimer's Disease Report stated that 50 million people worldwide have the disease, and it is the most common cause of dementia, accounting for 50%-70% of cases of dementia cases[24,25]. AD has been shown to cause intracellular formation of nerve fiber tangles caused by the deposition of β-amyloid (Aβ) peptides on the extracellular matrix between neurons and the accumulation of hyperphosphorylated tau proteins in neurons[26].

MSCs play a major role in the treatment of AD, such as immune regulation, reduction of Aβ plaque burden through internalization and Aβ degradation of endosomal–lysosomal pathway oligomers and neurotrophic/regenerative potential [25,27]. Injection of green fluorescent protein (GFP)-labeled bone marrow (BM) MSCs in the hippocampus of an AD animal model has been shown to reduce the size of Aβ plaques and regulate functional immunity[28]. Transplantation of MSCs was shown to increase neurogenesis as demonstrated by immunostaining brain sections with an anti-polysialylated form of the neural cell adhesion molecule and doublecortin antibodies [29]. It was also confirmed that MSCs labeled with PKH26-111 were injected into AD mice through the tail vein to reach the brain, and the radioactivity of BMSCs was significantly higher in the AD model than in the control group in the gamma counter and gamma camera imaging[30]. The effect of intravenous injection of BM MSCs in a mouse model of Alzheimer's disease was confirmed through β-amyloid positron emission tomography imaging, memory function studies, and histopathological evaluation[31]. Another technique for tissue repair involves paracrine effects using the secretion of extracellular vesicles from MSCs. The secretion of MSC extracellular vesicles can target Aβ deposition and is being studied as an important method for AD treatment, including siRNA and enzymes[29,32,33]. MSC-derived cytokines and vascular endothelial growth factors also showed regenerative effects in an AD model[34].

The mammalian brain has the capacity to repair itself through neurogenesis and gliogenesis to a limited degree; however, endogenous neurogenesis and gliogenesis decrease significantly with age and are unable to regenerate enough brain cells alone. Research using NSCs that express a phenotype similar to that of brain cells has great potential in the treatment of AD. Several recent studies have shown that NSCs can increase the survival and regeneration of endogenous neurons by producing neurotrophic factors, vascular endothelial growth factor (VEGF), and vessel density in the cortex[35], and that NSC-derived cholinergic neuron-like cells can also support a significant improvement in learning and memory ability with choline acetyltransferase (ChAT) activity[36]. Using a human source, NSC function was confirmed to significantly reduce cerebral Aβ42 Levels[37]. Park *et al*[38] found that a human NSC line encoding the ChAT gene was also transplanted into the amyloid precursor protein (APP) swe/PS1dE9 AD model mice and induced the proliferation of endogenous NSCs and the production of growth factors and neurotrophic factors.

Significant experimental and clinical progress has been made with PSCs since they were discovered 10 years ago. They are now widely used in the treatment of AD to regulate endogenous neurogenesis, neuronal loss, and pathological changes. Administration of PSCs derived from mouse skin fibroblasts by treating protein extracts of



	MSC (Mesenchymal stem cells)	NSC (Neural stem cells)	ESC (Embryonic stem cells)	PSC (Induced pluripotent stem cells)
Origin	Derived from adipose, bone marrow and peripheral blood	Restricted to neural lineages, especially from fetal brain	Derived from blastocyst inner cell mass	Reprogrammed adult somatic tissue
Function	Potential to transdifferentiate into neural lineages	Self-renewing; differentiate into neurons and glial cells	Pluripotent and self-renewing; Differentiated into NSCs	Alternative cells that function as ESCs
Advantage	Various cell sources; Broad immuno-regulatory properties	Lower risk of tumor formation; Possible for neural cell phenotypes	High proliferation capability; Potential to induce many specialized cells	Resistance to immune rejection; Easy isolation and handling
Disadvantage	Low efficacy; Donor site morbidity Tumor formation	Limited supply; Restricted potential to other cells except brain-related cells	Immune rejection; Carcinogenic potential; Ethical conflict	Limited efficiency of reprogramming; Free from ethical concern compare to ESCs
Application [References]	[16,17]	[18,19]	[20,21]	[22,23]

Figure 1 Stem cell types and their application to neurodegenerative diseases. MSC: Mesenchymal stem cell; NSC: Endogenous neural stem cell; ESC: Embryonic stem cell; PSC: Pluripotent stem cells.

ESCs has been shown to mitigate plaque deposition and cognitive dysfunction in a 5XFAD transgenic mouse model[39]. From human sources, PSC-or PSC-derived cells have been used to ameliorate degenerative disorders. Human iPSC-derived macrophage-like cells genetically modified to express neprilysin-2 or to mutate Tau Ex10+16, Aβ-degrading activity, differentiated into functional neurons, and reduced A β levels after xenograft administration to the 5XFAD or APP PS1 tg/wt NOD-SCID transgenic AD mouse model[40,41].

Stem cells and PD

PD is a common neurodegenerative disease characterized by impaired motor function, which is known to be caused by the selective loss of dopamine (DA) neurons in the human midbrain. Various studies have been conducted extensively on both motor and non-motor deficits. Cognitive impairment begins to develop motor impairment at an early stage and continues to progress. Non-motor symptoms are also a cause of deterioration in the quality of life of patients and treatments that can resolve cognitive impairment and dysfunction may be possible. Stem cells are generally used to consider neuroprotection, neuroplasticity, and immunomodulatory properties in PD pathogenesis.

Transplantation of human MSCs into 6-hydroxydopamine (6-OHDA)-induced lesions protected dopaminergic neurons and induced neurogenesis, resulting in therapeutic effects due to the release of soluble factors such as brain-derived neurotrophic factor (BDNF)[42]. MSCs were also found to regulate DA neuron apoptosis and oxidative stress and to improve motor function in the early stages of PD[43]. Intravenous administration of MSCs improved dysfunction and protected tyrosine hydroxylase-positive fibers in the striatum and substantia nigra pars compacta. It has been shown that the cytokine stromal cell-derived factor (SDF)-1α increases dopamine release from cells by inhibiting apoptosis in PC12 cells exposed to 6-OHDA, and confirmed that it is a principal component of the MSC-derived secre-tome[44]. To evaluate the effects of stem cell homing, Cerri *et al*[45] examined the intra-arterial infusion of MSCs in collaboration with a transient blood-brain barrier disruption by mannitol pretreatment. After 28 d of 6-OHDA induction, the progression of the damage site did not change; however, normalization of the pathological responsiveness of striatal neurons to dopaminergic stimulation was induced by MSC infusion. Furthermore, secretomes or exosomes derived from MSCs have been confirmed to have potential use in stem cells, including neuroprotective factors[46]. Conversely, the

inhibition of cell-to-cell transmission molecules, namely α-synuclein, in MSCs led to functional improvement of motor deficits based on a pro-survival effect on cortical and dopaminergic neurons[47].

NSC transplantation allowed parkinsonian rats to be recovered through the regulation of SDF-1/chemokine receptor 4 (CXCR4) expression. Intraperitoneal injection of the CXCR4 antagonist, AMD3100, increased mRNA and protein expression of SDF-1 and CXCR4 in the NSC-transplanted site of the right substantia nigra. Furthermore, apomorphine-induced rotational behavior was reduced significantly in a rat model of PD[48]. In the xenograft model, the characterization of PD sites was examined using a high-throughput quantitative proteomic approach at the SN, striatum, olfactory bulb, and SVZ after human NSC treatment. These effects demonstrated that the rescue of SVZ function and the elicitation of endogenous response were induced by an increase in neurotrophic factors[49]. For three-dimensional micro-engineered cell therapy, NSCs were cultivated in the Nichoids microscaffold, and then transplanted into lesions of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Mechanical reprogramming of NSCs and recovery of PD symptoms produced an enhanced therapeutic effect in a murine experimental model of PD[50].

In a study conducted 20 years ago, transplantation of low-dose undifferentiated mouse ESCs into mice increased the proliferation of differentiated DA neurons and restored cerebral function in a PD animal model[51]. Another study demonstrated that a highly enriched population of midbrain neural stem cells derived from ESCs improved the electrophysiological and behavioral properties in a rodent model of PD [52,53]. Recently, studies on ESC-derived DA neurons capable of translational use have been actively conducted. Using a two-step WNT signaling activation strategy, human ESCs were induced to midbrain DA neurons at the clinical-grade level, and engraftment of these cells upregulated their behavioral recovery of amphetamine-induced rotation in a 6-OHDA model[54]. Furthermore, clinical-grade midbrain DA neurons, named MSK-DA01, safely demonstrated survival of the transplanted cells and behavioral amelioration in parkinsonian rats under GLP conditions without adverse effects[55]. PSCs were also examined for the improvement of PD at the pre-clinical level. In a primate PD model, hPSC-derived DA neurons showed an improvement in long-term survival of cells and spontaneous movement, dopaminergic progenitors derived from a clinical-grade human PSC line were produced, and their therapeutic effects were confirmed in 6-OHDA-lesioned rats[56,57].

Stem cells and HD

HD is a common degenerative brain disease with autosomal dominant inheritance. It is the least researched of the three major neurodegenerative diseases[58]. HD, characterized by progressive neuronal death, has various symptoms such as cognitive decline, behavioral changes, motor dysfunction, weight loss, sleep disturbance, and mental disorders[59]. This situation begins in the striatal part of the basal ganglia by increasing the number of CAG repeats in exon 1 of the huntingtin (*HTT*) gene, which encodes the huntingtin protein, leading to an atypically long polyglutamine region at the protein N-terminus[60]. Numerous therapies are aimed at slowing disease symptoms; however, stem cell therapy has been continuously studied by many researchers to restore the atrophied tissue, which can replace some abilities of degenerated cells [61].

MSCs are a promising HD treatment because they are not only simple to acquire and cultivate, but also have unique nutritive activity and immunomodulatory functions. Simple treatment of human MSCs has been demonstrated to enhance neural differentiation capacity, neurotrophic factor stimulation, and anti-apoptotic effects using the R6/2-J2 animal model. Transplanted MSCs can integrate with host cells to increase the level of secretory factors such as von Willebrand factor, SDF-1, and CXCR4[62]. Both intranasal deliveries showed the possibility of improving the therapeutic efficacy of HD. MSCs pre-treated with lithium and valproic acid (VPA) enhanced that open motor function improved walking distance and average speed in N171-82Q transgenic mice. Lithium and VPA have been used as mood stabilizers to boost cell survival and efficacy[63]. The second study demonstrated that MSCs significantly increased the survival rate of R6/2 mice, simultaneously with improved sleep disturbances and motor performance. The PoC of MSCs was explained by detecting GFP-expressing cells in the olfactory bulb, midbrain, and striatum on day 5. Furthermore, increased expression of DARPP-32 and decreased expression of inflammatory modulators were found in the striatum for 7.5 wk[64]. Treatment methods using various molecules released from MSCs have also been proposed. MSC-secreted neurotrophic factors were injected with quinoline acid (QA), which functioned as excitotoxicity in the striatum, and changed striatal volume with QA lesion of the brain

in the rat HD model[65]. Two other studies demonstrated that MSCs that release *HTT142* mutant and shRNA or aquaporin 4 antisense oligonucleotides can expand the therapeutic abilities for HD therapy[66,67]. Recently, MSC-conditioned medium (CM) derived from the amniotic membrane has been reported to have a protective effect on striatal degeneration and motor deficits in the R6/2 mouse HD model. In this recovery process, a significant decrease in microglial activation and nitric oxide synthase induced by MSC-CM was observed, resulting in the modulation of inflammatory cells [68].

Pluripotent cell lines targeting HD have been developed by several research groups. The QA-lesioned HD rat model was monitored using an apomorphine-induced behavioral test and immunohistochemical staining after implantation of BDNF-overexpressing human NSCs (HB1.F3.BDNF) on the opposite side of the striatum. NSC PoC confirmed that the transplanted cells were moved to the QA lesion site with striatal GABAergic medium spiny neurons, containing DARPP-32 in the host brain[69]. A clonal conditionally immortalized NSC line (CTX0E03), which already showed safety and efficacy signals in patients with chronic ischemic stroke, was examined using the QA-lesioned HD model. Thirteen weeks post-transplantation, CTX0E03 survived in the striatum and cortex of the brain with QA lesions, differentiated into striatal neurons, and showed progenitor-palatal connections with the host tissue. Survived CTX0E03 reduced gliosis and host immune responses, but increased endogenous neurogenesis and angiogenesis[70]. The same research group also demonstrated the therapeutic potential of PSCs in a rodent model of HD. NSCs derived from a human PSC line (1231A3-NPCs) also showed reconstruction of the damaged neuronal connections and behavioral improvement for 12 wk post- transplantation[71]. Human embryonic stem cell-derived NSC lines were also reported in a therapeutic study in the striatum of R6/2 mouse HD fragment model (first confirmation) to confirm the efficacy of improving motor deficits and rescue synaptic alterations. The second confirmation for improving motor and late cognitive impairment was done using the Q140 knock-in mouse HD model[72]. Combination therapy related to transduction of *HTT* gene-regulated PSCs was also conducted. PSCs derived from fibroblast/dental pulp of wild or HD rhesus monkeys were transfected with shRNA targeting the *HTT* transcript and transplanted into the N171-82Q mouse model. The mutant *HTT*-PSC-transplanted group was encouraged in their lifespan counterpart, with motor function and pathological changes, including integration and differentiation[73].

STEM CELL TRACING IN DEGENERATIVE BRAINS

Histological analysis

To track transplanted stem cells, many preclinical studies on brain injury use bromodeoxyuridine, PKH26, and 4,6-diamidino-2-phenylindole as fluorophores[74-76]. Stem cells pre-labeled with fluorophores can be identified *via* immunohistochemistry in fixed tissue using anti-fluorescent-tagged antibodies or staining methods that use color-changing substrates. At present, histology is the gold standard to test whether transplanted stem cells survive or differentiate into tissue cells in an animal model study[77]. However, this requires the sacrifice of numerous animals and provides no longitudinal or whole-body monitoring. With a lack of information on stem cell behavior, *in vivo* longitudinal, non-invasive, and repeatable methods have been developed to monitor transplanted cells. In addition, it is crucial to track the capabilities of transplanted stem cells to reconstruct brain functions and biological roles.

Imaging technology

Stem cell imaging methods can be divided into direct and indirect cell labeling depending on the possibility of re-imaging over a long period of time. Direct cell labeling is the most frequently used method and consists of incubation prior to implantation and labeling cells *in vitro* using reporter probes containing fluorophores, radiotracers, or paramagnetic nanoparticles (Figure 2)[78,79-81]. These reporter probes can bind to specific epitopes on the cell membrane, such as copper-64-labeled antibody or zirconium-89-desferrioxamine-NCS (89Zr-DBN)[82,83], or can be absorbed by passive diffusion or transporters such as indium-111- and 89Zr-oxine or 2-[18F]-fluoro-2-deoxyglucose (18F-FDG)[30,79,84]. After incubation, cells are injected *in vivo* for monitoring by magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT), and optical imaging.

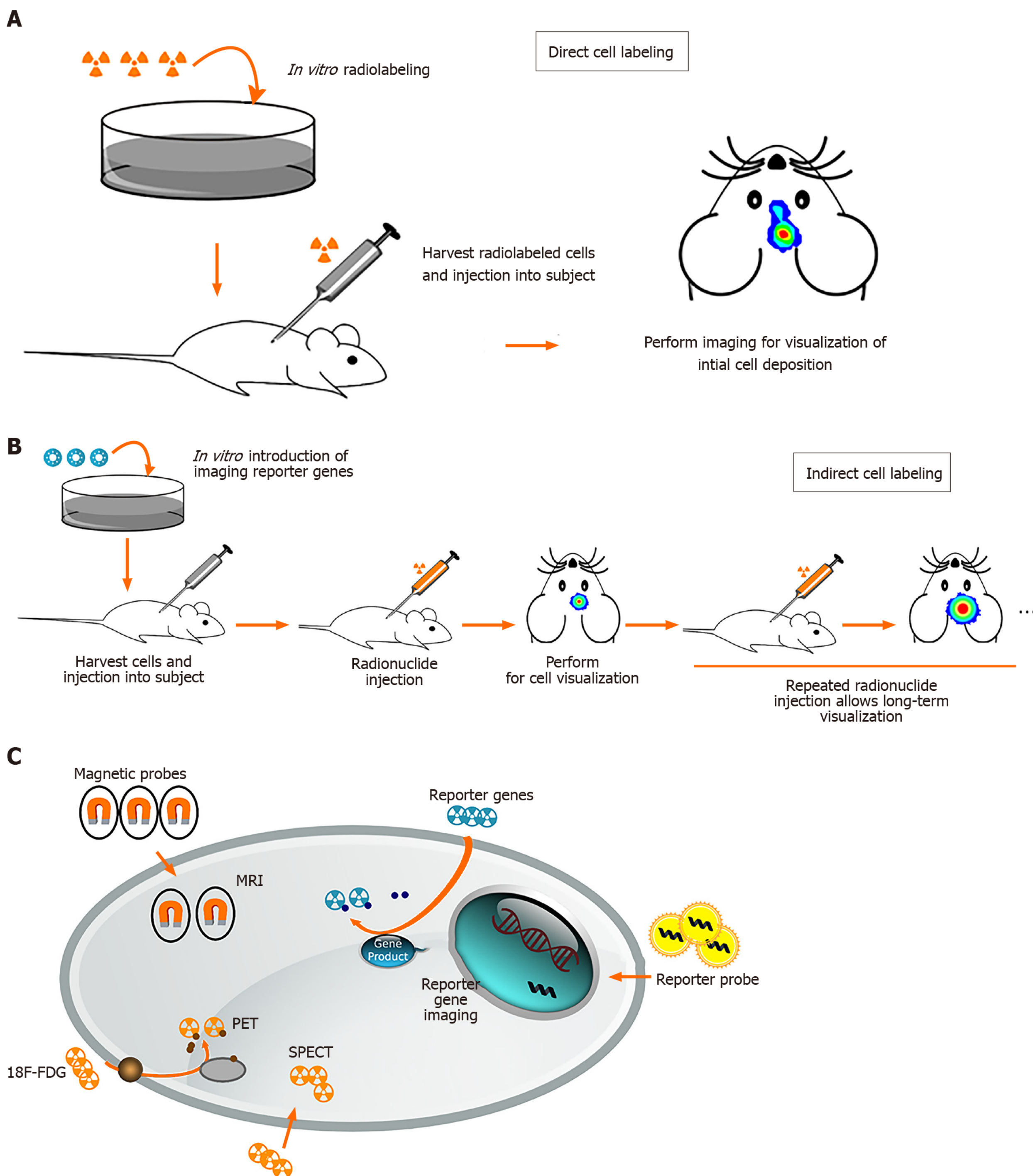


Figure 2 Schematic overview of different methods for stem cell labeling. A: Schematic overview of the processes for direct cell labeling; B: Schematic overview of the steps for indirect cell labeling; C: A diagram of different imaging method for tracking stem cells. This figure was modified from references[80,81]. MRI: Magnetic resonance imaging; PET: Positron emission tomography; SPECT: Single-photon emission computed tomography.

The first study of MR tracking of progenitor cells transplanted into the central nervous system was reported in 1992, and a superparamagnetic contrast agent was used for imaging rat brain cells[85]. Direct tracking through MRI offers benefits such as morphological characterization, high spatial resolution, lack of radiation, and long-term stem cell monitoring[86,87]. MRI requires the use of a contrast agent to visualize cells. For example, superparamagnetic iron oxide nanoparticles (SPIONs) have been shown to allow *in vivo* maintenance of neural progenitor cell viability, phenotype, proliferation, and differentiation[88,89]. Successful labeling of MSCs and progenitor cells with SPION has also been demonstrated in long-term, multimodal imaging and found no consequences on viability, differentiation capacity, or biological characteristics[90-92].

However, there are two limitations in labeling stem cells with magnetic contrast agents. After transplantation, the label was diluted because the stem cells continued to proliferate rapidly. Therefore, the MR signal decreased and lost rapidly over time because of cellular proliferation. In addition, SPIONs can be deposited in extracellular tissues when dead transplanted cells are entrapped by immune cells such as microglia in the central nervous system, leading to false signals in MRI[93].

PET and SPECT are nuclear medicine imaging techniques that represent promising imaging modality for tracking stem cells widely used in experimental trials. Before stem cells were transplanted into the host, radiotracers such as ^{18}F-FDG, lipophilic 99mTc-D,L-hexamethylene-propyleneamine oxime, and ^{111}In-oxine are required to label the stem cells to detect the transplanted cells *via* PET or SPECT scanner[79,94-96]. There was no difference in viability or differentiation ability after labeling with radiotracers. No microstructural changes were observed. The positron emitted from the radioactive isotope rapidly loses kinetic energy while traveling through the surrounding tissue, and then interacts with the electron to emit two high-energy photons of 511keVat (high-frequency photons) moving in almost the opposite direction. PET camera scanners can detect and image these photons. SPECT is very similar to PET in the use of radioactive tracers and the detection of gamma rays. These methods provide sensitivity in the picomolar range and the ability to use the same tracer across multiple species. SPECT imaging has the added advantage of having a lower false-positive signal compared to MRI. However, they do not provide anatomical information and must be used in conjunction with MRI, computerized tomography, or X-rays[97].

Optical imaging, compared to MRI, PET, and SPET, has the benefits of lower cost, rapid acquisition, no radiation toxicity, and relatively high sensitivity[98]. Semiconductor nanocrystals, also known as quantum dots (QDs), are a new class of biocompatible fluorescent materials that are relatively photostable and have a narrow luminescence band used for cell tracking. Near-infrared-emitting QDs may be particularly useful for tracking transplanted cells in the human brain, because longer wavelengths allow easier penetration of tissues such as bone and skin[99]. Bioluminescence imaging (BLI) has been widely applied in preclinical studies of stem cell imaging in the brain for several years. BLI has also been used to quantify gene expression and stem cell localization in mice and rats[100,101]. BLI is only limited to small animals, but not to large animals, because BLI can only penetrate a few centimeters of tissue.

Indirect cell labeling was modified by inserting an exogenous reporter gene into the cells. These reporter genes can produce specific proteins that function as radioactive probes, so the probe signal is not limited to the half-life of the tracer used and can be detected by PET, SPECT, and MRI for a long time. It not only allows long-term noninvasive imaging of stem cells, but also distinguishes between viable and nonviable cells. The most widely used imaging reporter gene is luciferase from firefly Photinus pyralis (Fluc), which catalyzes the oxidation of D-luciferin to oxyluciferin by emitting visible light (the principle of BLI) in the presence of oxygen, magnesium, and adenosine triphosphate. Integration of Fluc into NSCs and MSCs has been successfully performed for cell monitoring using BLI[102-105]. Another example of an indirect method of tracking consists of an exogenous reporter gene delivered to cells of interest, such as a ferritin heavy chain, which can act as a powerful MRI reporter to track cell distribution and migration in acute ischemic stroke[106]. However, the major drawback of reporter gene-based imaging is the incorporation of genomic material into the cell.

THREE-DIMENSIONAL IMAGING FOR STEM CELL TRACKING

Tissue optical clearing technique

Tissue imaging techniques for the depiction of three-dimensional structures and their molecular information are a growing trend that researchers need to facilitate volumetric imaging rather than the two-dimensional section used as the standard procedure. Due to the three-dimensional scalability of individual neurons and their interrelationships with brain cells, this imaging technique, namely Clear, Lipid-exchanged, Acrylamide-hybridized Rigid, Imaging/immunostaining compatible, Tissue hYdrogel (CLARITY), was developed by the Stanford University Research Group in 2013[107]. According to the tissue-reagent reaction principle, three major techniques are now available: hydrogel-, solvent- (hydrophobic), and aqueous-based (hydrophilic) approaches[108]. Descriptions of these techniques are provided in

Table 1 Comparison of major clearing techniques

	Hydrogel-based method	Solvent-based method	Aqueous-based method
Types	CLARITY, MAP, SHIELD, PACT, PARS	3DISCO, iDISCO, BABB	FocusClear, CUBIC, Scale12, SeeDB2
Component	FocusClear/80% glycerol or histodenz	Benzyl/alcohol series	Urea, glycerol or sucrose
Process	Hydrogel monomer infusion → hydrogel-tissue hybridization → Electrophoretic tissue clearing	Dehydration with lipid solvation → Clearing by RI matching	Decolorization by pigment removal → Delipidation using mild detergents → Expansion and RI matching
RI match	1.38–1.48	1.44–1.56	1.38–1.48
Features	Minimizing structural damage and loss of biomolecules	Fast and easy clearing. Permanent preservation of the endogenous fluorescent signal	Biocompatibility, biosafety and preservation of protein function. Penetrating more rapidly and deeply into tissues
Limitations	Expansion of tissue size. Longer incubation	Toxic nature of many solvents, substantial shrinkage of tissue (up to 50%)	Expansion of tissue size. Longer incubation
References	[107,109]	[110,111]	[112,113]

RI: Refractive index; CLARITY: Clear, lipid-exchanged, acrylamide-hybridized rigid, imaging/immunostaining compatible, tissue hYdrogel; MAP: Magnified analysis of proteome; SHIELD: Stabilization to harsh conditions *via* intramolecular epoxide linkages to prevent degradation; PACT: PAssive clarity technique; PARS: Perfusion-assisted agent release *in situ*; 3DISCO: 3D imaging of solvent-cleared organs; iDISCO: Immunolabelling-enabled DISCO; BABB: Benzyl alcohol/benzyl benzoate; CUBIC: Clear, unobstructed brain or body imaging cocktails and computational analysis; SeeDB: See deep brain.

Table 1[107,109-113].

Intact tissue clearing methods continue to grow for three-dimensional imaging of the brain, centered on labeling options and imaging analysis tools. It is expected that this process may prove the discovery of novel physiological and pathological mechanisms based on three-dimensional molecular information for neurodegenerative diseases. In the beginning, disconnected axons with APP accumulation and swelling were found in the traumatic mouse brain, revealing novel insights into three-dimensional axon degeneration of temporal progression after axonal injury[114]. To identify specific brain regions with early susceptibility to AD progression, Canter *et al* [115] created a spatiotemporal map of Aβ deposition by using whole-brain system-wide control of interaction time and kinetics of chemicals immunolabeling in the 5XFAD model, suggesting an understanding of the mechanisms of brain dysfunction and progressive memory loss[115]. Furthermore, neurons and mitochondrial proteins in the cerebellum of mouse and human brain tissues were first optimized by revealing mitochondrial disease. It has been demonstrated in a three-dimensional network that vascular, dendritic, or axonal networks finely determine the interrelationships between complex vascular structures and vasogenic factors in patients with mitochondrial disease[116].

Challenges and approaches for stem cell PoC

There are no PoC studies of three-dimensional stem cell tracing for treatment of neurodegenerative diseases. The only research on the existence of stem cells represented the spatial relationship with endogenous Gli1 positive MSCs in adult calvarial bones during postnatal craniofacial development, and indicated the osteogenesis mechanism for craniofacial research using the bone specific poly (ethylene glycol)- associated solvent system tissue clearing method[117].

The identification of the transplanted stem cells that can participate in the specific circuit and the host neurons that provide inputs to them may be critical for successful cell tracing for stem cell-based therapies for neurological disorders. To trace full or limited area projections in the brain, researchers need to be complemented by labeling or genetic manipulation *in vivo* before stem cell transplantation and by using high-resolution image system including *in vivo* multi-photon or light-sheet microscopy [118]. Neuro-specific proteins, DNA/RNA-conjugated fluorescent dyes, and viral/non-viral constructs have been used to explore the connectivity between reciprocal hosts and stem cell grafts[119]. For reliable three-dimensional analysis, the membrane-bound protein-specific phenotype of stem cells and the target circuits with strong can be set and verified in a genetic animal model of neurodegenerative disease [120,121]. Furthermore, stem cell labeling based on gene delivery can be important to define the correlation analysis in three dimensions between PoC phenotypes of endogenous or exogenous stem cells, state-modified/unmodified proteins, and state-

altering genes to understand the physiology and pathology of degenerative brains[108, 122].

CONCLUSION

For many decades, appropriate cell tracing strategies for PoC and the connectivity between host neurons and grafted stem cells have been observed using traditional two-dimensional tracing techniques. Through the development of tissue optical clearing techniques and their convergence technologies, however, it is possible to demonstrate tracing in three dimensions and to analyze the molecular pathological changes associated with endogenous cells functioning in neurodegenerative diseases. Studies on the ability of three-dimensional host-graft integration in diseases will help to serve from the basic application to the clinical monitoring of the potential strategies of stem cell therapy. An understanding of the three-dimensional imaging of stem cells may also help to approach fundamental questions regarding the cell conditions, that is, dose, time, phase, and disease mechanism, when regenerating naturally or therapeutically in neurodegenerative disease.

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REVIEW

Stem cell therapies in cardiac diseases: Current status and future possibilities

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Abstract

Cardiovascular diseases represent the world's leading cause of death. In this heterogeneous group of diseases, ischemic cardiomyopathies are the most devastating and prevalent, estimated to cause 17.9 million deaths per year. Despite all biomedical efforts, there are no effective treatments that can replace the myocytes lost during an ischemic event or progression of the disease to heart failure. In this context, cell therapy is an emerging therapeutic alternative to treat cardiovascular diseases by cell administration, aimed at cardiac regeneration and repair. In this review, we will cover more than 30 years of cell therapy in cardiology, presenting the main milestones and drawbacks in the field and signaling future challenges and perspectives. The outcomes of cardiac cell therapies are discussed in three distinct aspects: The search for remuscularization by replacement of lost cells by exogenous adult cells, the endogenous stem cell era, which pursued the isolation of a progenitor with the ability to induce heart repair, and the utilization of pluripotent stem cells as a rich and reliable source of cardiomyocytes. Acellular therapies using cell derivatives, such as microvesicles and exosomes, are presented as a promising cell-free therapeutic alternative.

Key Words: Stem cell; Cell therapy; Cardiac stem cell; Cardiovascular diseases; Progenitor cardiac cells; Pluripotent stem cells

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Core Tip: The challenge to regenerate an adult heart has stimulated the field of stem cell therapies to search for a therapeutic alternative to promote robust cardiac repair. In this review, we will discuss several types of cell therapy, which have been used in cardiology, such as adult somatic cells and endogenous progenitor cells, presenting future perspectives with the use of cardiomyocytes derived from pluripotent stem cells and their extracellular vesicles.

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INTRODUCTION

Cardiovascular diseases represent the world's leading cause of death, and in this heterogeneous group of diseases, ischemic cardiomyopathies are the most prevalent, accounting for 17.9 million deaths per year[1]. Coronary artery occlusion or reduction of blood flow results in irreversible death of cardiac cells with consequent functional cardiac impairment. Despite improvements in the clinical-surgical management of these patients, the main effect of ischemic heart disease — the death of cardiomyocytes — is not reversed. Acute interventions can restore blood flow, avoiding the death of more cardiac cells. However, this neither contributes to the recovery of the function of the damaged tissue nor stops the progression of ischemic disease[2]. Due to the limited cardiac regenerative capacity, the lost cardiomyocytes are replaced by fibrotic scarring, leading to cardiac remodeling and heart failure. The therapeutic option for heart failure patients is an organ transplant, but the demand is far greater than the availability of such organs[3]. This represents a significant public health problem, pushing researchers to look for effective alternative treatments.

In the 1990s, the idea to replace the cardiomyocytes lost due to ischemic diseases with an external cell source paved the way for cell therapies for regenerative medicine in cardiology[4]. In this review, we will discuss the various cell therapies that were applied to treat cardiac diseases, the questionable existence of an endogenous cardiac stem cell (CSC) and their putative cardiac regenerative potential, and, finally, what we envisage as the future in the field which is using cardiomyocytes derived from pluripotent stem cells or their derivatives, such as exosomes and microvesicles (Figure 1).

THE FIRST STEPS OF CARDIAC CELL THERAPY: SKELETAL MYOBLASTS

Skeletal myoblasts (SM) were the first cell type used to treat ischemic cardiac diseases. This was a natural choice as SM were contractile cells with autologous availability, simple isolation, high *in vitro* proliferation, and resistance to ischemia[5-7].

Preclinical studies showed that SM could survive and engraft after cell transplantation into ischemic hearts and differentiate into myotubules[4,7-10]. Although electromechanical coupling was not observed between the SM and resident cardiomyocytes, generating arrhythmogenic foci, an improvement in cardiac function was observed[11-13].

Phase I and II clinical trials failed to demonstrate the functional benefits observed in experimental studies, and the presence of arrhythmias was also observed in some patients[14]. These unfavorable outcomes stimulated the search for other cell types for cardiac cell therapy.

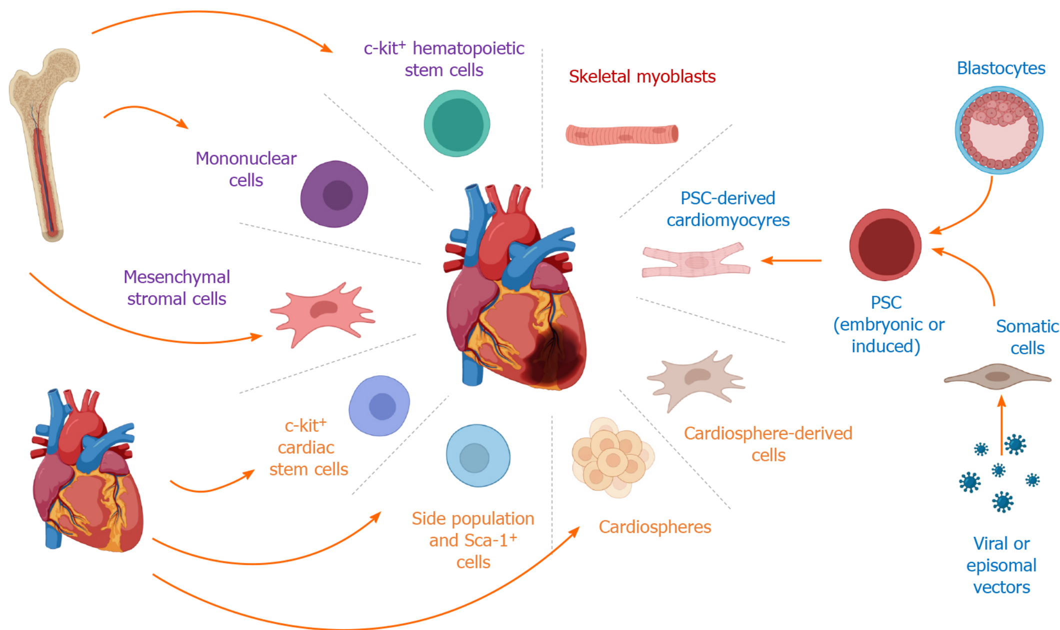

Figure 1 Cell-based therapies for ischemic heart disease. Cells can be obtained from several different sources. Skeletal myoblasts (red) were the first cell type used for cell therapies but induced life-threatening arrhythmias due to myocardial uncoupling. Bone marrow-derived cells (purple) include mononuclear cells, mesenchymal stromal cells, and c-kit+ hematopoietic stem cells, which can be used in autologous or allogeneic therapies. Unfortunately, promising results of bone marrow-derived cells in animal models were not reproduced in clinical trials. Several groups proposed the existence of cardiac stem cells (orange), and their therapeutic potential was tested in animal models and clinical trials. However, recent lineage tracing experiments demonstrate that myocardial regeneration occurs through cardiomyocyte proliferation. Thus, *de novo* cardiomyocyte generation from pluripotent stem cells (embryonic or induced) remains the most promising approach for cell-based therapies, provided that the field can overcome limitations such as immune rejection and the induction of arrhythmias. PSC: Pluripotent stem cell; Sca-1: Stem cell antigen-1.

THE NEXT STEP: BONE MARROW-DERIVED CELLS

Orlic *et al*[15], showed for the first time, that mouse c-kit+ progenitor bone marrow cells could transdifferentiate into cardiomyocytes and improve cardiac function in ischemic hearts. The group injected c-kit+ bone marrow-derived cells from a male GFP (green fluorescent protein) mouse into a wild-type infarcted female, claiming that the marrow-derived GFP cells expressed sarcomeric, endothelial, and smooth muscle proteins[15]. The same group achieved similar results by administering stem cell factor and granulocyte-colony stimulating factor into infarcted mice, showing bone marrow-derived cell homing into the infarcted area, differentiation into cardiomyocytes, and increased cardiac function[16].

The capability of mononuclear bone marrow cells to transdifferentiate into cardiomyocytes was disputed by independent groups, which showed that, after cell transplantation, these cells could only differentiate into mature blood cells[17,18]. In addition, some authors attributed the improvement in cardiac function after mononuclear bone marrow cell transplantation into ischemic hearts to a fusion process between bone marrow cells and cardiomyocytes[19,20]. However, this fusion mechanism was refuted since it was an extremely rare event that could not account for the improvement in function observed after bone marrow-derived cell therapy[21]. The currently accepted mechanism — the paracrine effect — consists of bone marrow cells releasing factors that could stimulate cellular proliferation, angiogenesis, modulate the inflammatory response, and promote ischemic tissue protection[22].

Equally important to understanding the mechanism involved in cardiac repair was the search for the subset of bone marrow cells that could be more effective. The potential for cardiac repair of mesenchymal stromal cells (MSC), a subpopulation of mononuclear bone marrow cells, was starting to be investigated. Initially, MSC transdifferentiation into cardiomyocytes was also proposed, but these data were also disputed, and the benefits of MSC treatments were also attributed to paracrine effects [22-26].

The preclinical results with bone marrow-derived cells drove several clinical trials to pursue the same positive results in the clinical setting. The clinical trials conducted to treat heart diseases using bone marrow cells were safe and feasible, but the results were controversial regarding the functional benefits. A large number of diverse clinical studies of different designs made the comparison of results difficult. They differed on

time to intervention and severity of the treated disease, subset of cells used, number of cells administered, injection pathway, endpoints, functional analyses methods, number of subjects, randomization, blinding, single or multicenter trials, among others. Due to this heterogeneity, even metanalyses were not able to draw definitive conclusions[27,28]. The only common aspect of these trials was that none could reproduce the highly significant preclinical improvements seen on cardiac function, nor could they demonstrate new cardiomyocyte generation. Therefore, scientists continued with their search for the ideal cell type to treat ischemic heart disease.

HEART REGENERATION

The regenerative capacity of the heart has been the subject of intense and controversial investigations for 150 years. However, the paradigm that the heart is a post-mitotic organ and therefore incapable of regenerating itself was challenged in 2009 when Bergmann *et al*[29] proved that the heart could regenerate itself during an individual's lifespan. Many nuclear tests were performed during the Cold War, leading to an increase in atmospheric Carbon 14 (C^{14}) concentration. The C^{14} was absorbed by plants and entered our food chain, marking the DNA of dividing cells. After the interruption of the nuclear tests in 1963, the atmospheric concentration of this isotope decreased dramatically. Bergmann's group compared the concentration of C^{14} present in cardiomyocyte DNA to the atmospheric C^{14} in the year that the individual was born, thus identifying the "age" of the cardiomyocytes. Using this elegant strategy, they showed that there was indeed cardiac cell renovation after birth and, by mathematical modeling, demonstrated that 50% of cardiomyocytes were renewed during the entire lifespan and that this renovation rate was age-dependent (1% per year at age 25, decreasing to 0.45% at age 75)[29].

This paradigm shift led researchers to pose a new question: what cell type was responsible for this cardiac regeneration capability? Hsieh *et al*[30] addressed this question using a double transgenic MerCreMer-ZEG mouse with the myosin heavy chain 6 promoter driving Cre-recombinase expression and constitutively expressing β-galactosidase (β-gal) flanked by loxP sequences followed by GFP. In this model, after administration of 4-hydroxy tamoxifen, most cardiomyocytes became GFP+ and non-cardiomyocytes remained β-gal+. Since stem cells were not labeled with GFP, if the percentage of β-gal+ cardiomyocytes increased, the group would assume that the new cardiomyocytes originated from resident stem cells. The proportion of GFP+/β-gal+ cardiomyocytes was evaluated during aging and after ischemic heart injury. One year after the tamoxifen pulse, the GFP+/β-gal+ ratio was not altered, suggesting that nonmyocytes (possibly stem cells) have no contribution to heart regeneration with aging. On the other hand, after myocardial infarction, the group observed a decrease in GFP+ and an increase of β-gal+ cardiomyocytes, suggesting that regeneration was due to resident stem cells[30]. This article supported, at that time, the idea that cardiac repair was promoted by endogenous CSC.

ENDOGENOUS CSC: THE C-KIT$^+$ CONTROVERSY

Beltrami *et al*[31] suggested that cardiac regeneration was driven by CSC located in special niches in the heart. These CSC had self-renewal properties, expressed the c-kit protein, had a clonal origin, and could differentiate *in vitro* into cardiomyocytes, endothelial and smooth muscle cells. When injected into an infarcted border zone, the c-kit$^+$ CSC differentiated *in vivo* into cardiomyocytes[31]. Human c-kit$^+$ CSC, when injected into immunodeficient mice and immunosuppressed rats, formed a chimeric heart improving the cardiac function of infarcted animals (increased ejection fraction and attenuated left ventricular dilation)[32]. The same group reported promising results of cell therapy using c-kit$^+$ CSC derived from rats, dogs, and humans[32-35].

They also reported an expressive cardiac renovation of the human adult heart dependent on c-kit$^+$ CSC. Contradicting the low regenerative rates demonstrated by Bergmann[29], Kajstura *et al*[36] reported that cardiomyocyte renovation rate was greater in women and increased with aging (10%-20% per year at age 20 and 40% per year at age 100), suggesting that all cardiomyocytes were replaced 15 times in women and 11 times in men during one's lifespan.

The regenerative potential of c-kit$^+$ CSC was evaluated in a phase I clinical trial coordinated by Bolli *et al*[37]. The c-kit$^+$ CSC were obtained from the appendage of the right atria of patients with chronic ischemic heart disease submitted to coronary artery

bypass surgery. The cells were isolated and cultured three weeks before injection. An increase in ejection fraction (30.3% to 38.5%) and ventricular mass (24% to 30%) of the c-kit-treated group was reported after four months of follow-up[37]. In addition, after 12 mo, the group reported a reduction of the infarcted area in the c-kit+ treated group compared to placebo[38].

Independent groups raised a cautionary note about the regenerative potential of c-kit$^+$ CSC. They failed to demonstrate the putative cardiac regeneration of c-kit$^+$ CSC in animal models and questioned the very existence of these endogenous cell populations in the adult heart[39,40]. Pouly *et al*[41] identified very few cells expressing c-kit on human atrial biopsies and these cells co-expressed CD45 and tryptase, indicating that they were not stem cells but rather mast cells. In transgenic mice expressing eGFP under the control of the c-kit promoter, c-kit-eGFP expression was observed during different stages of development in embryonic hearts, increasing in number until reaching maximum expression in the postnatal period (2 d of life). After this stage, the expression of c-kit-eGFP cells declined, and they were rarely found in adult hearts. In addition, Tallini *et al*[39] reported no evidence that adult c-kit$^+$ cells differentiated into cardiomyocytes, suggesting that the c-kit$^+$ expression shown by other groups after injury was only due to c-kit re-expression in preexisting cardiomyocytes. Jesty *et al*[42] evaluated the contribution of c-kit$^+$ CSC to cardiac regeneration by injecting c-kit$^+$-GFP CSC into infarcted neonatal and adult mice. They observed differentiation of c-kit$^+$-GFP CSC with cardiomyogenic fate only in neonatal animals. In adults, these cells did not contribute to tissue repair[42]. Zaruba *et al*[40] also showed that only c-kit$^+$ CSC derived from neonatal animals could differentiate into cardiomyocytes, promote cardiac repair and engraft in healthy hearts. They suggested that the c-kit$^+$ CSC potential to differentiate and repair has age limitations and was not present in c-kit$^+$ CSC derived from adults[40].

The controversy surrounding the existence of c-kit$^+$ CSC motivated groups to conduct lineage-tracing studies, considering that the main findings which sustained the cardiomyogenic ability of c-kit$^+$ CSC cells were based on immunofluorescence assays. These assays used an indirect strategy (primary and secondary antibodies) and therefore could produce false-positive results.

Ellison *et al*[43] used a lentiviral system that expressed cre-recombinase under the control of a c-kit promoter to evaluate myocardial repair in yellow fluorescent protein (YFP) reporter mice. After isoproterenol injury, the group showed the presence of new YFP$^+$ myocytes (3.4% to 7.7%). Since only cells infected by the virus and that expressed c-kit became YFP$^+$, the presence of YFP$^+$ cardiomyocytes led the group to state that adult c-kit$^+$ CSC were necessary and sufficient for functional cardiac regeneration and repair. They postulated that the key mechanism of cardiac regeneration after isoproterenol-induced heart injury was by c-kit$^+$ CSC differentiation into cardiomyocytes[43]. However, this study was criticized due to methodology issues, including the fact that the partial c-kit promoter used could not properly recapitulate c-kit regulatory elements[44,45].

Other groups used a Cre knockin c-kit transgene approach to identify the contribution of cardiac c-kit$^+$ cells for differentiation into cardiomyocytes during mouse development, aging, and after cardiac injury. van Berlo *et al*[46] showed that endogenous c-kit$^+$ CSC contributed minimally to generate cardiomyocytes (approximately 0.003% or less if cellular fusion was considered) and concluded that this rare phenomenon could not significantly impact cardiac function. Sultana *et al*[47] used multiple reporter genes in mice to show that there was no c-kit co-localized with Nkx2.5 — a gene expressed by cardiac progenitor cells — nor cardiac troponin T — a gene expressed by cardiomyocytes. The group showed that c-kit$^+$ cells in murine hearts are not cardiac progenitors but rather endothelial cell progenitors[47]. Liu *et al*[48] studied the cells immediately after Cre-recombinase induction using an instant c-kit lineage tracing model. Using this system, they described that 50% to 70% of labeled cardiomyocytes expressed c-kit 24 h to 48 h after myocardial injury. In this short time, it was unlikely that a progenitor could differentiate into a cardiomyocyte. Moreover, the group showed that c-kit was expressed by cardiomyocytes in adult hearts and concluded that new cardiomyocytes generated after injury were derived from preexisting c-kit$^+$ cardiomyocytes and not from CSC[48].

The technical limitations of the chosen tools and models to study c-kit biology, fate, and function are still subject to intense debate[49,50]. To add to the controversy, Vicinanza *et al*[51] argued that the previously reported Cre knockin c-kit models had major limitations, such as the inability to identify cells that express c-kit in low levels and the fact that these animal constructions caused haploinsufficiency of the c-kit gene, impairing normal biological regulation and causing a severe defect in CSC-dependent myogenesis[46-48,51].

To bypass these technical issues, He *et al*[52] developed a system that used two new kit-Cre drivers. This system allowed labeling of all c-kit+ cells (even those with low expression) and did not affect the endogenous c-kit gene expression. In agreement with previous studies, the group showed that neither in homeostasis nor after an injury did c-kit+ CSC contribute to the generation of new cardiomyocytes[46-48,52].

Thus, despite the many studies describing c-kit+ cardiac cells published in the last 20 years, the role of c-kit+ CSC in cardiac regeneration and even their existence in adult hearts is highly questionable. Therefore, cardiac regeneration by other endogenous CSC candidates would have to be explored.

CARDIOSPHERES AND CARDIOSPHERE-DERIVED CELLS

Cardiospheres and cardiosphere-derived cells (CDC) are a heterogeneous cell population obtained from explant culture of heart biopsies. Cardiospheres are originated from small phase bright cells, which detach spontaneously from the explants. These structures mimic the tridimensional tissue architecture and preserve the concept that resident stem cells are located in niches inside the organs. Messina *et al*[53] described them as clonal, with c-kit in the core and MSC at the periphery. Cardiospheres were obtained from mice and humans and, when injected in the peri-infarcted area in mice, induced cardiac regeneration, cell coupling, and improvement of cardiac function[53].

The expansion of cardiospheres as an adherent cell culture showed that a large number of CDC could be obtained from a small piece of heart biopsy, an important goal to translate CDC therapy to the clinical setting. The group reported that CDC differentiated into cardiomyocytes and presented spontaneous beating *in vitro* after ten days of co-culture with rat neonatal cardiomyocytes. When injected at the peri-infarcted heart zone, CDC improved the ejection fraction of infarcted animals by directly differentiating into cardiomyocytes and by paracrine effects[54-57]. CDC obtained from children, or newborn cardiac biopsies showed a superior regenerative cardiac capacity to treat infarcted animals compared to those obtained from adults[58,59].

Clinical trials using autologous CDC were conducted to treat patients who suffered recent myocardial infarctions (30 d), aiming to reverse ventricular dysfunction. The intracoronary administration of autologous CDC was safe and showed a discrete decrease of the fibrotic scar when analyzed by magnetic resonance imaging, accompanied by an increase in left ventricular mass with no effect on ejection fraction or end-diastolic or end-systolic volumes at four months and one year after treatment [60,61].

Negative results after administration of cardiospheres or CDC were also observed when utilizing this cell therapy to repair the heart. Li *et al*[62] failed to observe mouse cardiac improvement after administration of CDC derived from adult hearts. Takehara *et al*[63] treated four groups of infarcted pigs: (1) Treated with human CDC (hCDC); (2) Treated with a low release β-fibroblast growth factor (FGF) gel; (3) Treated with both; and (4) Treated with placebo. Group 2 showed cardiac regeneration and improved cardiac function. Group 3 had the same effects reported for group 2 but with higher magnitudes. The group that received isolated hCDC did not exhibit cardiac improvement[63]. In agreement with this, other groups showed that autologous CDC did not improve cardiac function in small or large animal models of myocardial infarction, with CDC, at most, attenuating cardiac remodeling[64-67]. Lineage-tracing of the cardiac explant-derived cells (EDC) was performed using the ventricular myosin light chain MLC2v-Cre/ZEG model. Transgenic EDC were analyzed *in vitro* by morphology and immunofluorescence for cardiac proteins and *in vivo* by engraftment and cardiac differentiation. EDC were engrafted into murine hearts but failed to generate cardiomyocytes, suggesting that the strategy to identify cardiac progenitor cells exclusively by morphology was inadequate[68].

Cardiospheres and CDC, similar to c-kit+ cells, generated controversial preclinical results and failed to demonstrate robust cardiomyocyte differentiation or improved cardiac function in clinical trials. Thus, the search for the identity of cardiac progenitors continued.

SIDE POPULATION

The capacity to extrude Hoechst33342 and the expression of cell surface ATP binding

cassette sub-family G member 2 (ABCG2) are characteristics shared among stem cells present in various tissues, called side population (SP) cells. These cells were also considered putative cardiac progenitor cells with cardiac, endothelial, and smooth muscle differentiation capabilities[69]. However, lineage-tracing using an ABCG2 CreER model demonstrated that this differentiation capacity was present only in embryonic phases and was lost in adulthood, refuting that these cells were CSC candidates responsible for homeostasis and injury response[70,71].

STEM CELL ANTIGEN-1$^+$

The presence of stem cell antigen-1 (Sca-1) in heart cells was first described by Oh *et al* [72]. This surface marker, also called lymphocyte activation protein-6A (Ly-6A), consists of a glycosylphosphatidylinositol-anchored cell surface protein (GP-AP) of the *Ly6* gene family that is a popular marker used to enrich samples with murine adult hematopoietic stem cells[73]. Sca-1$^+$ adult mouse heart cells were negative for CD45, CD34, c-kit, GATA-2, Lmo2, and Flk-1, presenting a distinct phenotype from hematopoietic stem cells, progenitor endothelial, and muscle satellite cells. In addition, Sca-1+ cultured cells were clonal, expressed contractile proteins, and presented spontaneous beating[72,74,75].

Transplantation of Sca-1$^+$ cardiac cells showed improved heart function in infarcted mice, promoted by direct differentiation into cardiomyocytes and the release of cytokines such as a soluble VCAM-1, which stimulated angiogenesis, migration, and survival *in vivo*[76].

The Sca-1$^+$ population was described as a heterogeneous cell population. The subpopulation that expressed Sca-1 in high levels did not differentiate into multiple cell types, while the Sca-1^{low} showed direct differentiation into endothelial cells and cardiomyocytes *in vitro* and *in vivo,* decreased infarct size, and preserved ventricular function in infarcted mice[77]. Another subpopulation, the Sca-1$^+$CD31$^-$, also showed cardiomyogenic potential in co-culture through a process mediated by cellular coupling with adult cardiomyocytes. This cell population could home to an ischemic heart area using the SDF-1α/CXCR4 pathway and attenuated post-infarct structural ventricular remodeling by direct endothelial and cardiomyocyte differentiation[78-80]. Similar properties were observed for Sca-1$^+$CD45$^-$Isl1$^+$ cells obtained from cardiospheres derived from middle-aged mice[81]. The Bmi1$^+$ cells, another Sca-1 subpopulation, demonstrated remarkable cardiac regeneration after cell therapy in infarcted mice. Approximately 14% of new mouse cardiomyocytes were observed after myocardial infarction[29,82], contradicting the low rates of cardiac regeneration previously demonstrated in humans.

Noseda *et al*[83] further refined the study of Sca-1$^+$ murine heart subpopulations using a single-cell expression profile to identify a definitive phenotype for the cardiac stem/progenitor cells. They evaluated the expression of Sca-1, CD31, PDGFRα, and the ability to extrude Hoechst33342 and identified that only SP$^+$ Sca-1$^+$ CD31$^-$ PDGFRα$^+$ cells were clonogenic cardiac progenitors.

Even though Sca-1 is not expressed in humans, Goumans *et al*[84] isolated cardiac progenitor cells from human fetal and adult cardiac biopsies using an antibody that recognized a mouse Sca-1 epitope as a target. These progenitor cells also differentiated *in vitro* into spontaneous beating cardiomyocytes and endothelial cells. Furthermore, when Sca-1$^+$ cells derived from fetal tissue were injected in immunodeficient infarcted mice, they also improved cardiac function by direct differentiation into cardiomyocytes[84].

Bailey *et al*[85] studied a Sca-1 knockout mouse to understand the role of Sca-1 in heart development and cardiac regeneration. These animals showed defects in ventricular contractility and repair, suggesting that the genetic deletion of Sca-1 compromised resident progenitor cells responsible for cardiac repair[85]. A triple transgenic mouse based on the Tet-off Cre system showed that Sca-1$^+$ cells played an important role in the generation of cardiomyocytes during homeostasis and after heart injury[86]. These data were not confirmed by independent groups using lineage-tracing and fate-mapping studies by multiple sophisticated tools used to genetically trace Sca-1$^+$ cells. They proved that these cells did not contribute to cardiac homeostasis or tissue repair by generating cardiomyocytes since Sca-1$^+$ cells mainly differentiated into fibroblasts and endothelial cells in response to stress[87-91].

Considering all these studies, it is unlikely that the beneficial results promoted by therapy using Sca-1$^+$ cells could be attributed to direct cardiomyocyte differentiation. It is more likely they are linked to angiogenesis stimulated by a paracrine effect. These

data reinforce the current leading theory that the generation of new cardiomyocytes during adult life is derived from the proliferation of preexisting cardiomyocytes than from progenitor cells, as already demonstrated in neonatal mice and zebrafish[92-94]. For more details, we suggest the review written by He *et al*[95].

IF RESIDENT CARDIAC STEM/PROGENITOR CELLS CANNOT BE FOUND: WHAT NOW?

All putative CSC listed in this review have been discarded as true cardiac stem/ progenitor cells by detailed lineage tracing experiments using sophisticated transgenic models. Furthermore, controversial results surrounded their proposed benefits in preclinical studies, and none resisted the test when applied in the clinical setting. The central idea to replace cardiomyocytes lost due to ischemic or chronic injury persists, but the efforts in the field are now redirected towards obtaining these cardiomyocytes *in vitro,* from pluripotent stem cells, and then transplant them into the injured heart.

CARDIOMYOCYTES-DERIVED FROM PLURIPOTENT STEM CELLS

Pluripotent stem cells are self-renewing cells that can differentiate into the three embryonic germ layers upon specific stimuli. Until 2006, the sole available source of pluripotent stem cells were embryonic stem cells (ESC). ESC was obtained from the blastocyst's internal mass and could be cultured *in vitro* as an immortalized lineage [96]. In 2006, Takahashi and Yamanaka[97] made a revolutionary discovery showing how to generate pluripotent stem cells from a somatic cell. They reprogramed fibroblasts, first from mice and, in the next year, from humans, by overexpressing *Oct-3/4, Sox-2, Klf-4,* and *c-Myc* transcription factors and obtained induced pluripotent stem cells (iPSC), which share the same unique ESC properties[97,98]. Furthermore, the differentiation protocols of pluripotent stem cells into adult cells were improved based on lessons learned from developmental biology[99]. These advances allowed pluripotent stem cells to be efficiently differentiated into cardiomyocytes *in vitro* by modulating the Wnt pathway, representing an almost inexhaustible source of animal-specific, including human, cardiomyocytes to be used for cell therapy[100].

Transplantation of cardiomyocytes derived from human ESC (hESC-CM) engrafted into infarcted hearts, partially remuscularized myocardial infarctions, improved cardiac performance, and attenuated the remodeling process in infarcted rats and guinea pigs[101,102]. Yu *et al*[103] proposed that the anti-inflammatory effect promoted by the administration of hESC-CM on immunodeficient female mice submitted to permanent ischemia was a therapeutic mechanism by which these cells improved cardiac function. Human ESC-CM were also evaluated in a non-human primate model submitted to ischemia and reperfusion injury to evaluate the safety, feasibility, and efficacy in a large animal model. As shown in small animals, the administration of 1 billion hESC-CM *via* intramyocardial injection improved cardiac function through remuscularization in the non-human primate model[104]. Arrhythmias are a significant concern in cardiac cell therapies. They can result either from the fetal-like phenotype of cardiomyocytes derived from pluripotent stem cells or from a dysfunctional electromechanical coupling between the graft and host cells — as seen with SM[14,105-107]. In this context, the maturation of grafted cells after three months of follow-up, the presence of electromechanical junctions leading to synchronic regular calcium transients between transplanted and host cells, and the absence of fatal ventricular arrhythmias were important observations by Chong *et al* [104].

The administration of human cardiomyocytes derived from iPSC (hiPSC-CM) also showed promising results in murine, porcine, and non-human primate ischemic cardiomyopathy models[108-110]. Kawamura *et al*[108] showed that hiPSC-CM therapy improved cardiac function and attenuated ventricular remodeling of immunosuppressed minipigs submitted to permanent occlusion of the left anterior descending coronary artery after eight weeks of administration of 25 million purified hiPSC-CM. This work suggested that a paracrine mechanism was responsible for the observed results instead of the direct muscularization observed by Chong *et al*[104]. This conclusion was based on blood flow increase at the infarcted myocardium border due to angiogenesis, probably induced by basic FGF and vascular endothelial growth factor secreted by hiPSC-CM. Also, the majority of the iPSC-CM survived for a short

time in infarcted hearts (2 wk), even though some were identified eight weeks after treatment. Therefore, the authors hypothesized that low engraftment was due to insufficient immunosuppressive therapy[108]. The low engraftment and paracrine activity of hiPSC-CM (release of proangiogenic and antiapoptotic cytokines) in the acute myocardial infarction model was corroborated by Ong *et al*[109], who also demonstrated improved cardiac function by magnetic resonance imaging.

A major concern in regenerative medicine is how to avoid immunologic rejection and how to induce immunologic tolerance to cells used in cell therapy, considering that autologous transplantation has to meet manufacture temporal challenges in a clinical setting. The use of major histocompatibility complex (MHC)-matched cells between host and graft was addressed by Shiba *et al*[110]. They treated five immunosuppressed infarcted heterozygous MHC HT4 monkeys with intramyocardial injection of 4×10^8 iPSC-CM per animal. The iPSCs were generated from an MHC haplotype (HT4) homozygous animal. After cell therapy, four of the five animals presented sustained ventricular tachycardia, peaking on day 14 post-transplant and decreasing spontaneously over time. The grafted cells coupled with host cells were still found in infarcted hearts 12 wk after cell treatment with no evidence of immune rejection. The authors suggested that cardiac contractile function was improved by the direct effect of adding new-force generating units. Still, they did not exclude a paracrine mechanism on heart regeneration promoted by allogeneic transplantation of iPSC-CM [110].

During myocardial infarction, aside from cardiomyocytes, other important cell types are also lost, such as endothelial, smooth muscle cells, and fibroblasts. Cell therapy using these cell types was successfully performed in a porcine model of acute myocardial infarction. Endothelial cells (hEC-ESC) and smooth muscle cells derived from human ESC (hSMC-ESC) were transplanted in a fibrin-gel patch into five infarcted pigs submitted to ischemia and reperfusion. Resonance magnetic imaging showed left ventricular functional improvement after a 4-wk follow-up period. The success of vascular cell therapy was attributed to direct neovascularization[111]. The combined use of cardiomyocytes, endothelial and smooth muscle cells derived from hiPSC to treat porcine myocardial infarction was also performed by Ye *et al*[112]. They showed that this cell combination resulted in cardiomyocyte engraftment and coupling to the host tissue, increased angiogenesis, improved left ventricular function and myocardial metabolism while reducing apoptosis and infarct size with no ventricular arrhythmias observed after four weeks of follow-up.

The end product of all these preclinical experiments with cardiomyocytes derived from pluripotent stem cells was that, for the first time, cardiomyocytes could be produced *in vitro* on a large scale to meet the requirements for cell therapy, presented robust engraftment of newly-generated units, and promoted angiogenesis, an important additional effect for cardiac regeneration, resulting in improved cardiac function. Despite these significant advances, there are still obstacles to overcome before moving to the clinic, such as managing the risk of arrhythmias, improving cell viability and consequently engraftment, eliminating immune rejection, and identifying the combination of secreted factors that could be responsible for the paracrine effect. Another major problem, not yet completely solved, is the maturation of the PSC-derived cardiomyocytes. Cardiomyocytes derived from pluripotent stem cells exhibit an immature phenotype that can be shifted to a more mature state by different approaches such as: *in vivo* grafting, three-dimensional constructs using scaffolds and electrostimulation, and manipulation of metabolic pathways. A detailed analysis of such maneuvers is beyond the scope of this review and can be found in Karbassi *et al* [113].

FUTURE POSSIBILITIES

Cardiac cell therapy has changed significantly in the past 30 years. We have lived through the rise and fall of the endogenous CSC paradigm, whose existence is still subject to debate. Cardiomyocytes derived from pluripotent stem cells emerged as a promising therapeutic alternative, and this cell technology should continue to be investigated to meet the required conditions for clinical application. IPSC technology in human diseases has already been demonstrated to be safe, feasible and showed exciting first results in a patient with macular degeneration[114].

As the paracrine effect has also been suggested as a possible cardiac function improvement mechanism produced by cell therapy, the soluble factors secreted by cells have been investigated. Microvesicles and exosomes — collectively known as extracellular vesicles — were described as small carriers of bioactive products (such as

RNA, DNA, proteins, lipids, and cytokines). They are released by cells in different contexts, exerting modulatory effects in diverse biological processes[115]. In ischemic cardiac diseases, extracellular vesicles derived from iPSC-cardiac progenitors and MSC have shown cardioprotective effects in infarcted mice by modulating the inflammatory response and promoting tissue regeneration *via* microRNAs[116-118].

Another emerging approach in cardiac therapy is the *in situ* direct reprogramming of fibroblasts into cardiomyocytes. The injection of transcription factors Gata4, Mef2c, and Tbx5 directly into mouse myocardium generated new induced cardiomyocytes (iCM), which coupled with the host's heart cells and decreased the infarct area. When the transcriptomes of iCM were compared to the adult heart cells, they were found to be more similar to those heart cells than to cardiomyocytes generated by the same method *in vitro*[119-120]. The direct reprogramming of human cells, which involves another molecular cocktail and epigenetic modulation, is still under investigation[121-124].

Strategies to stimulate the proliferation of endogenous cardiomyocytes are another possibility to regenerate infarcted hearts. MicroRNAs, such as the miR-15 family, regulate the cell cycle of cardiomyocytes. Treatment of infarcted mice with inhibitors of this family (anti-miR-15) has resulted in mitotic cardiomyocytes and improved cardiac function[125-127]. The potential of anti-miR-15, commercially named MGN-1374, is being evaluated in a clinical trial[128]. The small molecule MSI-1436 has also shown interesting results, accelerating heart regeneration in zebrafish and mouse infarction models by the same mechanism — stimulation of preexisting cardiomyocyte proliferation[129].

CONCLUSION

Thirty years of research have taken us a long way in the understanding of cardiac regeneration mechanisms. Unfortunately, this long journey has not yet resulted in the salutary effects of cell-based therapies in the clinical setting. Nonetheless, the long and winding road up to here has shown, as mentioned above, that many options still remain to be explored. Undoubtedly, learning from previous mistakes, we will reach efficacious cell-based therapies to repair and regenerate the injured heart.

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REVIEW

Current evidence on potential of adipose derived stem cells to enhance bone regeneration and future projection

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Abstract

Injuries to the postnatal skeleton are naturally repaired through successive steps involving specific cell types in a process collectively termed "bone regeneration". Although complex, bone regeneration occurs through a series of well-orchestrated stages wherein endogenous bone stem cells play a central role. In most situations, bone regeneration is successful; however, there are instances when it fails and creates non-healing injuries or fracture nonunion requiring surgical or therapeutic interventions. Transplantation of adult or mesenchymal stem cells (MSCs) defined by the International Society for Cell and Gene Therapy (ISCT) as CD105+-CD90+CD73+CD45-CD34-CD14orCD11b-CD79αorCD19-HLA-DR- is being investigated as an attractive therapy for bone regeneration throughout the world. MSCs isolated from adipose tissue, adipose-derived stem cells (ADSCs), are gaining increasing attention since this is the most abundant source of adult stem cells and the isolation process for ADSCs is straightforward. Currently, there is not a single Food and Drug Administration (FDA) approved ADSCs product for bone regeneration. Although the safety of ADSCs is established from their usage in numerous clinical trials, the bone-forming potential of ADSCs and MSCs, in general, is highly controversial. Growing evidence suggests that the ISCT defined phenotype may not represent bona fide osteoprogenitors. Transplantation of both ADSCs and the CD105- sub-population of ADSCs has been reported to induce

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bone regeneration. Most notably, cells expressing other markers such as CD146, AlphaV, CD200, PDPN, CD164, CXCR4, and PDGFRα have been shown to represent osteogenic sub-population within ADSCs. Amongst other strategies to improve the bone-forming ability of ADSCs, modulation of VEGF, TGF-β1 and BMP signaling pathways of ADSCs has shown promising results. The U.S. FDA reveals that 73% of Investigational New Drug applications for stem cell-based products rely on CD105 expression as the "positive" marker for adult stem cells. A concerted effort involving the scientific community, clinicians, industries, and regulatory bodies to redefine ADSCs using powerful selection markers and strategies to modulate signaling pathways of ADSCs will speed up the therapeutic use of ADSCs for bone regeneration.

Key Words: Mesenchymal stem cells; Adipose-derived stem cells; Endogenous stem cells; Skeletal stem cells; Bone regeneration

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Core Tip: This review systematically examined current progress and future projections of Adipose-derived Stem Cells (ADSCs) use in bone regeneration. Introduction covered the regulatory aspects of stem cell therapy and scientific concerns regarding stem cell use including ADSCs. We then analyze clinical and pre-clinical studies using ADSCs for the treatment of bone defects. We also evaluate the current understanding of ADSC's surface receptors and therapeutic subpopulations. Overall, we conclude that while mixed outcomes have been reported, a more rigorous definition of ADSCs, selection of osteogenic subpopulations, and understanding of signaling pathways will unleash ADSCs as a powerful tool in bone regeneration.

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INTRODUCTION

Of the 7.9 million fractures sustained each year in the United States, 5% to 20% result in non-union or delayed healings[1,2]. Since these fractures do not heal naturally, they require therapeutic interventions. Transplantation of multipotent stem cells, reportedly present in practically all postnatal tissues, is an attractive therapeutic option. Mesenchymal stem cells (MSCs) isolated from bone marrow [bone marrow-derived MSCs (BMMSCs)] are thought to be true, gold-standard osteoprogenitors[3]. To streamline investigations on MSCs, the International Society for Cell and Gene Therapy (ISCT) defined MSCs in 2006 as cells satisfying the following three criteria: Plastic adherent, CD105+CD90+CD73+CD45-CD34-CD14orCD11b-CD79αorCD19-HLA-DR-, and possessing the ability to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro*[4-6]. This remains the current definition of adult stem cells or MSCs. This school of thought suggests that MSCs exist in all adult tissues and can give rise to osteoblasts, chondrocytes, marrow stromal cells, and adipocytes. Accordingly, the U.S. Food and Drug Administration (FDA) revealed that 73% of Investigational New Drug applications for stem cell-based products rely on CD105 expression as the "positive" marker for adult stem cells[7]. The optimal utilization of MSCs has been prevented by the lack of ideal surface markers for selection and an incomplete understanding of the heterogeneity of MSCs and factors governing their bone-forming ability.

Clinical studies evaluating the exogenous addition of BMMSCs to enhance bone repair in segmental defects, nonunion of the tibia, and tibial osteotomy have shown increased healing rates[8-16]. However, several factors remain enigmatic for BMMSCs therapies, including impure cell preparations, the significant numbers of cells required to achieve satisfactory healing, supplementation of growth factors, the presence of

other cell types at a higher frequency than MSCs, and incomplete fracture healing in many patients, which suggest that more studies are required to fully understand MSCs therapy[8-10,14]. These shortcomings in addition to the invasive nature of isolating BMMSCs, their extremely low frequency in bone marrow, and the requirement of high numbers of MSCs to achieve enhancement of bone healing, diminish the enthusiasm for their therapeutic use.

In contrast, MSCs isolated from fat tissue [adipose-derived stem cells (ADSCs)] offer the following advantages over BMMSCs: ADSCs can be isolated in large numbers through a simple procedure, they possess higher proliferative capacity, their frequency is 500 times higher than BMMSCs, they are resistant to senescence, and they retain their differentiation potential for a longer period[17-25]. Given their clear clinical advantages compared to BMMSCs, ADSCs are believed by many researchers to hold great promises for implementation in regenerative medicine, specifically for the treatment of orthopedic conditions. Nonetheless, the current body of research on this topic yield confounding conclusions. The exact characterization of the osteoprogenitor population within ADSCs remains in dispute. At the same time, ADSCs utilization protocols vary greatly between different clinical and preclinical studies, which themselves are inconclusive on the nature of ADSCs' osteogenic capacity. Due to these limitations, there has been no ADSC-based orthopedic product suitable for widespread use. In this review, we attempt to capture the different aspects of current research on ADSC in the hope to highlights the importance of ADSCs for bone regeneration applications, current understanding of the subject, the obstacles facing researchers, and possible strategies to further realize ADSCs' potential as a therapeutic tool.

REGULATORY ASPECTS OF STEM CELLS THERAPY

Although there is general agreement in the scientific community that stem cell therapy holds great promise for bone repair and regenerative medicine applications, there is not much agreement on the definition of adult stem cells. Moreover, several leading experts in the field warn that the existing stem cell-based products are manufactured without vigorous testing and are not backed up by strong scientific evidence. An article titled "Clear up this stem-cell mess" published recently in *Nature* states that the confusion about MSCs is making it easier for industries to sell unproven treatments [26]. In agreement with this observation, another article in *Cell Stem Cell* comments that clinical trials using MSCs have been conducted for more than a generation, but the outcomes have fallen short of expectations[27].

A thorough understanding of the FDA guidelines is necessary for orthopedic surgeons to decide whether the stem cell-based products that they are using or being asked to use by industries are authenticated by the regulatory bodies. It is also necessary to clarify that the FDA guidelines do not establish legally enforceable responsibilities, but they describe FDA's current thinking and therefore should be viewed only as recommendations unless specific regulatory or statutory requirements are cited. This puts a greater responsibility on clinicians and scientists to make sure that the general public is aware of the effectiveness of stem cell therapy, and more importantly, the patients receiving stem cell therapy are aware of the risk to benefit ratio.

The current guidance issued by FDA is available under the docket number FDA-2017-D-6146 (https://www.fda.gov/media/109176/download). Adult stem cell-based products are regulated by the Center for Biologics Evaluation and Research, similar to human cells, tissues, and cellular- and tissue-based products (HCT/Ps). These regulations are provided by the FDA to HCT/P manufacturers, healthcare providers, and FDA staff, under Title 21 of the Code of Federal Regulations (CFR) Part 1271. These regulations explain the types of HCT/Ps that do not require premarket approval; and the registration, manufacturing, and reporting steps that must be taken to prevent the introduction, transmission, and spread of communicable disease by these HCT/Ps: (1) The product is minimally manipulated; (2) It is intended for homologous use and this is reflected by the labeling, advertising, and the manufacturer's objective intent; (3) The manufacture of the HCT/P does not involve the combination of the cells or tissues with another article (except for water, crystalloids, or a sterilizing, preserving, or storage agent, provided that these agents are safe); (4) The product is not dependent upon the metabolic activity of living cells for its primary function; and (5) If the product is dependent upon the metabolic activity of living cells or has a systemic effect then it must be only for autologous use

(cells isolated from the person transplanted back into the same person) or allogeneic use in a first-degree or second-degree blood relative or for reproductive use.

In a cautionary observation, Skovrlj *et al*[28] reported that all five commercially available cellular bone matrices for spine fusion, Osteocel Plus (NuVasive, San Diego, CA, United States), Trinity Evolution (Orthofix, Lewisville, TX, United States), Cellentra Viable Cell Bone Matrix (Biomet, Warsaw, IN, United States), AlloStem (AlloSource, Centennial, CO, United States), and Ovation (Osiris Therapeutics, Columbia, MD, United States), contain live, allogeneic MSCs but claim to meet the FDA criteria under Section 361, 21 CFR Part 1271, and have not undergone FDA premarket review. All of these products are composed of MSCs derived from freshly procured cadaveric bone marrow, cadaveric adipose tissue, or chorion layer of the placenta.

It is important to take notice of the fact that there is no stem cells-based product currently approved by the FDA that can be used for bone tissue engineering purposes or for the treatment of bone diseases. The list of all cell and gene therapy products approved by the FDA can be found on FDA's website: https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products. Thus, detailed investigation on bone-forming potential of stem cells *in vitro* and *in vivo* followed by non-industry sponsored clinical studies evaluating the efficacy of stem cells are required. Since ADSCs can be isolated in a non-invasive procedure, in abundant numbers, for autologous use, they offer a promising option for stem cell-based bone repair therapies.

THE CLINICAL TRIALS USING ADSCS

To elucidate the possible clinical benefits of using ADSCs, many clinical trials have been initiated. The clinical trials that will be used in our analysis were acquired from Clinicaltrials.gov in December 2020 using the combination of keywords "Orthopedic Disorder (Condition) - Adipose Stem Cells (Other terms)" and "Bone (Condition) - Adipose-Derived Stem Cells (Other terms). The first combination of keywords returned 74 registered trials while the second combination returned 17 trials. Following content review, we eliminated any overlapping trials, trials that did not directly involve orthopedic conditions, trials that did not include human patients, and trials that did not explicitly state the use of ADSCs. This resulted in 70 trials being included in this analysis. We found only four trials that addressed bone healing or bone regeneration using ADSCs: NCT02140528, NCT04340284, NCT03678467, and NCT03678467. While NCT03678467 is an ongoing trial, the results of the other three are not published. We, therefore, searched PUBMED using the keywords "adipose stem cells", "orthopedic", and "clinical" with the filter "Clinical Study" and found 10 relevant articles[29-38]. The general distribution of the 80 included clinical trials can be seen in Figure 1. The outcomes of clinical trials on bone regeneration are summarized in Table 1.

From the number of trials, it is clear that there is tremendous interest in ADSCs as a therapeutic tool for a variety of orthopedic disorders. The earliest trials were started in 2008. However, the number of initiated trials has been on an upward trend since this time. Moreover, only 37% of trials indicated as completed (total = 29). We will be seeing a large number of trials ending in 2021 (total = 17), which will have important implications for the field. The majority of the trials are in Phase 1 or 2, evaluating the safety and initial efficacy of treatment with ADSC. Only 6 trials (8%) are in phase 3 and one is in phase 4. Of the 29 completed trials, 19 corresponding publications could be found on PubMed using the National Clinical Trial registration number. Of these, we will review in detail 10 publications directly investigating bone regeneration using ADSCs.

SAFETY OF ADSCS ESTABLISHED IN CLINICAL TRIALS

In 2013, Pak *et al*[29] published the outcomes of long term follow up of 91 patients undergoing injections of autologous ADSCs with platelet-rich plasma in various joints to evaluate the safety of this treatment modality. Participants were observed for an average of 16 mo. During this time, magnetic resonance imaging (MRI) evaluation showed no evidence of neoplasm. Common adverse events included swelling of injected joints, tenosynovitis, and tendonitis, all of which were either successfully

Table 1 Summary of the clinical trials involving treatment of the bone defects using adipose-derived stem cells

Bone defect treated	Study duration and length of follow up	*n*	Intervention	ADSCs source	ADSCs number	Outcome	Ref.
Avascular necrosis of hip, osteoarthritis of hip/knee/ankle, spinal disc herniation	2009-2012, 30 mo	91	Intraarticular injection of SVF with PRP	Autologous SVF from abdominal tumescent liposuction	10 mL of SVF	No evidence of neoplasm, no serious adverse events, common adverse events (swelling of injected joints, tenosynovitis, and tendonitis) were either successfully managed or self-limited, established safety of ADSCs	Pak *et al*[29]
Upper arm fracture in elderly patients (62-84 yr)	2012-2014, 6 mo	8	SVF seeded porous silicated-hydroxyapatite microgranules with fibrin hydrogel implant	Autologous SVF from abdominal tumescent liposuction	800 microliters of SVF	Evidence of osteogenesis at graft site; circumstantial evidence for direct contribution of SVF cells to fracture healing	Saxer *et al*[30]
Large cranial defect	2008-2010, 12 mo	4	ADSCs-seeded β-tricalcium phosphate implant	Autologous ADSC from abdominal subcutaneous liposuction	15×10^6 cells	Noted equivalence between newly generated tissue and native bone	Thesleff *et al* [31]
Large cranial defect	2008-2016, approximately 7 yr	5	ADSCs-seeded β-tricalcium phosphate implant	Autologous ADSC from abdominal subcutaneous liposuction	15×10^6 cells	This study was long term follow up of Thesleff *et al*[31]; unsatisfactory long-term outcome with significant resorption	Thesleff *et al* [32]
Cranio-maxillofacial hard-tissue defects	2012-2014, up to 52 mo	13	ADSCs-seeded bioactive glass or β-tricalcium phosphate scaffolds, at times with recombinant hBMP-2	Autologous ADSC from anterior abdominal wall liposuction	Up to 160×10^6 cells	Majority of patients achieved satisfactory clinical and radiographic results; three experienced significant resorptions of the ADSCs graft	Sándor *et al* [33]
Long bone nonunion from bone tumor resection or pseudoarthrosis	2012-2014, 39 mo	6	ADSCs seeded decellularized bone matrix	Subcutaneous autologous ADSCs	Up to 200×10^6 cells	50% of the patients achieved bone regeneration and union	Dufrane *et al* [34]
Maxillary sinus floor elevation	2009-2015, 36 mo	10	SVF seeded β- tricalcium phosphate implant	Autologous SVF from abdominal tumescent lipo-aspiration	20×10^6 cells	Experimental group exhibited significantly more bone healing compared to control	Prins *et al*[35]
Alveolar cleft osteoplasty	2015-2016, 6 mo	10	Lateral ramus cortical bone plate with ADSCs-mounted natural bovine bone mineral	Autologous ADSCs from buccal fat pad	1.0×10^6	No significant different in bone regeneration found between experimental group and controls	Khojasteh *et al*[36]
Mandibular fracture	2010-2015, 12 wk	20	Direct application of ADSCs	Autologous ADSCs	Unreported	Significantly more osteogenesis in ADSCs-treated group compared to control	Castillo-Cardiel *et al* [37]
Nonunion following subtalar arthrodesis	2010-2016, 24 mo	140	ADSC-seeded partially demineralized bone matrix	Allograft ADSCs	Unreported	Inferior bone union rate in ADSCs treated group compared to autograft; equivalent clinical evaluations	Myerson *et al* [38]

ADSCs: Adipose-derived stem cells; SVF: Stromal Vascular Fraction; PRP: Platelet-rich plasma.

managed or self-limited. Evaluation of pain using VAS suggested that most patients experienced a significant reduction in pain three months post-operation.

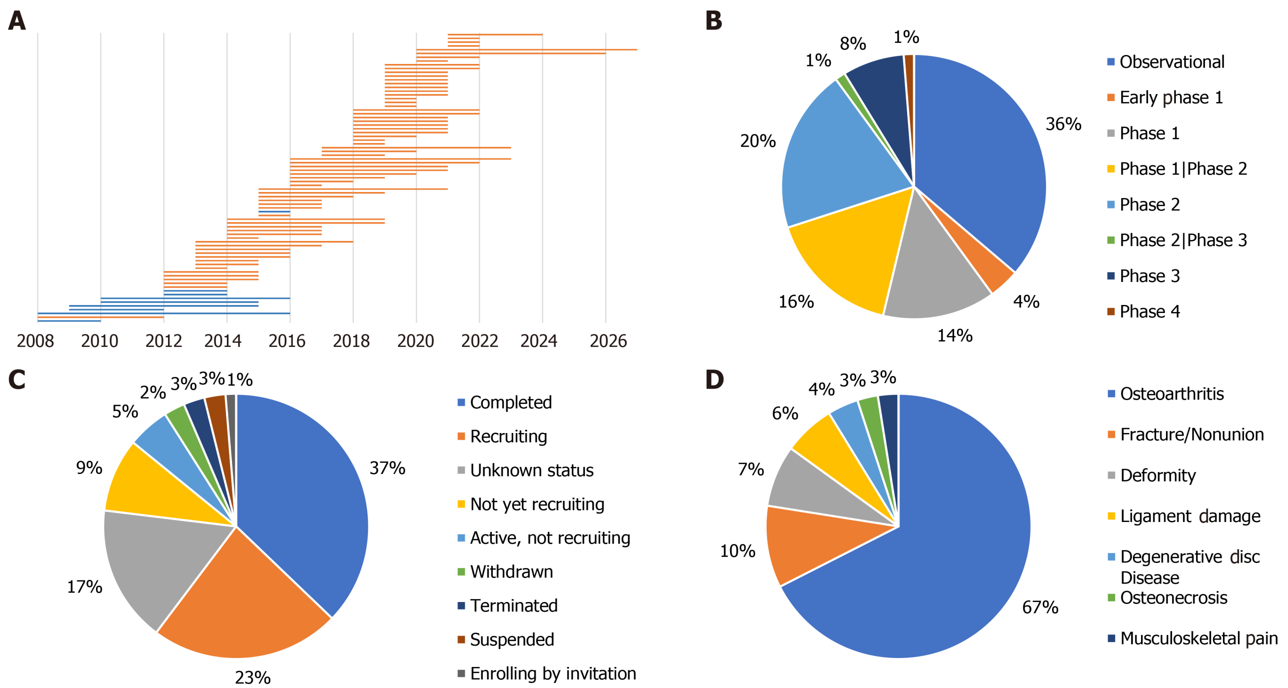

Figure 1 The clinical trials utilizing adipose-derived stem cells. The data were retrieved from clinical trials databases Clinicaltrials.gov and PUBMED. A: Distribution of adipose-derived stem cells (ADSCs) clinical trials' start and end dates. Clinical trials on bone regeneration with both identified start and end date are represented in blue. All others are represented in orange; B: Distribution of ADSCs clinical trials' status; C: Distribution of ADSCs clinical trials' phase; D: Distribution of ADSCs clinical trials' targeted condition.

OUTCOMES OF CLINICAL TRIALS USING ADSCS FOR BONE REGENERATION

Saxer *et al*[30] published in 2016 the results of a study investigating the safety and feasibility of a stromal vascular fraction (SVF) (construct in the treatment of proximal humeral fractures in elderly patients. The construct was made from unexpanded and undifferentiated human SVF derived from abdominal adipose tissue seeded onto a silicated-hydroxyapatite and fibrin hydrogel scaffold. The construct was tested first on male nude rats' 6 mm critical-sized femoral defects. Compared to cell-free control, the SVF-seeded construct was associated with significantly better mineralization and bone volume. Histological staining also confirmed *de novo* osteogenesis and angiogenesis in SVF-treated rats. The construct was subsequently tested on 8 patients aged 62-84 with displaced, low-energy, proximal humerus fractures who were followed prospectively for up to 12 mo post-surgery. The study confirmed that it was feasible for liposuction, SVF isolation, graft manufacturing, and implantation to all be completed intraoperatively. The implant was deposited into the void space created following open reduction and internal fixation. Over the follow-up period, the authors reported no adverse reaction that could be linked to the graft. Pain evaluation *via* VAS showed no lasting donor site pain and generally diminished operation site pain. Within one year of the surgery, five out of eight patients had their plates removed, which provided the opportunity for biopsy. The other three patients achieved subjective therapeutic goals and declined plate removal. Histological and micro-CT analysis confirmed osteogenesis at the graft site, either directly connected to or separated from the pre-existing bone. Bone ossicles were also found in scaffold pores. The authors considered these findings as circumstantial evidence for the direct contribution of SVF cells to fracture healing.

In 2011, Thesleff *et al*[31] presented the results of treatment of 4 patients with critical-size calvarial defects that underwent cranioplasty using grafts of ADSCs seeded on beta-tricalcium phosphate granules. ADSCs were obtained autologously from participants' subcutaneous abdominal fat, isolated, and expanded over three weeks. Participants were evaluated over a one-year follow-up period with computed tomography (CT) scans, which showed ossification. Hounsfield Unit measurements with CT scans showed approximate equivalence between normal bone and regenerated tissue. No serious adverse event was reported. In 2017, the same research group released the results of a 6-year follow-up on the same cohort of patients plus

one more[32]. Unfortunately, the authors determined that the long-term outcomes of ADSCs beta-TCP grafts for cranioplasty remained unsatisfactory. Among the five patients who underwent the procedure, four needed revision surgeries at 0.9, 2.0, 2.2, and 7.3 years following the original operation. Indications for re-operation included infect, partial resorption of graft, complete resorption, and meningioma recurrence. The grafts were then either removed and replaced with titanium, strengthened with titanium mesh, or the patient underwent craniotomy in the case of meningioma. Only one patient retained the original graft at the time of publication, but her skull X-ray did show some level of graft resorption.

Sándor *et al*[33] in 2014 reported a case series involving 13 patients with craniomaxillofacial bone defects, three of the frontal sinuses, five of the cranial bones, three of the mandibles, and two of the nasal septa. ADSCs from participants were harvested from abdominal subcutaneous fat, expanded, and seeded on either bioactive glass or beta-tricalcium phosphate scaffolds. In the three mandible cases, rhBMP-2 was also added. Follow-up periods ranged between 12 and 52 mo and showed satisfactory clinical and radiographic results for patients with mandibular, frontal sinus, nasal septum defects. Of the participants with cranial defects, two achieved clinically and radiographically satisfactory ossifications, while the other three experienced significant resorptions of the ADSC graft. One of the nasal septum defect patients resumed habitual nasal picking during follow-up and needed graft removal.

Dufrane *et al*[34] published a study in 2015 describing the treatment of 6 patients with long bone nonunion resulting from either bone malignancy or pseudoarthrosis. These patients were treated using 3D bone grafts produced from subcutaneous ADSCs, incubated in osteogenic media, and delivered using demineralized bone matrix (DBM) without scaffolding. During the follow-up period of 47 mo, no acute adverse events or tumorigenicity were reported, but there were two instances of infection. Three out of six patients achieved bone regeneration and union.

Prins *et al*[35] published a study in 2016 evaluating the use of autologous SVF, rich in ADSCs, seeded in calcium phosphate ceramics for maxillary sinus floor elevation. SVF was obtained from the participants' abdominal wall. A total of 10 participants received either bilateral implants, with one side being SVF with ceramics and one side being ceramics control or a unilateral implant of just SVF with ceramics. Follow-up over three years showed no serious adverse event. Follow-up biopsy and micro-CT showed active bone formation in the study arm with statistical differences in bone volume over control, most notably in SVF with β-tricalcium phosphate ceramics group.

In 2017, Khojasteh *et al*[36] published a phase I clinical trial following 7 patients with alveolar clefts treated with autogenous bone osteoplasty in combination with buccal fat pad derived ADSCs. Patients were divided into three treatment arms: Anterior iliac crest (AIC) spongy bone with a collagen membrane, lateral ramus cortical bone plate with ADSCs, and AIC spongy bone with ADSCs and collagen membrane. Results indicated bone generation in all three experimental arms, weakest in the AIC only group and strongest in the AIC with ADSCs group. However, the differences were not statistically significant. No serious adverse event was reported.

Castillo-Cardiel *et al*[37] published in 2017 the results of a single-blind, randomized, clinical trial involving 20 patients with mandibular angle fractures. Participants were separated into two groups, a control group receiving fracture reduction only and a stem cell treatment group receiving fracture reduction with application of ADSCs as well. ADSCs were obtained from abdominal fat 24 h prior to the mandibular procedure. Evaluation of bone regeneration over 12 wk showed statistically significant improvement in ossification in the ADSC group compared to control.

In 2019, Myerson *et al*[38] published a multicenter, randomized controlled study to compare safety and efficacy of ADSCs in subtalar arthrodesis (bone fusion of the subtalar joint involving ankle bone and heel bone) with classic bone autograft. This study included 140 patients enrolled in two study arms receiving either autologous bone grafts or ADSCs. Autologous bone grafts were obtained from either the iliac crest or the distal tibia. ADSCs were obtained autologously and deposited on partially demineralized cancellous bone. Patients were followed up for over two years using clinical scores such as AOFAS, SF-12, and FFI-R as well as radiographic evaluation for the fusion of the subtalar joint. Imaging showed a lower rate of fusion in the ADSCs group compared to autograft control. Nonetheless, both groups showed equivalent clinical evaluations.

There are three clinical trials registered at Clinicaltrials.gov with no published outcomes. NCT02140528 sought to evaluate the safety and efficacy of the injection of allogeneic ADSCs on the healing of tibial fractures in 40 patients. Patients were separated into two groups receiving either ADSCs injections or placebo. NCT04340284

is a retrospective report on the outcomes of 11 patients receiving fluoroscopic guided percutaneous injections of SVF to the site of long bone nonunion. Healing was evaluated over 12 mo using SF-12 and radiographic imaging. ADSCs were also considered for Spinal Cord Injury, which was investigated in trial NCT02981576. This study enrolled 14 participants separated into two arms receiving three intrathecal injections of either autologous ADSCs or autologous bone marrow-derived MSCs. Follow-up was done over 12 mo using ASIA impairment score as well as MRI imaging.

Finally, clinical trial NCT03678467 is an open-label trial using an autologous, anatomically shaped bone graft made from patients' own ADSCs specifically for patients' mandible injury or deformity. The main goal of the study is to assess the safety of the treatment. Six patients will be followed over 12 mo on the number of adverse events, quality of life, and bone regeneration with CT scans.

In summary, a total of ten different studies conducted on total of 307 patients suggest that the use of ADSCs is safe, but there is limited evidence that ADSCs can significantly enhance bone regeneration.

RESERVATIONS ABOUT USING ADSCS FOR BONE REGENERATION

Considering the abundant availability of ADSCs and ease of their isolation, several investigators have attempted to use ADSCs to enhance bone regeneration. These studies were conducted using conventional preparations of ADSCs satisfying the ISCT definition of adult stem cells or MSCs as CD105+CD90+CD73+CD45-CD34-CD14orCD11b-CD79αorCD19-HLA-DR- cells. These studies suggested a limited ability of ADSCs to induce bone formation or to enhance bone repair and raised serious doubts about their therapeutic utility. The outcomes of the investigations are summarized in this section.

Primary ADSCs failed to enhance bone healing, in defects created in rat calvaria and sheep tibia[39,40]. In a canine maxillary alveolar cleft model, autografts induced significantly higher bone formation than ADSCs-seeded on hydroxyapatite/beta-tricalcium phosphate scaffolds[41]. Godoy Zanicotti *et al*[42] used titanium surface as the scaffold for delivery of ovine ADSCs to repair sheep femur epicondyle defects. Histology and histomorphometry were used to evaluate the implants one month after surgery. Using PKH26 cell-tracking dye, the authors were able to confirm the persistence of ADSCs in the defect area at one month. Unfortunately, based on histomorphometry results, no significant difference in regenerated bone tissue was found among all experimental and control groups.

When human ADSCs (hADSCs) were implanted in immunodeficient animals, they failed to induce any ectopic bone formation in 8 wk[43-46]. Spheroids of human bone marrow-derived MSCs, but not hADSCs, could consistently induce ectopic bone formation in immunodeficient mice[47]. Surprisingly, hADSCs did not survive in the calvarial defects of nude mice after two weeks, although the recipient mice lacked T cells[48]. While the theory of paracrine factors released by hADSCs in these two weeks being sufficient for bone regeneration awaits more investigation, these data raise questions about the usefulness of ADSCs for bone regeneration in healthy (immuno-competent) recipients. Corroborating this notion, ADSCs could not enhance calvarial defect healing in immunocompetent rats[39].

Attempts by other investigators to improve the bone-forming ability of hADSCs, by the addition of BMP-2 have also failed in a femoral defect model in T-cell deficient nude rats[49]. This was proposed to be the consequence of the failure of hADSCs to respond to BMP-2 *in vitro*[50]. In agreement with this finding Runyan *et al*[51] found that recombinant human BMP-2 formed more bone than autologous ADSCs and recombinant human BMP-2 in combination in a porcine model of the periosteal envelope. Keibl *et al*[52] tested a fibrin scaffold embedded with ADSCs and BMP-2 in the treatment of a non-critical size rat femur defect model. At two- and four weeks post-treatment, the authors found no major difference among the groups indicating no effect of BMP-2 on ADSCs potential and ADSCs alone could not induce any bone repair. This questions the ability of ADSCs to induce bone formation and also their ability to respond to BMP-2. Interestingly, this problem could be overcome by overexpressing BMP-2 and BMP-7 both in ADSCs. Qing *et al*[53] reported that only the BMP-2/BMP-7 transduced ADSCs, but not non-transduced ADSCs, BMP-2 only ADSCs, and BMP-7 only ADSCs, showed complete filling of the defect area in rat femur defects. However, the combination of growth factors present in non-activated platelet-rich plasma (nPRP), such as PDGF, TGF-b, bFGF, and VEGF, did not show any

beneficial effect on ADSCs during rabbit calvarial defect healing[54]. There was little difference between the nPRP-ADSCs group, ADSCs alone, and PRP alone in terms of newly formed bone surface or volume.

Mazzoni *et al*[55] evaluated the osteogenic capacity of ADSCs on a hydroxyapatite-collagen hybrid scaffold in 50 patients undergoing malar augmentation. The authors reported the follow-up over three years which showed implant stability and osteointegration but histological samples from patients revealed osteogenesis and mature bone only in 70% of specimens.

Testing human stem cells in T-cell deficient animal models has been a regular practice but that may not be the ideal way to test the potential of ADSCs. Recent advances in the bone regeneration field suggest that certain T-cell subsets, CD4+CD25+FoxP3+ Treg cells being a prominent one, are required for stem cells to initiate the bone formation process. We believe that this could explain, at least partially, the inability of ADSCs to induce bone formation in T-cell lacking mice and rats.

PRECLINICAL STUDIES

Publications on preclinical studies utilizing ADSCs were obtained from OVID Medline using the search keywords: “ADSCs”, “Stem Cells”, “Animals”, “Mesenchymal Stem Cells”, “Tissue Engineering” and “bone regeneration”, which returned 90 studies. An additional 14 studies were included from past collections by the researchers. After the elimination of studies that either lacked *in vivo* experiments, lacked a focus on ADSCs, or overlapped with other sections, 52 pre-clinical studies, investigating the bone-forming ability of ADSCs using various animal models, are summarized in this section and in Table 2.

COMPARISON OF ADSCS WITH SVF AND MSCS

Kang *et al*[56] compared canine MSCs from adipose tissue, bone marrow, umbilical cord blood, and Wharton’s jelly in terms of their osteogenic potential *in vitro* and *in vivo*. ADSCs showed the highest proliferation capacity at all passages *in vitro*. Measured levels of ALP activity were highest in ADSC and umbilical cord blood-derived MSCs. When stem cells were mixed with β-TCP and implanted into the canine segmental defects created in the radial diaphysis, comparable bone healing was observed in all stem cells groups which were significantly higher than the scaffold control group as determined by radiographic union, histology analysis, and the ratio between newly formed bone over total defect size.

Toplu *et al*[57] created the bone defects on the bilateral zygomatic arches of 20 rats. On one side, the defect was left for secondary healing and on the other side, SVF was injected into the defect site. After 20 wk, Micro-CT analysis and histology confirmed a significantly larger volume of newly formed bone in the SVF-injected side[57].

GROUP 1: PRE-DIFFERENTIATED ADSCS

Kim *et al*[58] treated rabbit 20 mm mid-diaphyseal ulna bone defects using SVF on a PLGA scaffold. Animals were treated with scaffold alone, PLGA containing undifferentiated SVF cells, and PLGA with osteogenically induced SVF cells. Since the PLGA-osteogenic SVF group showed significantly higher bone volume, the authors concluded that osteogenic differentiation was necessary for optimal bone regeneration by SVF. Osteogenically induced ADSCs-seeded coral scaffold showed statistically significant more healing of the canine bilateral full-thickness parietal defect model in comparison with control scaffold[59].

Investigators have also explored pre-differentiating ADSCs into endothelial lineage. Shah *et al*[60] compared osteogenesis induced by ADSCs differentiated into osteogenic lineage with those differentiated into endothelial lineage. Undifferentiated control ADSCs and differentiated ADSCs were used to treat rats’ calvarial defects. The authors were not able to find any statistically significant difference in osteogenesis and angiogenesis among these groups. Sahar *et al*[61] also compared ADSCs differentiated into endothelial lineage with ADSCs differentiated into osteogenic lineage when implanted in a critical size rat calvarial defect model. The results showed that undiffer-

Table 2 Summary of the preclinical studies involving bone regeneration induced by transplantation of adipose-derived stem cells

Animal model	Scaffold used	ADSCs per implant	Time frame	Defect healing outcomes	Ref.
-Beagle Dogs; -Unilateral radial segmental defect-10 mm	β-TCP/poly l-lactide-co-glycolide-co-ε-caprolactone composite scaffold	1×10^6 canine ADSCs	20 wk	33.90 ± 4.31	Kang *et al* [56]
-Wistar albino rats; -Middle zygomatic arch defect; -3 mm wide	No scaffold	Rat inguinal fat pad derived SVF	20 wk	The average new bone growth in the experimental group was 1.1 mm, significantly higher than control	Toplu *et al* [57]
Group 1: Pre-differentiated ADSCs					
-New Zealand white rabbits; -Mid-diaphysis of left ulna; -20 mm long	Porous polylactic glycolic acid scaffold	1×10^6 rabbit SVF cells	8 wk	Approximately 55%	Kim *et al*[58]
-Beagle dogs; -Parietal bone; -20 mm × 20 mm full-thickness defect	Coral scaffold	60×10^6 of canine ADSCs	24 wk	84.19 ± 6.45	Cui *et al*[59]
-Lewis rats; -Calvarial defect -8 mm wide	Polylactic scaffold	0.1×10^6 rat ADSCs	8 wk	Coculture of endothelial- and osteoblast-induced ADSC showed no significant improvement over undifferentiated cells	Shah *et al*[60]
-Lewis rats; -Calvarial defect; -8 mm wide	Poly (D,L-Lactide) scaffold	0.1×10^6 rat ADSCs	8 wk	Osteogenic-induced ADSC generated 0.91 ± 0.65 mm^3 new bone, significantly higher than endothelial-induced ADSC	Sahar *et al* [61]
Group 2: FGF, VEGF, PDGF, and ADSCs					
-Osterix□mCherry reporter mice; -Closed transverse diaphysis fractures of the right femur	No scaffold	0.3×10^6 wild-type mice ADSCs	35 d	The experimental group induced significantly larger mineralized surface and bone callus compared to cell-free and non-transduced controls.	Zhang *et al* [62]
-Balb/c nude mice; -Parietal bone defect; -4 mm wide	Whitlockite□reinforced gelatin/heparin cryogels	1×10^6 human ADSCs	8 wk	> 16%	Kim *et al*[63]
-CD1 nude mice; -Parietal bone defect; -4 mm wide	Coral scaffold	1.5×10^6 human ADSCs	8 wk	95.40%	Behr *et al*[64]
-Sprague Dawley rats; -Distal femoral cancellous bone -3.5 mm wide and 5 mm deep defect	Trimodal mesoporous bioactive glass scaffold	20×10^6 cell/mL until saturation; rat ADSCs	8 wk	14.25 ± 3.57	Du *et al*[65]
-Nu/Nu J mice; -Parietal bone; -4 mm wide	Polycaprolactone - fibrin scaffold containing heparin-conjugated decellularized bone	0.2×10^6 human ADSCs	12 wk	The experimental group induced a significantly larger new bone volume compared to the control without PDGF	Rindone *et al* [66]
Group 3: BMP and ADSCs					
-Sprague Dawley rats; -Full-thickness parietal bone defect -5 mm wide	Polylactic glycolic acid scaffold	0.0025×10^6 human ADSCs	8 wk	33.3 ± 29.0	Park *et al*[67]
-Chinese white rabbits; -Full-thickness calvarial defects; -8 mm	Fibrin gel matrix	3×10^6 rabbit ADSCs	12 wk	Approximately 48	Lin *et al*[68]
-Japanese white rabbits; -Segmental radial defect; -15 mm	Nano-hydroxyapatite/recombinant human-like collagen/poly (lactic acid) scaffold	2×10^6 cells/ml; rabbit ADSCs	12 wk	97.25 ± 2.06	Hao *et al*[69]

-Taiwan Lee-Sung minipigs; -Mid-shaft left femur defect; -30 mm long	Apatite coated poly (L-lactide-co-glycolide) scaffolds	100 × 10^6 cells/animal; minipig ADSCs	12 wk	Experimental group's new bone formation showed equivalent density and volume compared to native bone and is significantly better than non-transduced control	Lin *et al*[70]
-CD-1 nude mice; -Full-thickness parietal bone defect -3 mm wide	Porous poly(lactic-co- glycolic acid) scaffold	3 × 10^6 cells/mL; ADSC from C57BL/6 mouse	6 wk	77%	Fan *et al*[71]
-Nude mice; -Parietal bone defect; -4 mm wide	Polylactic glycolic acid scaffold	5 × 10^5 human ADSCs	12 wk	83%	Li *et al*[72]
-Nude mice; -Subcutaneous implantation	Porous poly(lactic-co- glycolic acid) scaffold	0.01 × 10^6 rat ADSCs	4 wk	Transduced ADSC construct induced more bone and vessel formation compared to cell-free and non-transduced control	Weimin *et al* [73]
-CD□1 nude mice; -Right parietal bone defect; -4 mm wide	Polylactic glycolic acid scaffold	0.15 × 10^6 human ADSCs	6 wk	Up to 100%	Levi *et al*[74]
-Athymic nude rat; -Mandible defect; -5 × 5 mm	Chitosan/chondroitin sulfate scaffold	0.25 × 10^6 ADSCs from C57BL/6 mouse	8 wk	Approximately 43%	Fan *et al*[75]
Group 4: Genetically manipulated ADSCs					
-BALB/c nude mice; -Subcutaneous implantation	β-tricalcium phosphate scaffold	2 × 10^6 human ADSCs	8 wk	Approximately 30%	Wang *et al* [76]
-Sprague Dawley rats; -Calvarial defect; -8 mm wide and 1 mm thick	Poly (sebacoyl diglyceride) scaffold	Rat ADSCs	8 wk	50.53 ± 4.45	Xie *et al*[77]
Group 5: Engineered scaffolds and ADSCs					
-C57BL6/J mice; -Mid femur defect; -2 mm	Strontium-substituted hydroxyapatite poly (γ-benzyl-l-glutamate) scaffold	5 × 10^6 C57BL6/J mice ADSCs	8 wk	Approximately 38%	Gao *et al*[78]
-Sprague Dawley rats; -Full-thickness femur defect; -4 mm wide	NaB/polylactic glycolic acid scaffold	1 × 10^6 rat ADSCs	4 wk	ADSC-seeded poly lactic glycolic acid scaffold with 0.05% NaB induced the highest bone density, compared to cell-free control and other concentration of NaB	Doğan *et al* [79]
-Balb/c nude mice; -Cranium defect; -4 mm wide	SiRNA lipidoid nanoparticle immobilized on polydopamine coated PLGA scaffold	1.0 × 10^6 human ADSCs	8 wk	Approximately 75%	Shin *et al*[80]
-Sprague Dawley rats; -Calvarial defect; -5 mm wide	Collagen-resveratrol scaffold	0.05 × 10^6 human ADSCs	2 wk	Undifferentiated ADSC-seeded construct exhibited better osteogenesis compared to controls and osteoinduced ADSC seeded scaffold	Wang *et al* [81]
-Athymic nu/nu mice; -Subcutaneous implantation	Alginate microspheres	0.5 × 10^6 rabbit ADSC	12 wk	Approximately 41%	Man *et al*[82]
Group 6: Manipulation of recipient host and ADSCs					
-Sprague-Dawley rats; -Calvarial defect; -7 mm wide	Polylactic glycolic acid scaffold	1 × 10^6 human ADSCs	12 wk	Approximately 60%	Wang *et al* [83]
-C57 black/DBA mice; -Supracondylar right femur defect -0.9 mm wide	Hydrogel	0.3 × 10^6 mice ADSC	8 wk	Approximately 50%	Deng *et al* [84]
-Osteoporotic Sprague-Dawley female rats; -Distal epiphysis left femur defect; -3 mm wide	Gelatin	2 × 10^6 rat ADSCs	5 wk	Approximately 23%	Li *et al*[85]

Group 7: Allogeneic ADSCs					
-New Zealand white rabbits; -Ulna defect; -15 mm	Demineralized bone matrix	60 × 10^6 rabbit ADSCs	12 wk	Both allogeneic and autologous ADSC seeded construct induced almost complete defect repair while cell-free control remained unrepaired	Gu *et al*[86]
-Sprague Dawley rats; -Ulna defect; -8 mm long	Demineralized bone matrix	60 × 10^6 rat ADSCs	24 wk	Radiographs and histology confirmed superior bone healing in the experimental group compared to cell-free control	Wen *et al*[87]
-Beagle Dogs; -Parietal bone defect; -20 × 20 mm	Coral scaffold	60 × 10^6 of canine ADSC	24 wk	Approximately 70%	Liu *et al*[88]
-Wistar rats; -Left radius defect; -4 mm long	Heterogeneous deproteinized bone	0.1 × 10^6 rat ADSCs	8 wk	Radiographs and histology confirmed improved healing in osteoinduced ADSC/scaffold group compared to undifferentiated ADSC, cell-free, and blank controls	Liu *et al*[89]
Group 8: Non-manipulated or unaltered ADSCs					
Decellularized matrices					
-CD1 nude mice; -Distal femur defect - 3 mm	Human cancellous bone scaffold	0.5 × 10^6 human ADSCs	8 wk	hADSCs-seeded scaffold induced significantly superior defect healing compared to cell-free scaffold	Wagner *et al*[90]
-C57BL/6 mice; -Calvarial defect; -4 mm wide	Extracellular matrix deposited on porcine small intestinal submucosa	0.0025 × 10^6 of human ADSCs	4 wk	21.77 ± 6.99	Zhang *et al*[91]
-Institute of Cancer Research mice; -Full-thickness parietal defect; -4 mm wide	Decellularized tendon	1.0 × 10^6 human ADSCs	8 wk	86%	Ko *et al*[92]
-Sprague Dawley rats; -Two-wall periodontal intrabony defect; -2.6 × 2.0 × 2.0 mm	Amniotic membrane	0.3 × 10^6 human ADSCs	3 wk	ADSC-seeded scaffold resulted in a significantly smaller defect size than the control	Wu *et al*[93]
Ceramics					
-Sheep; -Tibia; -3.2 cm long defect	Hydroxyapatite-based particle in a semi-solid milieu	56 × 10^6 human ADSCs	12 wk	The experimental group showed bridging and significantly better healing compared to control	Ben-David *et al*[94]
-New Zealand White rabbits; -Full-thickness proximal medial tibia defect; -8 mm wide	Hydroxyapatite	0.2 × 10^6 rabbit ADSCs	8 wk	The new bone area was equivalent between seeded and unseeded scaffold; however, ADSC seeded construct represented preferable histological characteristics	Arrigoni *et al*[95]
-New Zealand White rabbits; -Full-thickness proximal medial tibia; -8 mm in diameter	Hydroxyapatite	1.5 × 10^6 rabbit ADSCs	8 wk	ADSC-seeded scaffold exhibited better scaffold resorption than cell-free scaffold and superior histological characteristics compared to all controls	De Girolamo *et al*[96]
-Fisher 344 rats; -Calvarial defect; -5 mm wide	Hydroxyapatite	0.4 × 10^5 rat ADSCs	8 wk	16.88 ± 1.52	Xia *et al*[97]
-T and B cell-deficient NOD SCID mice; -Subcutaneous implantation	Type I collagen (30%) and magnesium-enriched hydroxyapatite	1 × 10^6 human ADSCs	8 wk	hADSC-seeded presented improved osteogenesis and angiogenesis compared to cell-free scaffold control	Calabrese *et al*[98]
-Miniature Pigs; -Mandibular defect -3 cm × 1 cm × 2 cm	Tri-calcium phosphate- poly (D,L-lactide-co-glycolide) scaffolds	5 × 10^6 porcine ADSCs	12 wk	34.8 ± 4.80	Probst *et al*[99]

Bioactive glass					
-Wistar rats; -Full-thickness calvarial defect; -8 mm wide	Bioactive glass	0.5 × 10^6 rat ADSCs	12 wk	ADSC-seeded scaffold group exhibited significantly more bone repair and higher bone density compared to blank control. ADSC construct's result was equivalent to that of autologous bone graft	Saçak *et al*[100]
-Sprague Dawley rats; -Parietal bone defect; -8 mm wide	Icariin doped bioactive glass	0.5 × 10^6 rat ADSCs	12 wk	The experimental group saw the complete repair of the defect while all controls showed various degrees of incomplete healing; repair in the experimental group is characterized by mature bone and complete scaffold resorption	Jing *et al*[101]
Polymers					
-Wistar rats; -Calvarial defect; -5 mm wide	Polycaprolactone scaffold	0.05 × 10^6 human ADSCs	8 wk	Both undifferentiated and osteo-induced ADSC-seeded scaffold resulted in preferable histological features and higher expression of osteogenesis and angiogenesis markers	Caetano *et al*[102]
Platelet-rich plasma as carrier material					
-Beagle dogs; -Tibial defects; -10 mm wide	Activated platelet-rich plasma	1.0 × 10^6 human ADSCs	6 wk	68.97 ± 0.91	Cruz *et al*[103]
-F344 rat; -Calvarial defect; -5 mm wide	Activated platelet-rich plasma	0.2 × 10^6 rat ADSCs	8 wk	95.60	Tajima *et al*[104]
Hybrid materials					
-New Zealand white rabbits; -Calvarial defect; -10 mm wide	Hyaluronic acid-g-chitosan-g-poly (N-isopropylacrylamide) embedded with biphasic calcium phosphate microparticles and PRP	0.1 × 10^6 rabbit ADSCs	16 wk	The experimental group induced obvious significant bone formation and defect bridging. Cell-free scaffold control showed negligible defect repair	Liao *et al*[105]
-Sprague Dawley rats; -Parietal defect; -5 mm wide	Multi-layered stacking of electrospun polycaprolactone/gelatin membranes	0.006 × 10^6 rat ADSCs	12 wk	Up to 90%	Wan *et al*[106]
-Balb/c nude mice; -Calvarial defect; -4 mm wide	1H,1H,2H,2H-per- fluorodecyl acrylate (97%) and glycidyl methacrylate coated paper scaffold	1.0 × 10^6 cells/paper human ADSCs	8 wk	92%	Park *et al*[107]

ADSCs: Adipose-derived stem cells; SVF: Stromal Vascular Fraction; PRP: Platelet-rich plasma.

entiated ADSC or osteogenic ADSC induced a significantly higher amount of bone tissue than endothelial ADSCs group which was equivalent to acellular control.

GROUP 2: FGF, VEGF, PDGF, AND ADSCS

Modulation of the bone-forming ability of ADSCs by expressing genes of FGF, VEGF, PDGF or by tethering these proteins to scaffolds has been reported. Zhang *et al*[62] created bone defects in mouse femurs and injected control ADSCs or ADSCs transduced with bFGF intramuscularly adjacent to the fracture site. While no significant improvement was observed in the ADSCs group, improved bone healing

was observed in the ADSCs-bFGF group. Interestingly, using GFP-assisted observation, the authors identified that at day 21, only a very small fraction of the originally implanted ADSCs remained in the healing callus. This supported the idea that ADSCs' role in bone healing is more reliant on its paracrine function rather than direct cell replacement[62].

Kim *et al*[63] studied VEGF-transduced ADSCs for the treatment of mouse calvarial bone defects. At week 8, microCT and histology revealed that groups with ADSCs exhibited faster bone regeneration. In that, the VEFG overexpression group was found to have significantly more bone healing. hADSCs pre-treated with VEGF also showed beneficial effects. Behr *et al*[64] implanted hADSCs that were pre-treated with 2 μg VEGF into critical-size calvarial defects of nude mice using coral scaffold. Quantification of defect filling at week 8 by microCT showed that the VEGFA treated ADSCs group yielded significantly better bone regeneration than all other groups including untreated ADSCs. VEGF is likely to have a direct effect on the differentiation of ADSCs and it might also increase the bone healing rates indirectly by improving angiogenesis during bone regeneration. Du *et al*[65] have reported that the bioactive glass implant pre-vascularized *in vitro* for 7 d using endothelial cells and then seeded with ADSCs was associated with enhanced angiogenesis and significantly more bone regeneration in rat femur critical size defect compared to acellular scaffold and non-vascularized ADSC-seeded implant.

Similar to FGF and VEGF modulation, PDGF has been reported to improve the osteogenic ability of ADSCs. Rindone *et al*[66] designed a scaffold in which PDGF-BB was anchored using heparin-conjugation and simple electrostatic force. When implanted in murine calvarial defects, the experimental group containing ADSCs and PDGF-BB showed significantly higher bone formation compared to controls without PDGF-BB.

GROUP 3: BMP

BMP-2 is FDA-approved for the treatment of bone injuries and is currently being widely used to treat bone defects. It is known to govern osteogenic differentiation of stem cells. Naturally, it has been the focus of investigation for improving the bone-forming potential of ADSCs.

Park *et al*[67] investigated the ability of BMP-2 transduced ADSCs for the treatment of full-thickness parietal bone defects in rats. Similar to control receiving no implant, scaffold only group and scaffold with osteogenically induced ADSCs group showed either no or incomplete filling. However, mice receiving BMP-2 transduced ADSCs showed complete healing at week 8. Lin *et al*[68] compared bone formation induced by BMSCs and ADSCs in rabbit calvarial defect model. BMSCs and ADSCs were transduced with a BMP4-carrying-adenovirus vector and seeded on a fibrin gel scaffold. Both transduced BMSC and ADSC groups showed a significantly higher amount of newly regenerated bone tissue compared to their respective non-transduced control. No difference was identified between transduced BMSC and transduced ADSC groups. Hao *et al*[69] investigated the potential of BMP-2 overexpressing ADSCs in a rabbit critical size radial segmental defect. The authors reported that animals treated with transfected ADSCs-seeded scaffolds demonstrated recanalization of the radial medulla, bone contour modeling, and scaffold degradation. No significant defect repair was found in either scaffold only or scaffold with non-transfected ADSCs groups.

Lin *et al*[70] overexpressed BMP-2 and VEGF genes in ADSCs and the resulting cells were seeded on a PLGA scaffold which was implanted in a minipig massive segmental left femoral defect model. Bone regeneration in the experimental group was observed as early as two weeks post-procedure and progressively increased to complete union at 12 wk. PET evaluation also revealed improved angiogenesis in the experimental group compared to the control.

Strategies that promote BMP signaling in ADSCs have also been used successfully. Fan *et al*[71] coated PLGA scaffolds with Phenamil and BMP-2 and then seeded the scaffold with ADSCs. Phenamil is a derivative of the diuretic Amiloride, that acts as a powerful stimulator of BMP-2 signaling. The authors hypothesized that using Phenamil would allow optimal osteogenesis while reducing the needed BMP-2 dose to avoid adverse effects. The construct was tested on a mouse calvarial bone defect model. The authors reported that ADSCs-loaded scaffold treated with both Phenamil and BMP-2 induced significantly improved bone regeneration compared to ADSCs-loaded scaffold with BMP-2 alone group as measured by micro-CT. Li *et al*[72]

transduced ADSCs to upregulate expression of BMP-2 and miR-148b using a Cre/LoxP-based baculovirus hybrid before seeding onto gelatin-coated PLGA scaffold. miR-148b is a miRNA identified for its osteogenic property when acting with BMP-2. When this construct was used to treat critical-sized calvarial bone defects in nude mice, the authors found that at 12 wk post-procedure, the experimental group showed significantly improved bone healing compared to controls with either no transduction, transduction with only miR-148b, or only BMP-2. Weimin *et al*[73] expressed LIM mineralization protein 1 (LMP-1) and hypoxia-inducible factor 1 (HIF-1 α) genes in ADSCs to promote osteogenesis. LMP-1 was recognized as a positive intracellular regulator of osteogenesis, upstream of BMP-2, while HIF-1a initiated angiogenesis[41]. After lentiviral expression of genes in ADSCs, the resulting cells were seeded onto a PLGA scaffold and tested on the dorsal subcutaneous pockets of nude mice. Based on histological analysis, the authors claimed that there was more neo-osteogenesis found in LMP-1 and HIF-1a expressing ADSCs than found in controls.

Lentiviral delivery of shRNA to inhibit expression of Noggin, an inhibitor of BMP-2 signaling, in ADSCs has been shown to improve their osteogenic potential[74]. The transduced ADSCs induced more rapid and complete healing of the calvaria defect in nude mice in comparison with non-transduced ADSCs. BMP-2 addition and Noggin inhibition together are known to further improve bone healing outcomes. Fan *et al*[75] transfected ADSCs with lentivirus silencing the expression of noggin and loaded them onto a chitosan and chondroitin sulfate scaffold, coated with apatite to ensure BMP-2 attachment and controlled release. The construct induced significantly more bone repair in a rat mandibular defect model in comparison with blank scaffold, scaffold with BMP-2, or scaffold with ADSCs (Nog-/-) at 8 wk.

GROUP 4: GENETICALLY MANIPULATED ADSCS

Wang *et al*[76] found that Prostaglandin G/H synthase 1 (PTGS1) is expressed in ADSCs in response to TNF-α in inflammatory conditions and that PTGS1 knockout ADSCs showed higher osteogenic potential. When PTSG1 knockout ADSCs were mixed with Synthograft (Bicon), a commercial beta-tricalcium phosphate product, and were transplanted into the dorsal subcutaneous tissue of mice, they induced significantly more bone formation at week 8 compared to control[76]. Xie *et al*[77] used lentivirus to transduce ADSCs to upregulate the expression of miR-135, a microRNA recognized for its role in regulating osteogenesis. Transduced ADSCs were implanted in rats' critical-sized calvarial bone defect model. The authors observed almost complete sealing of defect area when treated with miR-135 transduced ADSCs. All other groups showed from nonexistent to incomplete filling of the defect.

GROUP 5: ENGINEERED SCAFFOLDS

Gao *et al*[78] developed a microcarrier from Strontium (Sr)-substituted hydroxyapatite, which was found to release Sr ions, known activators of the Wnt/b-catenin pathway, consistently at the right concentration. When these microcarriers were seeded with ADSCs and implanted into mouse femur nonunion defect, they were found to stimulate significantly more bone formation than control at 8 wk. Doğan *et al*[79] incorporated boron into PLGA scaffold (NaB/PLGA) and seeded ADSCs onto it to test this implant in a rat femur defect model. One month after implantation of ADSC-NaB/PLGA, the ADSC-NaB/PLGA group showed the highest Hounsfield units which represented superior bone regeneration compared to all other groups.

Shin *et al*[80] constructed a system in which siRNA lipidoid nanoparticles, designed to target and silence the osteogenesis inhibitor guanine nucleotide-binding protein alpha-stimulating activity polypeptide (GNAS), were immobilized on PLGA scaffolds, and hADSCs were seeded onto this PLGA scaffold for treating mouse critical-sized calvarial defect. The authors hypothesized that using this system, hADSCs could undergo genetic modification and osteogenic induction after being seeded onto the scaffold, eliminating the need for activation using culture-based protocols. At eight weeks post-procedure, the experimental group showed significantly more bone regeneration in comparison with no treatment control, construct without siRNA, and construct with scrambled siRNA.

Wang *et al*[81] combined collagen scaffold with Resveratrol (RSV), an antioxidant with anti-inflammatory and osteogenic properties, and seeded ADSCs on the construct. The authors reported that hADSCs-seeded collagen-RSV scaffold was the most effective in bone regeneration in a rat calvaria defect model when compared with other groups: collagen scaffold only, collagen scaffold with resveratrol, ADSCs seeded collagen scaffold, and ADSCs seeded collagen scaffold with resveratrol, based on their micro-CT results at 12 wk.

Man *et al*[82] evaluated the effect of PRP on the osteogenic potential of ADSCs encapsulated in alginate microspheres. The microspheres were combined with 5% PRP, 10% PRP or 15% PRP and injected subcutaneously in athymic nude mice. Only groups receiving ADSC-Alginate with 10% and 15% PRP showed mineralization at 1 and 3 mo with the 15% PRP group showing a dose-dependent increase.

GROUP 6: MANIPULATION OF RECIPIENT HOST

Wang *et al*[83] used the hADSC-seeded PLGA constructs for the treatment of rat critical-size calvarial defect and also evaluated the impact of locally injected Alendronate (Aln), a bisphosphonate often used for the treatment of osteoporosis. At 12 wk the acellular groups (control and PLGA-Aln) showed limited bone formation while both PLGA-ADSCs and PLGA-ADSC-Aln showed abundant mature neo-osteogenesis. Complete bridging of the defect was observed only in the PLGA-ADSC-Aln group.

Deng *et al*[84] found that exendin-4 enhanced the ability of ADSC to induce bone regeneration in a mouse femur metaphyseal defect model. Exendin-4 is a glucagon-like peptide 1 receptor agonist previously recognized for its role in glycemic control, control of bone resorption, and increased bone mass[84]. After creating the femur metaphyseal defect, the authors planted hydrogels with ADSCs into the defect site followed by daily intraperitoneal exendin-4. This experimental group was compared with wild-type non-defective bone, defect bone without treatment, and defect bone treated with ADSC only. The results of bone regeneration after 8 wk showed that this experimental group exhibited significantly more repair than ADSCs only group as well as the controls.

Li *et al*[85] reported that miR-214 targeted the Wnt pathway to favor adipogenesis in ADSCs isolated from osteoporotic ovariectomized rats and this microRNA was found at a high level in aged osteoporotic patients as well. Implantation of ADSCs genetically manipulated to silence miR-214, but not unaltered ADSCs isolated from osteoporotic rats, could lead to complete healing of critical size femoral metaphyseal defects in ovariectomized rats when delivered using a gelatin scaffold.

GROUP 7: ALLOGENEIC ADSCS

The use of allogeneic stem cells is currently prohibited by FDA. However, there is limited data available that allogeneic ADSCs can be as effective as autologous ADSCs in rabbit, canine, and rat bone defect models.

Gu *et al*[86] investigated the osteogenic capacity of ADSCs-seeded DBM to treat critical-sized ulna defects in a rabbit model. Micro-CT was used to compare three experimental groups: Allogenic ADSC-seeded DBM, Autologous ADSC-seeded DBM, and DBM only. The authors reported that both Allogenic and Autologous ADSC groups showed bone formation that bridged the defect gap. DBM alone group, on the other hand, did not show bridging of the defect but only loose fibrous tissue. Wen *et al* [87] also used a DBM as a scaffold for allogeneic rat ADSCs to promote bone healing in rat critical-sized ulnar bone defect model. At 24 wk, superior osteogenesis in defects treated with ADSCs-DBM was recognized grossly and radiographically. ADSCs-DBM treatment was also associated with significantly higher ulnar bone strength than those treated with DBM only. Allogeneic ADSCs were shown to be as effective as autologous ADSCs for the treatment of cranial critical-sized defects in canine models as well[88]. There was no significant systemic immune reaction as measured by the ratio of CD4/CD8 as well as serum levels of IL-2, IL-4, IL-10, IFN-g, and TGF-β1. MicroCT evaluation showed equivalent bone regeneration between allogeneic and autologous groups with both groups inducing significantly better healing than the scaffold-only group. When GFP-positive ADSCs were implanted, they could still be detected in osteocyte lacunae and bone matrix at 24 wk, pointing to their direct role in osteogenesis.

Liu *et al*[88] found that allogeneic ADSCs require pre-differentiation to be effective. Undifferentiated allogeneic ADSCs failed to induce bone formation. They seeded allogeneic ADSCs on heterogeneous deproteinized bone (HDB) and delivered the construct for the treatment of critical-sized bone defects in a rat radius model[89]. The authors investigated four groups: no implant, HDB implant only, non-induced ADSCs seeded on HDB or osteogenically induced ADSCs on HDB scaffold. It was found that at 8 wk, the group treated with osteogenic ADSCs on HDB showed evident bridging with new bone completely filling the defect area. All other controls, on the other hand, did not improve healing at 8 wk.

GROUP 8: SCAFFOLD TYPES USED FOR NON-MANIPULATED OR UNALTERED ADSCS

To this end, many different materials have been experimented with *in vitro* and *in vivo* in combination with ADSCs. Commonly used materials include decellularized tissues used as matrix, ceramics, polymers, as well as hybrid materials.

Decellularized matrices

Various natural matrices such as human cancellous bone, porcine small intestinal submucosa, bovine tendon, human amniotic membrane, have been used successfully, after their decellularization, for delivery of unaltered ADSCs and this approach has attained reasonable success in enhancing bone regeneration.

Wagner *et al*[90] investigated the combination of hADSCs and freeze-dried human cancellous bone for treatment of femur critical-sized defect in rats. They optimized the seeding density of ADSCs and found that a cell number of 250000 cells (84600 cells/100mm$_3$) was optimal. At 4 wk, the authors observed a significant elevation of bone regeneration in the ADSCs group compared to unseeded control. Zhang *et al*[91] explored a scaffold made from the extracellular matrix (ECM) deposited on porcine small intestinal submucosa (SIS). This porcine SIS construct was cultured with osteoblasts to induce deposition of osteogenic ECM, followed by decellularization and ADSCs seeding. The ADSCs-seeded ECM-SIS scaffold induced significantly more healing of mouse critical-sized calvarial defects than SIS only, ECM-SIS, ADSC-SIS groups. Ko *et al*[92] evaluated decellularized, bovine Achilles and neck tendons as the scaffold for osteogenically induced hADSCs to evaluate bone regeneration in a mouse calvarial bone defect model. The implant was placed in two layers into the calvarial bone defects and its capacity for bone regeneration was evaluated. Results at 4 and 8 wk showed significantly better filling of the defect in the experimental group compared to all controls. Wu *et al*[93] obtained amniotic membranes (AM) during cesarian delivery, decellularized them, and co-cultured with ADSCs to initiate seeding. This construct was used to treat two-wall periodontal bone defects in rats. CT imaging of the defect 29 d after implantation showed a smaller defect volume in the ADSCs-AM group compared to no treatment control, AM only, and ADSCs only.

Ceramics

Hydroxyapatite and β-tricalcium phosphate are the two most widely used matrices in this group and have shown successful outcomes in supporting ADSCs-mediated bone regeneration. ADSCs seeded and grown on hydroxyapatite-based mineral particles could successfully treat full cortex segmental tibial defect in sheep[94]. Following implantation of the ADSCs-seeded particles, statistically higher newly formed bone volume was observed in the treatment group compared to the control. Arrigoni *et al* [95] compared bone regeneration in a rabbit critical-sized tibial defect model mediated by hydroxyapatite alone and ADSCs-seeded hydroxyapatite implant. The authors reported that the ADSCs-seeded group displayed superior performance. de Girolamo *et al*[96] also reported somewhat similar findings in the rabbit model when they used autologous ADSCs seeded hydroxyapatite scaffold to treat full-thickness defects in New Zealand rabbit's proximal tibial epiphysis. At 8 wk, the authors reported that there were no significant differences in defect filling and bone mineral density, but the ADSCs-scaffold group induced the most mature bone that was quite similar to native tissue. The structure of hydroxyapatite is reported to play an important role. Based on *in vitro* results and data from the healing of the rats' bilateral calvarial defects, the micro-nano-hybrid structure, which is a hybrid of nanorod and microrod, was found to be the most effective surface topography for the delivery of ADSCs[97]. Calabrese *et al*[98] evaluated the ectopic bone formation induced by hADSC seeded on a collagen-

hydroxyapatite scaffold through subcutaneous implantation in mice. The scaffolds seeded with ADSCs exhibited faster hydroxyapatite formation and increased vascular generation, both statistically significant in comparison with scaffold control.

Probst *et al*[99] examined the efficacy of pig ADSCs with tri-calcium phosphate poly (lactic-co-glycolic) acid scaffold for regeneration of critical-sized mandibular bone defects in minipigs. ADSCs were induced with an osteogenic medium prior to seeding. MicroCT showed a significantly higher ratio of bone volume to total volume in the ADSCs group in comparison with control but even in the test group, the regenerated bone volume was only about one-third of the defect size.

Bioactive glass

Saçak *et al*[100] investigated bone regeneration in calvarial bone defect of mice using ADSCs seeded bioactive glass. The authors divided the animals into four groups either untreated, treated with autologous bone graft, treated with bioactive glass, or treated with ADSC-seeded bioactive glass. Bone regeneration in the ADSC-seeded bioactive glass group and autologous bone graft treatments were equivalent.

Jing *et al*[101] doped 45S5 Bioglass with Icariin, a flavonoid glucoside isolated from the plant Herba Epimedii and then seeded the implant with ADSCs. Implantation of the Icariin-doped, ADSC-seeded scaffold resulted in the complete repair of the rat calvarial defect in 12 wk. Groups receiving no scaffold, Bioglass only, and ADSC-seeded Bioglass without Icariin only exhibited partial repair. The authors reported that when cultured with Icariin, ADSCs upregulate their expression of VEGF, thus promoting angiogenesis which was the mechanism behind their enhanced osteogenic potential.

Polymers

Caetano *et al*[102] evaluated the use of polycaprolactone, a semi-crystalline biodegradable polymer, as a scaffold for human ADSCs to treat critical-size calvarial bone defects in rats. They compared undifferentiated hADSCs with hADSCs cultured in osteogenic conditions. The authors found osteoid tissue larger in size and more organized in groups treated with both types of ADSCs. Immunohistochemical staining revealed that the undifferentiated ADSCs group showed the highest percentage of cells with BMP-2 expression. The two groups with ADSCs showed equivalent angiogenesis, assessed *via* CD31 staining, which was significantly higher than no ADSCs groups.

Platelet-rich plasma as carrier material

Cruz *et al*[103] evaluated the use of platelet-rich plasma activated with calcium chloride as the carrier for ADSCs to treat 10 mm wide, beagle dog tibial bone defects. Four defects were introduced in each animal. The defects were treated with clot, PRP only, autogenous bone graft, or ADSCs-seeded PRP. Histological analysis showed that the PRP-ADSCs group induced significantly more bone formation when compared to control, autogenous bone graft, and PRP only.

Tajima *et al*[104] similarly explored activated PRP as the scaffold for delivering ADSCs to rat calvarial defects. Based on micro-CT results at 4 and 8 wk following the surgery, the authors found that ADSCs-seeded PRP demonstrated significantly larger regenerated bone area and volume compared to treatment with ADSCs-seeded type 1 collagen, PRP only, type 1 collagen only, and PBS control. The authors also confirmed that ADSCs transplanted by this manner differentiated into osteoblasts, by creating a construct using GFP expressing ADSCs-seeded PRP and observing cells staining positive for both GFP as well as osteocalcin.

Hybrid materials

Liao *et al*[105] used hyaluronic acid-g-chitosan-g-poly(N-isopropylacrylamide) (HA-CPN) embedded with biphasic calcium phosphate microparticles and PRP as the organic matrix for delivery of rabbit ADSCs to critical-size cranial bone defects in rabbits. This experimental implant induced significant bone formation, almost completely covering the defect area while the control showed only negligible bone formation at 16 wk.

Wan *et al*[106] designed a construct involving multi-layer stacking of three ADSCs-seeded polycaprolactone/gelatin electro-spun membranes. The construct was implanted into a model of calvarial defects in rats on bilateral parietal bones. The authors reported that the ADSCs seeded multilayer membrane group showed significantly more bone regeneration at higher density than those found in control and scaffold-only groups.

Park *et al*[107] evaluated a paper-based multi-layer scaffold for delivery of ADSCs to a mouse calvarial defect model. Based on their *in vitro* results, the authors determined that a scaffold of commercial weighing paper coated with 1H,1H,2H,2H-perfluorodecyl acrylate (97%) and glycidyl methacrylate was most suitable. The authors compared two stacks of scaffolds only, two stacks of ADSCs-seeded scaffolds, three stacks of alternating ADSCs-seeded scaffolds (A) and HUVEC seeded scaffolds (H), and finally five stacks of alternating A-H-A-H-A scaffolds. All ADSCs seeded scaffolds increased bone healing after 8 wk compared to the blank control and scaffold-only groups. HUVEC-seeding did not show any statistically significant difference but there was a trend of increased bone healing.

SURFACE RECEPTORS EXPRESSION-BASED SELECTION OF SUB-POPULATIONS OF ADSCS AS A RELIABLE STRATEGY TO IMPROVE THE THERAPEUTIC POTENTIAL OF ADSCS

Since investigations utilizing ADSCs in their un-purified and unaltered form have given mixed results, strategies to create ADSCs-based formulation that can enhance bone healing, unambiguously and reliably are necessary. Therefore, the search for the osteogenic sub-population of ADSCs has been initiated worldwide. Since a common molecular marker for all osteogenic progenitors has not been discovered and the precise identity of true skeletal stem cells, required for homeostasis and repair of the postnatal skeleton, remains elusive, investigators have used various surface markers for the selection of osteogenic sub-populations of ADSCs. The results of this investigation are summarized in this section and in Table 3.

CULTURED CELLS *VS* UNCULTURED SVF

Culture-expanded, horse ADSCs showed superior osteogenic ability when implanted in nude rats than that induced by the uncultured SVF[108].

CD146

Interestingly, a mixture of two distinct FACS-purified hADSCs populations ($CD146^+CD34^-$ and $CD146^-CD34^+$) induced ectopic bone formation and also healed 60% of calvarial defect created in immunodeficient mice[109-111]. When FACS-purified $CD146^+CD34^-$ cells were compared with unpurified SVF for their bone-forming ability using the ectopic bone formation assay and in the calvarial defect model, cells showed greater bone formation. Moreover, BMP2 treated cells showed more bone formation but with a massive adipogenic response. Usage of Nel-1 in place of BMP2 avoided adipogenesis to selectively promote only bone formation[110]. A study has shown that both $CD146^+CD34^-$ and $CD146^-CD34^+$ subpopulations from hADSCs undergo tri-lineage differentiation and express adult stem cell markers CD105, CD90, CD73. $CD34^+$ cells pre-cultured in an osteogenic medium for 3 d could induce bone formation in calvarial defects[112].

CD90

FACS-purified $CD90^+$ hADSCs, but not unpurified hADSCs, induced bone healing in calvarial defects of nude mice[113]. However, when $CD90^+CD34^+$ hADSCs were implanted in nude mice using a collagen sponge, they generated only adipose tissue after 4 wk[114]. This indicates the relevance of CD34 expression. In another study, mADSCs were FACS-purified into $CD90^+CD105^-$, $CD90^+CD105^+$, $CD90^-CD105^-$, and $CD90^-CD105^+$ populations. Marker expression of cells in basal medium, in osteogenic medium, and BMP2 transfected cells were determined. BMP2 transfection and culturing in an osteogenic medium were found to decrease the expression of CD105 [115]. $CD105^{low}$ and $CD90^+$ subpopulations were purified from hADSCs and compared with each other for their osteogenic potential. $CD90^+$ cells were found to be more osteogenic compared to $CD105^{low}$ cells *in vitro* as measured by ALP, Alizarin Red staining, and mRNA expression of Runx2, Ocn, Opn. When sorted cells were

Table 3 Specific markers used for selection of sub-populations of adipose derived stem cells showing superior bone forming ability

Ref.	Marker	Study outcome and salient findings
	CD146	
James *et al*[110]	$CD146^+CD34^-CD45^-$ (Pericytes) + $CD146^-CD34^+CD45^-$ (Adventitial cells)	Intramuscular ectopic bone formation in SCID mice; FACS purified, human, pericytes + adventitial cells produced significantly more ectopic bone formation than SVF; BMP2 enhanced osteogenic as well as adipogenic differentiation, whereas Nel-1 promoted only bone formation when tested in ectopic bone formation assay; 250000 cells were implanted intramuscularly in SCID mice for 4 wk using collagen sponge or DBX+ β-TCP + 3.5 -11.25 µg of BMP2 or 350 µg Nel-1
James *et al*[109]	$CD146^+CD34^-CD45^-$ (Pericytes) + $CD146^-CD34^+CD45^-$ (Adventitial cells)	Human pericytes + adventitial cells together make up around 40% of SVF from human lipoaspirate (60 patients tested) both types representing around 20% and these numbers do not change with age, gender, or body mass index; FACS purified, human, pericytes + adventitial cells induce significantly more healing in mouse calvarial defect than SVF; 250000 cells were implanted to critical size (3 mm) calvarial defect in SCID mice for 8 wk using PLGA
Meyers *et al*[112]	$CD146^+CD34^-CD45^-$ (Pericytes) + $CD146^-CD34^+CD45^-$ (Adventitial cells)	It was feasible to purify human pericytes + adventitial cells using a multi-column approach of magnetic beads; Purified pericytes + adventitial cells could enhance critical size (4 mm) calvarial defect created in SCID mice; 250000 cells were implanted to critical size (4 mm) calvarial defect in SCID mice for 8 wk using PLGA
	CD90	
Chung *et al*[115]	$CD90^+$	$CD90^+$ cells induced almost complete healing of critical size (4 mm) calvarial defect in nude mice compared to $CD105^{low}$ (approximately 75%), $CD105^{high}$ - (approximately 65%), and $CD90^-$ (40%) by micro-CT; Taken together $CD90^+$ cells are more osteogenic compared to $CD105^{low}$ cells; 150000 cells were implanted to critical size (4 mm) calvarial defect in SCID mice for 8 wk using PLGA
Ferraro *et al*[113]	$CD90^+CD34^+$	Implantation of human $CD90^+CD34^+$ ADSCs in nude mice resulted in the formation of only fat tissue surrounded by loose connective tissue; 250000 cells were implanted subcutaneously in nude mice for 4 wk using a collagen sponge
	CD105	
Levi *et al* [120]	$CD105^{low}$	FACS-sorted, human, $CD105^{low}$ sub-population of ADSCs significantly enhanced bone regeneration (> 95%) in critical size (4 mm) calvarial defect in CD1-nude mice compared to $CD105^{high}$ (approximately 40%) and unsorted ADSCs (50%-60%); Knockdown of CD105 in ADSCs (shCD105) resulted in improving their ability to induce bone formation (> 60%) compared to ADSCs transfected with control shRNA (30%); 150000 cells were implanted to critical size (4 mm) calvarial defect in nude mice for 8 wk using PLGA-HA
Madhu *et al*[123]	$CD105^+CD34^-$; $CD105^+CD34^+$; $CD105^-CD34^+$; $CD105^-CD34^-$	FACS-purified, mouse, $CD105^+CD34^-$ ADSCs that responded maximally to BMPs *in vitro* failed to induce ectopic bone formation upon their sub□cutaneous implantation immunocompetent syngeneic mice; FACS-purified $CD105^-CD34^-$ ADSCs responded the least to BMPs *in vitro*. A bone marrow-derived, clonal, osteoprogenitor population showing the similar phenotype of CD105-CD34- induced robust bone formation; OM preconditioned 1 × 10^6 cells were implanted subcutaneously in Balb/c mice for 4 wk using Matrigel
Chan *et al*[128]	$AlphaV^+CD200^+CD105^-D90^-$	Mouse skeletal stem cells that give rise to bone were identified as $AlphaV^+CD200^+CD105^-D90^-$ cells and were present in the femoral growth plate; They were not present in adipose tissue; however, when a collagen sponge loaded with BMP-2 was implanted in adipose tissue, the authors reported de novo formation of $AlphaV^+CD200^+CD105^-D90^-$ cells in the adipose tissue; Subcutaneous implantation of 10 µg BMP2+ Collagen Sponge in nude mice for 4 wk
Chan *et al*[131]	$PDPN^+CD164^+CD73^+CD146^-$	The human counterpart of mSSC was discovered and was found to be of phenotype $PDPN^+$ $CD164^+CD73^+$ $CD146^-$; Human adipose stroma did not naturally contain these cells but when it was mixed with BMP-2 and injected sub-cutaneously it led to skeletal reprogramming and induced formation of $PDPN^+$ $CD164^+CD73^+$ $CD146^-$ human skeletal stem cells; 10 × 10^6 cells with 10 µg BMP2 + Matrigel were subcutaneously implanted in nude mice for 4 wk
	CXCR4	
Xu *et al* [133]	$CXCR4^+$	$CD146^+CD34^-CD45^-$ cells were FACS-purified from hard (human periosteum) and soft (adipose and dermal tissue). Cells isolated from hard tissue but not the soft tissues showed a strikingly high tendency for skeletogenesis; This corresponded to high CXCR4 signaling in periosteal cells; Inhibition of CXCR4 signaling abrogated bone-forming potential of $CD146^+CD34^-CD45^-$ periosteal cells; $CXCR4^+$ cells from soft tissue (adipose) derived $CD146^+CD34^-CD45^-$ cells represented osteoblastic/non-adipocytic precursor cells; 1 × 10^6 cells were implanted intramuscularly in nude mice for 4 wk using DBM putty
	PDGFRα	
Wang *et al*[134]	PDGFRα+	Lineage tracing using PDGFRα reporter mice showed that PDGFRα expression marks different sub-populations in the adipose tissue; $PDGFR\alpha^+$ and $PDGFR\alpha^-$ fractions both are multipotent progenitor cells, however, $PDGFR\alpha^+$ ADSCs-derived ectopic implants ossify to a greater degree than $PDGFR\alpha^-$ cell fractions; 1 × 10^6 PDGFRα+ or PDGFRα- cells were implanted intramuscularly in nude mice for 8 wk using HA-β-TCP; Or Subcutaneous implantation of 2.5 µg BMP2 + Matrigel into the inguinal fat pad of PDGFRα+ -CreER for 8 wk

ADSC: Adipose-derived stem cells; FACS: Fluorescence-activated cell sorting; SCID: Severe combined immunodeficiency; BMP: Bone morphogenetic protein; TCP: Tricalcium phosphate; PLGA: Polylactic glycolic acid; HA: Hydroxyapatite; DBM: Demineralized bone matrix.

implanted into calvarial defects of nude mice, $CD90^+$ cells showed more bone formation[113].

CD105, TGF-Β1 SIGNALING AND BMP-RESPONSIVENESS

While ADSCs have been conventionally characterized by positive expression of CD105 [39,40,59,116,117], many groups have also observed considerable amounts of phenotypic variability within ADSCs during *in vitro* expansion[118,119]. Our group and other laboratories are actively investigating the role of CD105 in determining the osteogenic potential of ADSCs. CD105 is the co-receptor of the TGF-β1 signaling pathway and is known to enhance signaling of the main receptors Alk1 and Alk5 through phosphorylation of the downstream mediators - Smads2/3.

It has been shown that FACs-purified $CD105^{low}$ hADSCs possess more osteogenic differentiation potential than $CD105^{high}$ and unsorted cells *in vitro*, and also show decreased TGF-β1 and Smad2 phosphorylation. Treatment with TGF-β1 significantly reduces the osteogenic differentiation of $CD105^{low}$ ADSCs *in vitro*. In contrast, treatment with the Alk5 inhibitor enhances osteogenic differentiation. Moreover, CD105 knockdown promoted the bone-forming potential of ADSCs in immunodeficient animals *in vivo*[120-122].

We FACS-purified 4 different sub-populations of mADSCs; $CD105^+CD34^-$, $CD105^+CD34^+$, $CD105^-CD34^+$ and $CD105^-CD34^-$ and tested their BMP-responsiveness *in vitro*. Only $CD105^+CD34^-$ cells, showing the classical MSCs phenotype, responded to BMPs while others did not show significant response. We hypothesized that the ADSCs population maximally responding to BMPs *in vitro* would possess the ability to induce bone formation, and therefore investigated the bone-forming potential of $CD105^+CD34^-$ ADSCs in immunocompetent mice. Our hypothesis was clearly refuted and $CD105^+CD34^-$ ADSCs could not induce any bone formation[123]. Although we did not test the bone-forming ability of other three FACS-purified populations in that study, we found that bone marrow-derived D1 osteoprogenitor cells isolated from the same Balb/c mouse strain, did not express CD105 and did not respond to BMPs *in vitro*, but showed robust ability to induce bone formation[123,124]. Data from our group and others suggest that $CD105^-$ population represents true osteoprogenitors and inhibition of TGF-β1 signaling can improve the bone-forming ability of ADSCs. However, the bone-forming ability of $CD105^-$ ADSCS is not yet established in immunocompetent hosts. FACS purified $CD105^-$ human bone marrow-derived MSCs showed superior osteogenic efficacy when compared to $CD105^+$ cells *in vitro*. In critical-size defects created in the tibia of canine, $CD105^-$ MSCs implantation led to superior bone healing with complete bone remodeling, while $CD105^+$ MSCs implants failed to remodel resulting in the defect site filled with fibrocartilaginous tissue[125]. In sum, these studies showed that $CD105^-$ cells have more osteogenic potential *in vitro* as well as *in vivo*.

We have shown that simultaneously inhibiting TGF and BMP signaling pathways by using small chemical inhibitors induces neuronal differentiation of hADSCs *in vitro* and neurite outgrowth *in vivo*[126]. Previously this was demonstrated in ESCs and iPSCs, but not in adult ADSCs. It is well established that activin/nodal signaling contributes to the maintenance of pluripotency of hESCs. Activin/nodal/TGF-β and BMP pathways naturally antagonize each other because they compete for a common signal transducer Smad4. Inhibition of activin/nodal/ TGF-β signaling results in trophoblast differentiation, similar to induction of trophoblast differentiation by BMP-4[127]. These findings reveal the crucial roles of TGF-β and BMP signaling in deciding the fate of ADSCs.

In a recent discovery, the phenotype of mouse skeletal cells (mSSC) has been described as the $CD45^-Tier119^-Tie2^-AlphaV^+Thy^-6C3^-CD105^-CD200^+$ cells which were isolated from femoral growth plates of the mice[128]. These $CD105^-$ cells were able to form bone *in vivo* when implanted beneath the kidney capsule of T-cell deficient mice. Surprisingly, these cells were not efficiently engrafted, suggesting their requirement for a supportive niche. When these cells were transplanted with unsorted cells, they could form both bone and cartilage. Blocking VEGF signaling promoted chondrogenesis. Subcutaneous implantation of BMP2 in a collagen sponge in mouse inguinal pad formed ectopic bone; however, it did not originate from circulating SSCs recruited to implanted sites but SSCs formation was induced in the adipose tissue. It is not clear whether the $CD105^+$ or $CD105^-$ population of adipose tissue contributed to SSCs formation and this ectopic bone formation. Co-delivery of BMP2 with VEGF inhibitor into adipose tissue favored cartilage formation over bone[128]. We have shown that the crosstalk between BMP and VEGF signaling pathways enhances osteogenic differentiation of hADSCs through the p38 signaling pathway. Mineralization was abrogated when the p38 signaling pathway was inhibited[129]. We also found that VEGF could crosstalk with a downstream signal mediator of BMP, LIM mineralization protein 1 (LMP1) to enhance cell mineralization and ectopic bone formation mediated

by osteoprogenitors[130]. Similar to mSSCs, human skeletal stem cells (hSSCs) formation has also been reported, by the discoverers of mSSCs, in BMP2 treated adipose tissue. hSSCs displayed the phenotype PDPN$^+$CD73$^+$CD164$^+$CD146$^-$[131].

CD271

CD34$^+$CD271$^+$ hADSCs showed increased osteogenic differentiation compared to CD34$^+$CD271$^-$ and SVF whereas adipogenic and chondrogenic differentiation were similar[132].

CXCR4

FACS purified CD146$^+$CD31$^-$CD45$^-$ hADSCs isolated from different origins such as the periosteum, adipose, and dermal tissue display different degrees of osteogenic capabilities. Periosteal cells also express standard adult stem cell markers (CD105, CD90, CD73), Gli1, PDGFRα, and CXCR4; and are known to be more osteogenic *in vitro* as well as *in vivo* unlike soft tissue-derived CD146$^+$CD31$^-$CD45$^-$ ADSCs. Inhibition of CXCR4 expression abolishes the ability of these ADSCs to induce ectopic bone formation. Unsorted ADSCs as well as CD146$^+$ ADSCs further selected for CXCR4+ show enhanced osteogenic potential *in vitro* and *in vivo*[133].

PDGFRA

PDGFRα$^+$CD34$^+$, PDGFRα$^+$CD34$^-$, PDGFRα$^-$CD34$^+$, and PDGFRα$^-$CD34$^-$ were sorted from SVF of mouse adipose tissue from PDGFRα$^+$CreER and PDGFRα$^-$CreER mice. The authors found that PDGFRα$^+$CD34$^+$ ADSCs displayed more osteogenic potential *in vitro*. They also found that subcutaneously implantation of PDGFRα$^+$ cells and subcutaneous implantation of BMP2 into inguinal fat pads of PDGFRα$^-$CreER mice formed more bone as compared to controls[134].

CD105 AND SSEA3 EXPRESSING MUSE CELLS

Multilineage-differentiating stress-enduring (Muse) cells were first identified from bone marrow, which are of interest. These cells are positive for mesenchymal and embryonic stem cell markers CD105 and SSEA3. Muse cells comprise a small population of MSCs in BM-MSCs (1%-2%) and ADSCs (5%). 250000-500000 cells can be obtained from one gram of lipoaspirate. Adipose-derived Muse cells spontaneously differentiate into all three germ layers: mesodermal, endodermal, and ectodermal cell lineages and have non-tumorigenic and immunomodulatory properties. Muse cells have been successfully used for regeneration of skin, muscle, liver, kidney in different animal disease models however it has not been tested for its osteogenic differentiation potential[135].

Thus, the selection of subpopulations of ADSCs can harness abundantly available ADSCs for applications in bone regeneration.

CONCLUSION

The safety of ADSCs is reasonably established since they have been tested in 79 clinical trials including 580 patients total and there have been no serious adverse events reported. However, the clinical trials, as well as the pre-clinical studies investigating the potential of ADSCs in enhancing bone regeneration, have given confounding outcomes. In some cases, they were reported to enhance bone healing whereas, in others, they have failed to do so. It is also difficult to compare outcomes of different studies as investigators have used different animal models, delivery methods, and genetic manipulation of ADSCs. In many of the pre-clinical studies, T cell-deficient hosts were used. This transplant scenario is unlikely to provide a realistic picture of the osteogenic potential of ADSCs since T cells are likely to modulate bone regeneration induced by exogenously added adult stem cells. After careful review of

all the published reports, it is safe to conclude that ADSCs in their unaltered and unpurified form cannot be considered as reliable therapy for bone repair yet. Two major steps can be taken to solve this problem - first is to develop potency assays for each batch of ADSCs used in clinical and pre-clinical studies to allow comparison of outcomes of different studies and second is to search for a unique and reliable set of surface markers to define ADSCs. The current definition of adult stem cells can no longer be applied to ADSCs since both $CD105^-$ as well as $CD105^+$ fractions of ADSCs have been shown to possess bone forming potential. Surface markers such as CD146, AlphaV, CD200, PDPN, CD164, CXCR4, and PDGFRα will play an important role in defining osteogenic population within ADSCs in coming years. Areas such as the role of endogenous bone-progenitors in bone regeneration induced by exogenously added ADSCs and BMP-responsiveness of ADSCs also need immediate attention. Most of the studies published so far have not evaluated the survival and differentiation of transplanted ADSCs as well as recruitment of endogenous bone-progenitors to investigate whether the regenerated bone is donor stem cells-derived or originates from endogenous precursors. While BMPs are thought to promote differentiation of stem cells into the osteogenic lineage and BMP-overexpression has increased bone-forming potential of ADSCs in certain animal models, some investigators have also reported that ADSCs do not respond to BMPs. This observation and recent findings that implantation of BMP in adipose stroma leads to skeletal reprogramming and de novo formation of skeletal stem cells in adipose tissue, together, demand urgent attention of the scientific community to signaling pathways of ADSCs during osteogenic differentiation and after BMP stimulation. VEGF, BMP and TGF-β signaling pathways are the most important ones in this regard. Although the current clinically tested ADSC therapies do not yet appear to induce bone repair reliably, the ADSC optimizations described in this manuscript, based on cell subset purification and stimulus/activation, show great promise, and could potentially dominate stem cell-based therapies such as bone regeneration in the future.

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MINIREVIEWS

Neural stem cell therapy for brain disease

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Abstract

Brain diseases, including brain tumors, neurodegenerative disorders, cerebrovascular diseases, and traumatic brain injuries, are among the major disorders influencing human health, currently with no effective therapy. Due to the low regeneration capacity of neurons, insufficient secretion of neurotrophic factors, and the aggravation of ischemia and hypoxia after nerve injury, irreversible loss of functional neurons and nerve tissue damage occurs. This damage is difficult to repair and regenerate the central nervous system after injury. Neural stem cells (NSCs) are pluripotent stem cells that only exist in the central nervous system. They have good self-renewal potential and ability to differentiate into neurons, astrocytes, and oligodendrocytes and improve the cellular microenvironment. NSC transplantation approaches have been made for various neurodegenerative disorders based on their regenerative potential. This review summarizes and discusses the characteristics of NSCs, and the advantages and effects of NSCs in the treatment of brain diseases and limitations of NSC transplantation that need to be addressed for the treatment of brain diseases in the future.

Key Words: Neural stem cell; Brain disease; Therapy; Animal experiment; Clinical trial; Cellular therapy

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Core Tip: In this review, we elaborate on the characteristics of neural stem cells (NSCs) and their effects on the treatment of traumatic brain injury, hypoxic-ischemic brain injury, Alzheimer's disease and Parkinson's disease. At the same time, we discuss the

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INTRODUCTION

Brain diseases are among the major disorders influencing human health. The main types of brain diseases include brain tumors, neurodegenerative disorders, cerebrovascular diseases, and traumatic brain injury (TBI). Previous studies have suggested that repair and regeneration is a complex process and is challenging due to the following reasons: (1) Nerve cells, including neurons, are highly differentiated terminal cells, with very low regenerative capability; (2) Insufficient secretion of neurotrophic factors is unable to sustain the homeostasis of local environment results in the failure to repair damaged nerve system; and (3) Following injury, the secretion of inflammatory factors and various cytokines is upregulated, which inhibits synaptic regeneration and aggravates hypoxia and ischemia. The major cause of nerve regeneration disorders is the scar formation at the injuries, which may act as a physical and chemical barrier, suppress nerve regeneration, and dysregulate the extension and growth of synapses. Therefore, various physiological processes, including the supply of neurotrophic factors, regeneration of axons, plasticity of synapses, and the microenvironment, are involved in the repair and regeneration of the central nervous system (CNS) after injuries, and the underlying mechanisms are very complex.

Cellular therapy uses neurogenic or non-neurogenic cells to replace, repair, or improve the functions of the injured nerve system, which are implemented mainly through transplantation of cells into the system. Stem cell transplantation therapy has been widely applied in treating CNS diseases because of its ability of regeneration in nerve repair and tissue damage. The mechanisms underlying the treatment of brain diseases with stem cell transplantation are similar: facilitating the local microenvironment, promoting blood vessel development, supporting neuron regeneration, and reducing inflammatory responses. The commonly used stem cells include neural stem cells (NSCs), mesenchymal stem cells (MSCs), adipose mesenchymal stem cells, and human-derived umbilical cord blood stem cells, among which NSCs have been widely used and has unique advantages in the treatment of brain disease.

In this review, we discuss the role and generation of NSCs for various neurodegenerative disorders. Recent studies using different types of NSCs and transplantation approaches have been discussed in detail, and the limitations of NSCs for neurodegenerative disorders are also discussed.

BASIC CHARACTERISTICS OF NSCS

During development, the brain and spinal cord are generated from a small number of NSCs lining the neural tube. These cells are undifferentiated cells and can differentiate into different cells[1]. The subgranular zone (SGZ) of the dentate gyrus (DG) and subventricular zone (SVZ) in adult brains are two neurogenic regions for neurogenesis [2]. The neurogenic regions, especially the hippocampus, participate in cell renewal by developing new neurons from the neural progenitor cells[3]. Several sources can be used for NSCs. They can be collected from brain tissue, reprogrammed from somatic cells[4,5], or differentiated from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)[6,7]. In addition, NSCs can differentiate into lineage-specific cells, such as neurons, oligodendrocytes and astrocytes[8]. They exist in highly-specific microenvironments, consisting of cell and extracellular components, such as ependymal cells, vasculature, extracellular matrix proteins, soluble factors, astrocytes, microglia, and pericytes[9,10]. Interaction of cells, transcription factors, neurotrophins, cytokines (such as growth factors, neurotransmitters, hormones and signaling molecules) have a crucial role in the proliferation and differentiation of NSCs. Cytokines (TNF-α) has been shown to induce proliferation of neural stem cells *via*

IKK/NF-κB signaling. While BMP4/LIF has been shown to induce neuronal stem cells in monkeys, it was shown to induce astrocyte-like differentiation of monkey NSCs[11-14]. Neural stem cells are involved in various biological functions and continue to play their role throughout the lifespan of an organism. Both intra and extracellular signals regulate the functional properties of NSCs. Sox2 is one of the major regulators among transcription factors that serve as molecular switches[15]. The association of NSCs and migration in blood vessels were recently studied and shown that blood vessels play a significant role in neuronal migration during brain development. Moreover, NSCs can migrate to designated regions, such as injured regions, following injury[16].

Preclinical studies on treating brain diseases with NSCs have reported promising results, while clinical trials in patients are still ongoing. Nevertheless, experiments on animal models or *in vitro* studies have shown that NSCs may be induced and activated to differentiate into neurons, consequently replacing the lost neurons, improving the local microenvironment, promoting blood vessel development, regulating inflammatory responses, and restored homeostasis of the brain.

NSCS AND ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a progressive multifactorial brain disorder characterized by the amyloid-β (Aβ) deposition, as insoluble deposits or inclusions of proteins, accumulations of neurofibrillary tangles, and intracellular tau aggregation. It is the most common cause of dementia that slowly destroys memory and thinking skills. More than 26 million people are living with AD worldwide, and this number is expected to increase to 100 million over the next 35 years[17,18].

Targeting Aβ levels has been the central strategy to halt, retard, and reverse or cure AD pathology progression. Though great efforts have been made to cure AD symptoms and delay its progression, limited treatment options are available. Only four cholinesterase inhibitors (tacrine, donepezil, galantamine and rivastigmine are rarely prescribed due to its possible side effects) and NMDAR antagonists (memantine) have only been approved by United States Food and Drug Administration for AD. There is not a single drug approved in the last two decades. The available drugs (cholinesterase inhibitors), can only reduce the acetylcholinesterase activity to prevent the buildup of acetylcholine levels synaptic region. However, neither drug design to reverse the AD pathology nor immunotherapy that targets amyloid or Tau is the ultimate solution for Alzheimer's. Several lines of evidence have shown the successful approach of neural stem cells for the treatment of neurodegenerative disorders, including AD, amyotrophic lateral sclerosis and PD[19].

This approach of NSCs transplantation offers a tremendous therapeutic potential to cure neurodegenerative disorders based on its self-renewal ability and differentiate into neuronal, oligodendrocytes and astrocytes cells[20]. Tg2576 neural stem cells isolated from mice represent an Alzheimer disease model related to Aβ plaque. Tg2576 derived cells showed a disease model with reduced neuronal growth and MAP-2 expression. This model has been studied in various studies and offers to screen new molecules for the treatment of AD[21].

Ager *et al*[22] used NSCs derived from the fetal brain tissue and transplanted to the hippocampus of 3xTg-AD murine models and found that the transplanted NSCs improve the cognitive functions and enhanced synaptogenesis. The human neural stem cell population, HuCNS-SC, has been clinically tested before for different neurodegenerative disorders. Transplantation of HuCNS-SCs has been shown to improve cognition in two different models of neurodegeneration. Migration and differentiation of HuCNS-SC into immature neurons and glial cells were observed. Researchers have found the association of significant synaptic increase and other growth-associated markers were found in both 3xTg-AD and CaM/Tet-DTA mice models.

The hippocampus, which is critical for learning new memories, is normally affected at earlier stages of AD. Disruption of metabolic activities in hippocampal neurons has been demonstrated in earlier studies in AD[23]. The following diagram shows the different mechanisms of stem cells associated with AD (Figure 1).

A study conducted by Li *et al*[24], 2016 showed that metabolic activity was increased in the frontal cortex and hippocampal neurons. The human brain-derived NSCs (hNSCs) were transplanted into the hippocampus transgenic mouse model of AD to assess the role of hNSC on behavior and Alzheimer's pathology. Six weeks later, transplanted hNSCs migrated in different brain regions and slowly differentiated into neuronal cell types of CNS. These transplanted cells rescue AD symptoms, including

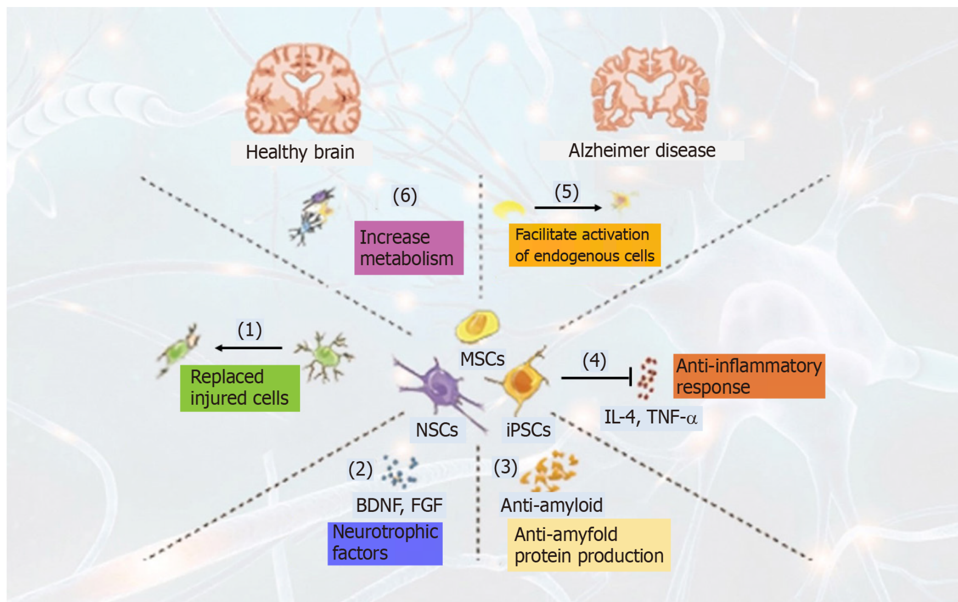

Figure 1 Mechanism of action of stem cells in Alzheimer disease. (1) Replaced injured or lost cells; (2) Enhanced secretion of neurotrophic factors (BDNF, GDNF, FGF, *etc*.); (3) Anti-amyloid protein production; (4) Inhibit inflammatory response; (5) Facilitate activation of endogenous cells; and (6) Enhanced metabolic activity of neuronal cells in the brain.

cognitive defects, learning and memory impairment, by increasing neuronal connectivity and metabolic activity. This study suggests the role of hNSCs in modulating the metabolism of neuronal cells and validates the association between hippocampal neuronal metabolism and AD symptoms[24].

Chronic inflammation has a significant role and contributes to AD pathology in the brain. Transplantation of NSCs has been assessed to inhibit inflammatory processes. Researchers have shown that NSCs transplantation into the hippocampus attenuates inflammatory reactions and supports a neuroprotective role in beta-amyloid 42 (Aβ -42) peptide injected rat hippocampus, suggests an important role of NSCs in the inhibition of inflammatory reactions[25]. Neural stem cells are making a dominant appearance because of it neurogenic abilities, based on the recent findings that neurogenesis reduces significantly in AD patients compared to healthy subjects[26]. Progress is currently being made to differentiate the transplanted NSCs into cholinergic neurons, to compensate for the loss of injured neurons, the main research focuses on the treatment of AD.

A summary of preclinical studies of stem cells of different sources in rat and mice models of AD was showed in Table 1[27-37]. Ibotenic acid lesion or APP/PS1 transgenic mice were used in most of the AD model. Stem cells of different origin were used, which include rat, mouse and also from human. Genetically modified stem cells are also used in some studies, which have increased capacity to migrate from transplantation sites. Damage neuron replaced by transplanted stem cells. Stem cells migrate to the lesion site and differentiate to specific neurons *e.g.*, cholinergic neuron, clear beta-amyloid, and produced anti-inflammatory effects. These studies showed that transplantation of stem cells (ECS-derived, NSCs, and MSCs) improved or restored learning and memory in AD-model rats.

NSCS AND PARKINSON'S DISEASE

Parkinson's disease (PD) is a complex neurodegenerative disease that result from the loss of dopaminergic neurons in the substantia nigra, pars compacta (SNc) and mesencephalon, and the formation of α-synuclein-containing Lewy bodies, which consequently induce motor disorders[38]. The stem cell approach offers a significant therapeutic output to a wide range of neurodegenerative disorders including PD, because of the regenerative potential to renew the cells and replace the affected cells. Several studies have reported using neural stem cell approach to find a cure and explore the disease mechanism.

Table 1 Therapeutic potential of stem cell transplantation in Alzheimer's disease models

	Animal model	Transplanted cells	Density of transplanted cells	Transplantation site	Therapeutic effects	Unique features	Results	Ref.
1	Mice (Transgenic 3 x Tg- AD and Thy1-APP)	NSCs	100000 cells in 2 µL	Hippocampus	Aβ-clearance, increased synaptic density	Neprilysin gene transfer	Not assessed	Blurton-Jones *et al* [37]
2	Mouse (NBM lesion)	ESC-derived neurosphere	400 µL/injection, 1-5 × 10^4 cells/µL	Prefrontal and parietal cortices	ChAT and serotonin-positive neurons	ChAT + cells↑	Working memory ↑	Wang *et al* [27]
3	Rat (Forebrain), Okadaic acid	NSC (rat)	5 µL /injection site (2 injections) 2 × 10^4 cells/mL	Hippocampus and cerebral cortex	replace damaged or lost neuron	NGF(human), gene transfer	Memory ↑	Wu *et al*[28]
4	Mice (Transgenic Tg2576)	MSCs from human UCB	100000 cells/ Mouse (i.v.)	Systematic	Anti-inflammatory, anti-amyloidogenic	None	Not assessed	Nikolic *et al* [29]
5	Rat (NBM lesion) Ibotenic acid	ESC-derived NPC (mouse)	2 × 10^5 cells in 2 µL	Forebrain specially NBM	Forming cholinergic cell phenotype	Shh-primed	Water maze ↑; Spatial probe↑	Moghadam *et al*[30]
6	Mouse (3X TG-AD)	NSC (mouse)	100000 murine NSCs	Hippocampus	Neurotropic effects	BDNF-mediated effect	Working memory↑	Blurton-Jones *et al* [31]
7	Rat (Hippocampus) Kainic acid	Immortalized NSC (human, HB1.F3)	1 × 10^6 cells/rat	Hippocampal CA3 region	Migrate to injured site differentiate into neurons overexpressing ChAT	ChAT (human), gene transfer	Water maze ↑; Spatial probe↑	Park *et al*., 2012a[33]
8	Rat (NBM lesion) AF64A toxin	Immortalized NSC (human, HB1.F3)	1 × 10^6 cells/rat	ICV	migrate to various brain regions including cerebral cortex and hippocampus	ChAT (human) gene transfer	Water maze ↑; Spatial probe↑	Park *et al*[32]
9	Mice (Transgenic APP/PS1)	MSCs from human UCB	1 × 10^5 cells in 3 µL(3 injection once after 2 wk)	Hippocampus	Anti-inflammatory, anti-amyloidogenic, anti-phosphorylation of tau	None	Improved learning and memory	Lee *et al*[34]
10	Mouse (Hippocampus) Ibotenic acid	Immortalized NSC (human, HB1.F3)	2 × 10^5 cell suspension 2 µL	Hippocampus	migrated to lesion sites and differentiated into neurons and astrocytes	NGF (human); Gene transfer	Water maze ↑; Spatial probe↑	Lee *et al*[35]
11	Mice (Transgenic APP/PS1)	MSCs from human UCB	2 × 10^4 cells per head	Hippocampus, cortical region	Anti-inflammatory, Aβ-clearance	-	Not assessed	Kim *et al*[36]

NBM: Nucleus basalis of Meynert; ESC: Embryonic stem cell; NGF: Nerve growth factor; 3XTG: Triple transgenic/APP-presenilin-tau; BDNF: Brain-derived growth factor; ChAT: Choline acetyltransferase; NPC: Neural precursor cell; NSC: Neural stem cell; SHH: Sonic hedgehog protein; UCB: Umbilical cord blood; Aβ: Beta-amyloid; MSCs: Mesenchymal stem cells; APP: Amyloid-β precursor protein; ICV: Intra-cerebro ventricular.

Induced neural stem cells (iNSCs) exhibited different stem cell biomarkers with self-renewal properties and has shown the potential to differentiate into dopaminergic (DA) neurons. Researchers have shown the role of grafted cells for the neuronal network by assessing synaptic markers. Analysis of 4 wk of post-transplantation showed an extensive network of presynaptic neurons. hESC-derived neural cells has been reported to reduce the tumorigenicity and function of DA neurons in a prolonged mature culture. The transfer of such grafts in monkeys improved behavior for 12 mo period, reflecting the significance of matured hESCs that can act as a source for DA neurons[39].

Studies have shown that transplantation of iNSCs transformed from somatic cells into PD mice brains improves motor manifestation behavior. Wernig *et al*[40] shown that iPS cells efficiently differentiate into neural precursor cells, further giving rise to neuronal and glial cells. Transplantation of iNSCs into the brain of fetal mice shows the potential of stem cell migration into different brain regions and its differentiation into glia and neurons, including glutamatergic, catecholamines and GABaergic subtypes. Moreover, induced iPS cells were differentiated into DA neurons after transplantation into the adult brain.

Researchers have shown that steroli cells can be directly converted into iPS cells, which exhibit different stem cell biomarkers with self-renewal properties and can differentiate into DA neurons. These grafted cells were validated for a matured neuronal network by assessing synaptic markers. Analysis of 4 wk of post-transplantation showed an extensive network of presynaptic neurons, suggest a crucial role of steroli based iNSCs may provide a source of replacement of affected cells with new fresh cells[41]. iNSCs derived from fibroblasts have been shown to improve PD symptoms. Transplantation of iNSC into the 6-hydroxydopamine (6-OHDA)-injected mice striatum shows substantial reduction in apomorphine mediated rotational symmetry. The engrafted iNSCs show the differentiation pattern to all neuronal lineages and differentiate to DA neurons[42].

Yang *et al*[43] shows that neural stem cells transplantation into a 6-hydroxydo-pamine-lesioned rat, migrate to the striatum and express dopaminergic traits. Studies demonstrated the role of single factors, (Platelet-derived growth factor (DGF-AA), -AB, and –BB) which plays a role in the differentiation of primary stem cells derived from fetal and adult CNS, differentiate C17.2 cells *in vitro*, suggesting its significance that C17.2 NSCs lead to the development of dopaminergic neurons and a source for transplantation[44].

Nurr1 is a transcription factor and is specifically required to induce DA neurons in the midbrain region[45,46]. However, later in another separate study, Wagner *et al*[47] used the same stem cell line C17.2 and demonstrated that Nurr1 alone was unable to induce the differentiation of C17.2 cells into dopaminergic neurons. While, in a combination of other factors derived from local type 1 astrocytes, overexpression of Nurr1 in NSCs (C17.2) generates dopaminergic neurons (Figure 2).

A summary of preclinical studies of stem cells of different sources in rat, mice and monkey models of PD was showed in Table 2[42,48-54]. OHDA (rats and mice) and MPTP (monkey) drugs were used to create Parkinson’s model in these studies. Transplantation cells of different origin were used, which include rat, monkeys and from human. Genetically modified stem cells are also used in some studies, which had unique features. Results of these studies showed that transplantation of stem cells in different cell densities (ECS-derived, NSCs and MSCs) in striatum decreased rotation and improved motor function in PD model.

NSCS AND TBI

Traumatic brain injury (TBI) refers to a disruption of normal function of the brain and/or pathological injuries of brain tissues caused by external forces instead of disorders of brain tissues. TBI has a complex pathological condition, which includes breakage of the blood-brain barrier, massive neuroinflammation, axonal injury and lesions[55]. It has been estimated that about 50-60 million patients globally are newly diagnosed with TBI every year. In developing countries, TBI is mainly caused by traffic accidents, while in developed countries, by the falling of the elderly[56]. Based on the population census in 2013, TBI mortality rates in China were 13/100000, while in the 27 United States, TBI accounted for 30% of all trauma-induced deaths. In the United States, about 5.3 million individuals are living with TBI-related disabilities[57,58].

Despite having the higher frequency of TBI, a large proportion of molecular mechanisms and the basis of cognitive deficits and brain insults remain unknown.

Over the recent years, studies have demonstrated that neurogenesis in SVZ and SGZ was enhanced after TBI[59]. Endogenous NSCs get activated and migrate to regions of nerve injuries, which differentiate into neuroglial cells or oligodendrocytes and integrate into the injured local neurovascular network, promote the secretion of neurotrophic factors, and participate in nerve repair. Therefore, activating endogenous neurogenesis following TBI to contribute to post-injury functions may be a potential therapeutic approach[60,61]. On one hand, neurogenesis and nerve migration in human beings mainly exist in neonates younger than 18 mo but drastically decrease in adults, suggesting that neurogenesis following TBI in middle-aged and elderly people is substantially lower than in adolescents. While, glial scars have been reported to prevent the regeneration of axons and directly limit the repair of injuries in the late stage of TBI[62,63]. In addition, massive cell death and inflammatory responses in the late stage of TBI may disturb the local microenvironment, reduce the survival rate of new endogenous NSCs, and limit injury repair.

Transplantation of pre-differentiated human endogenous neural stem cells (ENSCs) has been reported to increase angiogenesis and neuronal survival in the lesion area

Table 2 Therapeutic potentials of stem cell transplantation in Parkinson's disease models

	Disease model	Source of transplanted cells	Transplantation location	Density of transplanted cells	Unique feature or treatment	Results	Ref.
1	Rat, 6-OHDA	Immortalized NSC (mouse, C17-2)	Striatum	10^6 cells	TH/GTPCH1; Gene transfer	Rotation↓	Ryu *et al*[48]
2	Monkey, MPTP	ESC (monkey)	Bilateral putamen	3 x 10^5-6 x 10^5 cells per side	Stromal cell (mouse) feeder	PFS-parkinsonian factor score↓	Takagi *et al* [49]
3	Rat, 6-OHDA	Immortalized NSC (human, HB1.F3)	Striatum	3 × 10^5 cells/3 μL	TH/GTPCH1 gene transfer	Rotation↓	Kim *et al*[50]
4	Rat, 6-OHDA	Immortalized NSC (human, HB1.F3)	Striatum	2 x 10^5/3μL	NSC migration	Rotation↓	Yasuhara *et al* [51]
5	Rat, 6-OHDA	MSCs from human UCB	Striatum	1 × 10^5 cells/10 μL	FGF8/SHH	Rotation↓	Fu *et al*[52]
6	Rat, 6-OHDA	DA neurons from ESC (human)	Striatum	5 x 10^5 cells	None	Rotation↓, beam walking↓	Cho *et al*[53]
7	Mice, 6-OHDA	DA neurons from ESC (human)	Striatum	1.5 × 10^5 cells /1.5 μL	Wnt signal; SHH	Rotation↓	Kriks *et al*[54]
8	Mice, 6-OHDA	iNSCs (rat)	Striatum	1 × 10^5 cells	Tripotential differentiation capacity	Rotation↓	Choi *et al*[42]

6-OHDA: 6-hydroxydopamine; MSC: Mesenchymal stem cell; ESC: Embryonic stem cell; FGF8: Fibroblast growth factor 8; GTPCH-1: GTP cyclohydrolyrase-1; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; iNSC: Induced neural stem cell; TH: Tyrosine hydrpxylase; NTN: Neurturin; UC: Umbilical cord blood; SHH: Sonic hedgehog protein; CN: Caudate nucleus; SN: Substantia nigra.

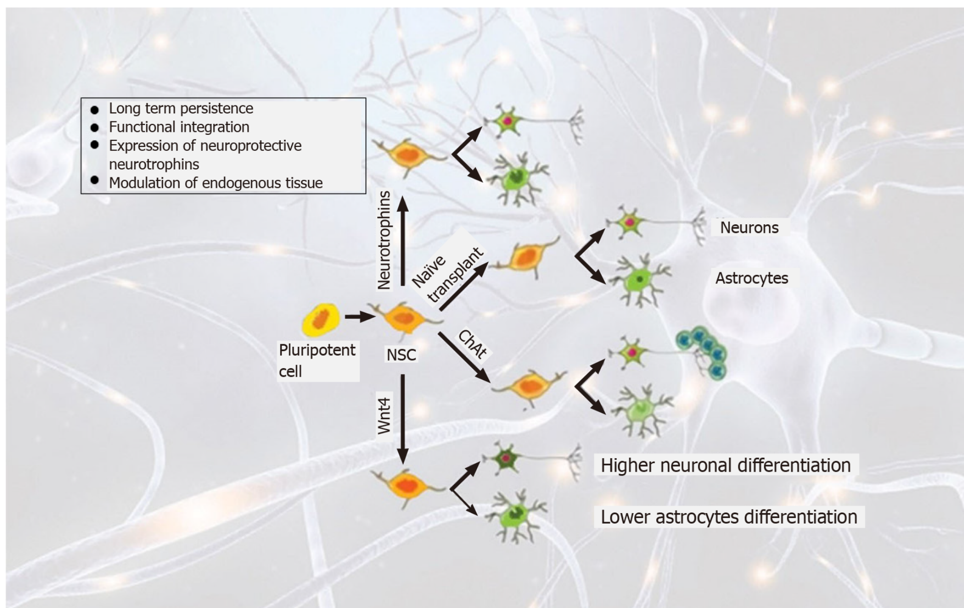

Figure 2 Overview of lineages of stem cells and transplantation strategies in Parkinson diseases. Pluripotent stem cells are directly converted to stem cells that can be further differentiated to long-term survival neurons by overexpressing neurotrophins. Wnt4 overexpression drives differentiation into neuronal cells while reducing glial scar formation.

and decrease astrogliosis, resulting in improved motor functions[64,65]. Moreover, researchers have shown that immediate transplantation of embryonic cortical neurons in the adult cortex after injury facilitates the restoration of injured motor pathways and supports the development of neuronal projections[66,67] (Figure 3).

Exogenous NSC transplantation can compensate for the disadvantage of insufficient endogenous NSCs to a certain degree and has a significant impact on the treatment of TBI[68,69]. Experiments in mice and rats have been demonstrated that, upon NSCs transplantation, the transplanted stem cells survive in affected regions and differentiate into mature astrocytes, oligodendrocytes, and neurons, which can then be

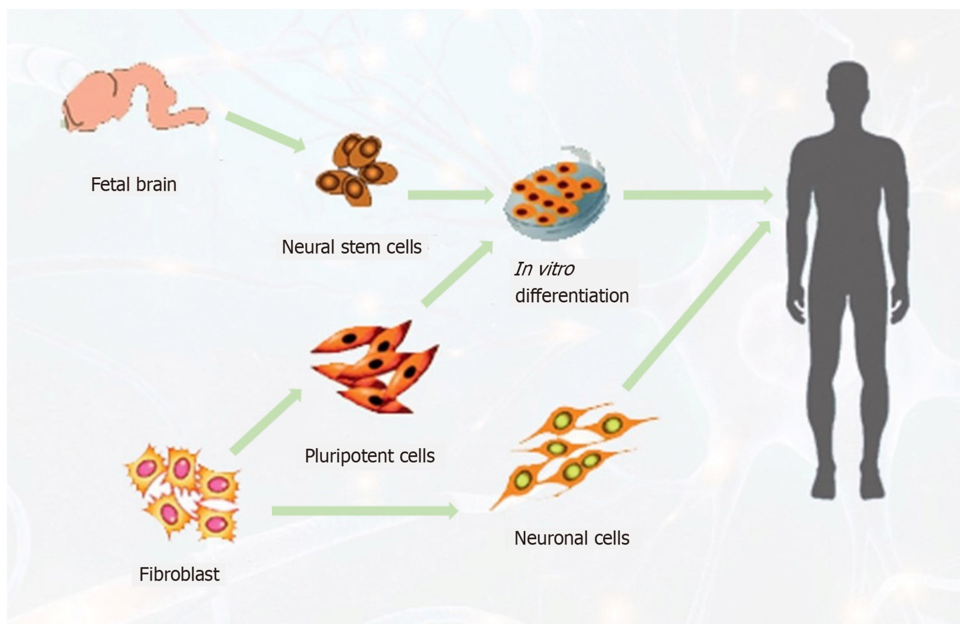

Figure 3 Schematic diagram of possible sources of neural stem cells to target stroke patients. (1) Neural stem cells from the fetal brain, differentiated to neuronal cells; (2) Neuronal cells directly generated from fibroblast cells, expanded to neuronal cells to replace the lost cells.

integrated into the neural circuit of the host to improve the injury-related cognitive and motor disorders[70,71]. When transferring human fetal NSCs to the hippocampus of TBI rats at 24 h post-injury, the transplanted cells survived. In addition, treating *in vitro* cultured NSCs with basic fibroblast growth factor, heparin, and laminin promote its differentiation into neurons at the injured area and the expression and secretion of glial-cell-line-derived neurotrophic factor *in vivo* from the transplanted cells, thus improving the internal environment of the brain, promoting the endogenous repair, and finally improving the cognitive functions of TBI rats[72]. The approach of cell therapy by transplanting ENSCs reduces neuroinflammation and supports neurogenesis in the adult injured cortex of the controlled cortical impact mouse model [69].

NSCS AND HYPOXIC ISCHEMIC BRAIN INJURY

Cerebrovascular disease is a global health issue, where the incidence and mortality rate of ischemic stroke are high levels. Thrombolytic therapy is considered the best treatment procedure for ischemic stroke[73,74]. Though it is not safe and tissue damage is usually inevitable. It is a complex process, which involves oxidative damage and apoptosis of neurons[73,75].

The sub-ventricular zone and dentate gyrus are the primary sites of endogenous NSCs. Exogenous NSCs are mainly extracted from three main sources for therapeutic purposes: extraction from brain tissue, differentiation from IPSc, and trans-differentiation from somatic cells[76]. Studies have been reported the methods of generating different types of NSCs and its applications in neurodegenerative diseases[76,77]. The SVZ NSCs have been shown its association with glioma progression and its occurrence. Effect of conditioned medium derived from NSCs has confirmed its association with SVZ NSCs, and found that conditioned medium from NSCs promote the glioma proliferation and invasion[78]. Earlier studies reported the characteristics of exogenous NSCs that it can migrate into ischemic brain regions, and differentiate into neurons and glial cells and facilitate endogenous NSCs differentiation and proliferation[79-81]. Transplantation of human NSCs in a stroke model of rats showed neuroprotective effects by enhancing dendrites branching, increasing corticospinal tract projections and inhibited inflammation[82,83]. It has been demonstrated that NSCs improved the condition of stroke rats when transplanted, suggesting a role of NSCs mediated regulation of angiogenesis and formation of brain microvasculature because of increased activity of proangiogenic factors[84].

Researchers conducted a small Phase 1 translational study and demonstrate the role of CTCoE3 human NSCs in stroke patients. Upon implantation of human NSCs into

the putamen, they found patients safe even for 2 years after transplantation and no side effects showed. However, a slight improvement showed in the NIH stroke scale [85]. The use of primary human tissue is limited because of the ethical and logistic complications to obtain large quantities of fetal neurons. Therefore, much effort is required to develop alternate sources of human cells for use in transplantation. One source is the NT2/D1 human embryonic carcinoma-derived cell line. These cells can proliferate and differentiate into human neuronal cells (LBS-Neurons) upon treatment with retinoic acid. These neuronal cells have been reported to survive, express neurotransmitters and regulate functional synapses.

Despite its significant role of NSCs in treating most neurodegenerative diseases, there are still some limitations. Modulation of cell dose is a critical factor, as low dose cannot provide therapeutic outcomes. While transplantation of high cell dose of tissue-derived NSCs can clot *in vivo* and may have a poor survival rate[2]. Furthermore, understanding molecular mechanisms of endogenous NSCs regulation largely remain unknown in patients with ischemic brain injury[86].

Due to the effectiveness of NSCs in animal models of cerebral stroke, clinical trials using NSCs have been conducted for the treatment of chronic ischemic cerebral stroke [87]. Although over 50 clinical trials have been registered for the treatment of cerebral stroke by stem cells, only human neural precursor cell line NT2/D1 and immortal human NSC line CTX have progressed to stage 1 and stage 2 phases. NT2/D1 cell, also known as NT2 cell, is a human teratoma-derived pluripotent embryonic carcinoma stem cell line, considered a neural precursor cell line. Treating NT2/D1 cells with tretinoin induces mitosis of anaphase neuron-like cell NT2N neuron (trade name: LBS-Neurons). A phase 1 clinical trial investigated the effects of NT2N neurons in basal ganglia stroke patients with severe motor disturbance. The 18-mo serum or imaging evaluations confirmed the safety and applicability of brain neuron transplantation in cerebral infarction patients with motor disturbance[88-90].

CTX0E03 is an immortalized human NSC line derived from human embryo brain tissues. CTX0E03 has been used as a clinical-grade NSC, based on which the commercial product CTX-DP was developed to treat chronic cerebral stroke (the ReNeuron PISCES trial)[90]. The 5-year follow up findings of phase 1 clinical trial of CTX0E03 in chronic cerebral stroke patients (PISCES I, NCT01151124) showed the following results: no immune or cell-related adverse events occurred, and only adverse influences from surgical procedures or complications were found; the overall NIHSS score improved by 2 points at 2 years after transplantation, which was associated with the improvement of neurological functions[85]. In another phase 2 clinical trial of CTX0E03 (PISCES II, NCT02117635), the 12-mo follow-up showed no cell-related safety events, while clinical related function improvement was found in 15 patients. CTX0E03 PISCESIII (NCT03629275), has already been approved, is a randomized, controlled, phase 2b clinical trial that aims to evaluate the safety and effectiveness of CTX cells in patients with chronic cerebral stroke (Figure 4).

CONCLUSION

The stem cells approach offers a significant output to a wide range of disorders, including neurodegenerative disorders, because of the regenerative potential to renew the cells and replace the affected cells. Neural stem cells are making a dominant appearance because of it neurogenic abilities, that neurogenesis reduces significantly in neurodegenerative patients compared to healthy subjects. Although studies on brain diseases with NSCs-based therapy are continuously increasing, and the NSC treatment strategy has provided an exciting and promising treatment method for brain diseases, there are still various uncertainties and potential risks involved in NSC transplantation, similar to the treatments with other stem cells: (1) Modulation of cell dose is a critical factor, as low dose is unable to provide the therapeutic outcomes. While transplantation of high cell dose of tissue derived NSCs can clot *in vivo*, and may have a poor survival rate; (2) Furthermore, understanding molecular mechanisms of endogenous NSCs regulation largely remain unknown in patients with neurodegenerative disorders; (3) Transplantation approaches can be improved by region specific regulation of local microenvironment in the brain: precise regulation of the microenvironment through genetic engineering techniques and combination transplantation may promote the proliferation and differentiation of transplanted NSCs, and greatly increase the treatment efficacy; and (4) Methods, timing, and doses of transplantation: strategies should be made to improve the transplantation methods to favor the aggregation of NSCs to the injured regions.

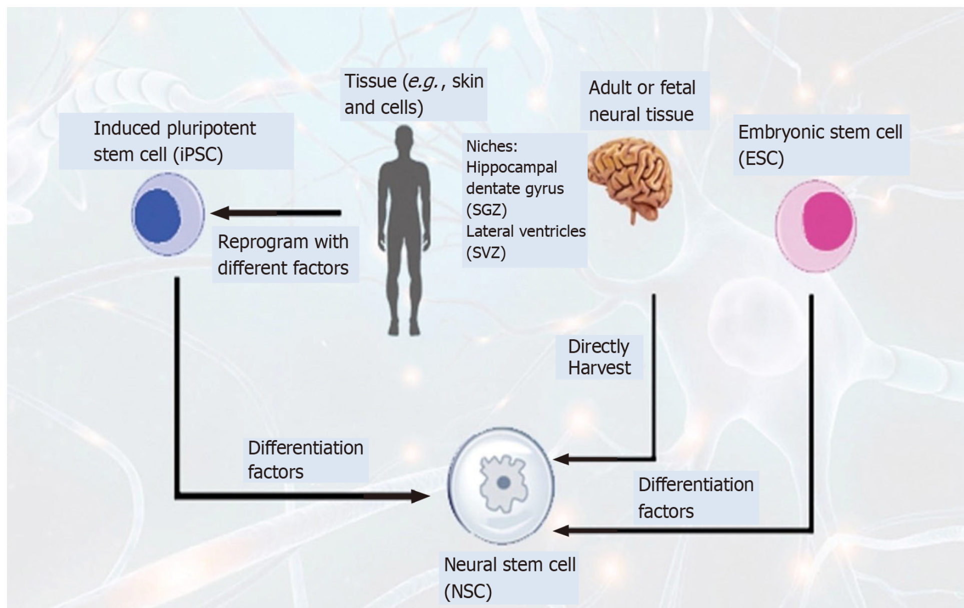

Figure 4 Schematic diagram of generation of neural stem cells *via* different methods to treat neurodegenerative disorders. Neural stem cells (NSCs) can be generated by extracting directly from the subgranular zone of the hippocampal dentate gyrus and subventricular zone of the lateral ventricles from fetal or adult brain. NSCs isolation from patients can be reprogrammed by using different factors such as transcription factors, small molecules, microRNAs, and other morphogens. NSCs can also be generated from blastocyst-derived embryonic stem cells by using differentiation factors. SGZ: Subgranular zone; SVZ: Subventricular zone.

However, based on the shortcomings of various *in vitro* and *in vivo* neurodegenerative disease models, the translational effects of NSCs into human patients remains unknown. Thus, a more definite role of NSCs in various transplantation settings further needs to be explored. Many studies provided the evidence of the association of cognitive improvement with increase in synaptic activity, which is closely correlated with increase in neuronal and glial cells. NSCs transplantations supports behavioral and cognitive functions. Although specific cell types that associate with improvements, that NSCs need to differentiate into, remains unknown. The selection of the best time window for stem cell treatment is closely associated with the clinical prognosis of patients; however, thus far, no studies have reported the best treatment time window. The differentiation potential of NSCs derived from different sources may also vary, and how to determine the doses of transplanted cells is, therefore, an important issue for future research studies. There are still great challenges in preventing immunological rejection responses, improving the survival rate of transplanted NSCs, and consequently obtaining activated young stem cells with a clinically effective grade.

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MINIREVIEWS

Empty nose syndrome pathogenesis and cell-based biotechnology products as a new option for treatment

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Abstract

Empty nose syndrome (ENS) is a rare complication that develops after partial or complete turbinectomy. The main feature of ENS is paradoxical nasal obstruction feeling despite objectively wide nasal airway. ENS pathogenesis is multifactorial and includes changes in laminar physiological airflow, disruption of mucosa functions and deficient neural sensation. This leads to the development of ENS symptomatology such as dyspnea, nasal dryness, nasal burning, nasal obstruction, feeling of suffocation and even comorbid psychiatric disorders that significantly impairs life quality. Specific effective treatment of ENS does not exist up to date. In this review we outline existing biomaterial for surgical reconstitution of nasal anatomy and discuss the perspective of stem cell-based technologies in ENS management. The main focus is directed to justification of rationality application of adult mesenchymal stem cells (MSCs) from different tissues origin and neural crest-derived stem cells (NCSCs) based on their intrinsic biological properties. MSCs transplantation may stimulate mucosa tissue regeneration *via* trophic factors secretion, direct transdifferentiation into epithelial cells and pronounced immunosuppressive effect. From the other hand, NCSCs based on their high neuroprotective properties may reconstitute nerve structure and functioning leading to normal sensation in ENS patients. We postulate that application of cell-based and tissue-engineered products can help to significantly improve ENS symptomatology only as complex approach aimed at reconstitution of nasal anatomy, recovery the nasal mucosa functionality and neural tissue sensation.

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Core Tip: Empty nose syndrome is a complex disease, which pathogenesis is associated not only with changes in the architecture of the nasal passages, but also with changes in the receptor status of the nasal mucosa and disorders of perception of external stimuli at the systemic level. Due to the lack of effective convencial protocols for the treatment of this disease, there is a need to find new approaches to restoring the normal structure of the architecture of the nasal passages and return the functionality of the nasal mucosa. In addition, the proposed methods should be minimally invasive, implemented by injection. The advancement of regenerative medicine and biotechnology contributes to the development of new cell-based products in combination with various materials, which in the future will be able to help develop protocols for treatment patients with empty nose syndrome.

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INTRODUCTION

For the first time, the term "empty nose syndrome" (ENS) was introduced into medical practice by E. Kern and M. Stenkvist in 1994 to describe empty space in the place of inferior and middle turbinate on computed tomography in patients who had partial or total turbinectomy[1]. Today, ENS can be defined as a rare rhinological disease developed as complication of turbinate surgery and characterized by paradoxical obstruction feeling despite an objectively broad nasal airway[2]. ENS symptoms development is usually delayed, from months to years postoperatively, and the symptoms severity is not strictly dependent on the resected tissue volume[3]. ENS frequency is not known exactly, several studies reported about 8%-22% ENS occurrence rate in patients who have undergone turbinate resection[4]. Submucosal cautery, submucosal resection, laser therapy, and cryosurgery can also lead to ENS development if performed in an overly aggressive manner[5]. Existing methods of ENS diagnostics and treatment have some drawbacks as ENS pathogenesis is not fully understood. In this review we focus on the latest concept of ENS pathogenesis and discuss cell-based technologies as new therapeutic option for ENS management.

ETIOLOGY AND PATHOGENESIS

ENS is divided into at least four subtypes depending on the turbinate resected: ENS inferior turbinate (ENS-IT), ENS middle turbinate (ENS-MT), and ENS-both, which means both the inferior and middle turbinates removal; and ENS after turbinate-sparing procedures (ENS-type)[6]. ENS-IT is the most common type[7]. Patients with ENS frequently report symptoms of dyspnea, nasal dryness, nasal burning, nasal obstruction, and feelings of suffocation. Other symptoms may be sensation of excessive airflow; lack of sensation of nasal airflow; hypersensitivity to cold air; dyspnea (also paradoxical), breathlessness, hyperventilation; nasal pain of variable, sometimes pseudoneuralgic, types; headache; nasal and pharyngeal dryness; difficulty falling asleep, general fatigue (Figure 1)[8]. Anxiety, depression, loss of productivity are main psychological symptoms of ENS[9]. Depression was reported in 71 % of ENS patients, but there was no correlation between nasal cavity volume and depression severity[10]. ENS diagnosis is mainly based on the patient's subjective symptoms since no reliable objective tests exist. This makes ENS diagnostics and treatment effectivity

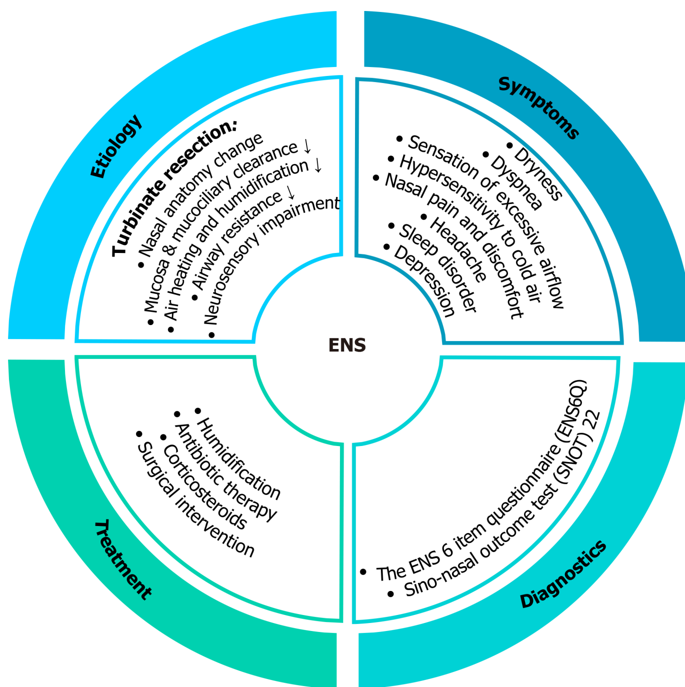

Figure 1 Empty nose syndrome: Causes, symptoms, diagnostics and current treatment strategy. ENS: Empty nose syndrome.

evaluation very challenging.

The nasal patency depends on adequate mucosa cooling and appropriate number of functioning TRPM8 (transient receptor potential cation channel subfamily M member 8) thermoreceptors[11]. In case of ENS, turbinate resection leads to reducing general nasal surface area causing changes in laminar airflow characteristics. The mucosa reduction leads to decreasing in number of TRPM8 receptors and violation of inspired air heating and humidification due to increased nasal cavity, reduced contact between air and mucosa, and the lowered nasal airway resistance[12]. Complete removal of the inferior turbinate can reduce heating and humidity by 23%[2]. This leads to increased mucosa heating as a compensatory mechanism. Consequently, effective mucosal cooling does not occur and TRPM8 is not activated causing restricted signaling to respiratory center which is misinterpreted as obstruction or apnea, and, in turn, leads to increased breathing efforts. It is important to note that resection of inferior turbinate is associated with higher risk of ENS development than middle turbinate removal. Thus it is recommended to preserve at least 50% of inferior turbinate during any surgical procedure that allows to save sufficient mucosa and keep moderate nasal volume essential for normal airflow and in this way minimize ENS occurrence[13].

Reduction of mucosal tissue after surgery causes significant compensatory structure changes that result in squamous metaplasia, a higher rate of submucosal fibrosis, goblet cell metaplasia and lower number of submucosal glands in ENS patients. However, in some cases, normal respiratory epithelium similar to atrophic rhinitis and absence of inflammation can be preserved[14].

Taking together, ENS pathogenesis is multifactorial including changes in anatomical turbinate structure, disruption of normal nasal mucosa function and neural sensation deficiency.

CURRENT MANAGEMENT STRATEGY

Taking into consideration the polygenic pathophysiology of ENS, the effective treatment strategy should be complex and overcome the three main challenges: (1) Reconstitute the nasal anatomical structure that will allow the nasal airway resistance increasing; (2) Recover damaged nasal mucosa that will result in edema decreasing, normalizing air heating and humidifying; and (3) Stimulate nerve recovery and renew tissue sensitivity.

The arsenal of conservative ENS treatment methods is limited today to measures that increase humidification (nasal saline irrigation, nasal saline sprays, moisturizing agents, and humidifiers), local antibiotics and corticosteroids application (Figure 1)[2].

Psychological symptoms in ENS patients could be successfully treated by inhibitors of serotonin and norepinephrine reuptake inhibitors or cognitive behavior therapy[15]. The main goal of surgical techniques in ENS treatment is to reconstitute anatomical structure of nasal cavity to restore physiological airflow. Positioning an implant on the septum, floor or lateral wall is a basic principle in ENS surgery[8]. Unfortunately, the results of surgery with different types of biomaterial implantation are not unambiguous and require further investigation and improvements.

OVERVIEW OF IMPLANT MATERIALS

What type of implant could be used for nasal cavity reconstitution? Synthetic or natural materials that are used in medicine to treat, augment or replace tissues and organs are called biomaterials[16]. Biomaterials could be used for implant construction or as a scaffold for stem cells seeding to replace damaged tissue[17]. Physical and chemical properties of biomaterials are varied, but their medicine application imposes certain requirements as biocompatibility, biofunctionality, biodegradation, bioresorbability, non-toxicity. Namely, save and maximally effective biomaterial should safely integrate with host cells and tissue without any adverse effects, its mechanical properties should correspond to those of replaced part of the body, it should not produce any toxic compounds from the surface, pores or during degradation[18]. The most common synthetic biomaterials that are used in tissue engineering are synthetic polymers, which include relatively hydrophobic materials such as the α-hydroxy acids, polyanhydrides, and others[19]. However, synthetic biomaterials have some disadvantages since their composition and structure differ significantly from tissues/organ nature, so their ability to induce tissue remodeling is low[20]. Collagen, gelatin, silk, cellulose, chitin/chitosan, decellularized dermal matrix are examples of natural biomaterials that are widely used as instruments of regenerative medicine.

Not all implant materials can be used to repair empty nose syndrome. From one side biomaterial should be inert, have good biocompatibility, compact and dense enough to avoid extrusion or shrinkage, keep shape well, should not induce inflammation and rejection[21]. The most common biomaterials used in surgical inferior turbinate reconstruction in ENS patients are porous polyethylene (Medpor), cartilage, and acellular dermis (Alloderm)[22].

Commercialized porous polyethylene (Medpor) is an alloplastic biomaterial, medical-grade, high density polyethylene in the form of a flexible framework of interconnecting pores[23]. Pore size in Medpor is more than 150 μm in diameter that allows host tissues, blood vessels, and nerve ingrowth, together with collagen deposition thus forming stable complex which reconstitutes absent part of tissue/organ. Submucosal implantation of Medpor to the ENS patients showed optimistic results. All studies described significant improvement based on SNOT (sino-nasal outcome test) score, level of depression and anxiety symptoms (Table 1)[14,24-28]. The results of Medpor transplantation were stable at least one year after surgery[14,24-27]. Huang *et al*[14] noted absence of nasal crusting and facial pain/pressure improvement after Medpor transplantation. At the same time, a serious drawback of Medpor was partial implant extrusion in some patients 6 mo after surgery[24,25]. Besides, it was reported that chronic atrophic rhinitis has been developed in 1 from 16 patients 4 years after Medpor implantation[25].

The conchal cartilage implantation for turbinate reconstitution may provide the best resistance to infection and a low degree of resorption compared to synthetic biomaterial[29]. From the other hand, usage of autologous cartilage requires additional surgery that is traumatic and results in longer post-operative period. Submucoperiosteal implantation of autologus conchal cartilage into lateral nasal wall showed significant decreasing of SNOT-25 score and good mucosal healing in ENS patients for up to 12 mo[30]. However, difficulties in autologus conchal cartilage harvesting and restricted data about its effectivity after implantation limit conchal cartilage usage for ENS treatment.

Another popular defect reconstitution material in ENS is Alloderm®, a cell-free dermal allograft comprised of structurally integrated basement membrane complex and extracellular matrix in which collagen bundles and elastic fibers are the main components[21]. Since alien cells are not present in Alloderm, it does not cause inflammation and immune rejection compared to other allografts. Alloderm implantation showed improvement of ENS patients' symptoms based on SNOT-20/25 score[5,31,32]. However, similar to Medpor implant, Allodem showed partial extrusion in some cases. Besides, it is subject to shrinkage in time. Taken together, these are significant

Table 1 Comparative analysis of existing empty nose syndrome treatment approaches, clinical results

No.	No. of patients	Type of study	Material used for transplantation (implant material?)	Treatment details	ENS score before surgical intervention	ENS score after surgical intervention	Other effects	Complications, undesired effects	Follow-up time	Ref.
1	19	Prospective self-controlled study	Porous polyethylene (Medpor)	Submucosal implantation into the inferior turbinate and (or) lateral nasal wall and (or) nasal septum opposite the nasal concha	50.1 ± 18.7 (SNOT-20)	22.6 ± 15.8 (3 mo; *P* = 0.037); 20.4 ± 18.9 (6 mo; *P* = 0.007); 37.7 ± 7.6 (12 mo; *P* = 0.736) (SNOT-20)	Improvements of nasal resistance, nasal volume, and minimum cross-sectional area (*P* < 0.05) (CT, acoustic rhinometry); mucociliary clearance improved non-significantly	Partial implant extrusion in one patient at 6 mo follow-up	3-18 mo	[24]
2	16	Retrospective clinical study	Porous polyethylene (Medpor)	Submucosal implantation mainly in septum and nasal floor, unilaterally or bilaterally	39.25 ± 21.44 (SNOT-22)	19.81 ± 16.17 (3 mo, *P* < 0.05); 16.19 ± 13.98 (1 yr, *P* < 0.05) (SNOT-22)	Not evaluated	Chronic hypertrophic rhinitis in 1 patient 4 yr after implantation. Implant protrusion in 1 patient 6 mo after surgery	3 mo-1 yr	[25]
3	13	Prospective cohort study in a tertiary medical center	Porous polyethylene (Medpor)	Submucosal implantation into nasal floor	19 (BAI); 24.4 (BDI-II)	6.8 (BAI); 6.25 (BDI-II)	Mucosa covering the graft was smooth and intact on the lateral nasal wall (endoscopy)	Not reported	1 yr	[26]
4	14	Retrospective study in a tertiary medical center	Porous polyethylene (Medpor)	Inferior nasal wall submucosal implantation	40.7 ± 23.4 (SNOT-22); 22.6 ± 27.0 (BDI-II); 13.8 ± 19.5(BAI)	21.1 (P = 0.002) (SNOT-22); BDI-II decrease (*P* = 0.031)BAI decrease (*P* = 0.004)	Not evaluated	Not reported	1 yr	[27]
5	18 (16 followed-up)	Retrospective study in a tertiary medical center	Porous polyethylene (Medpor)	Lateral nasal wall submucosal implantation	49.3 ± 20.5 (SNOT-22); 20.5 ± 15.1 (BDI-II); 20.1 ± 15.2 (BAI)	11.8 (*P* < 0.001) (SNOT-22); BDI-II decrease (*P* < 0.001); BAI decrease (*P* < 0.001)	Not evaluated	Not reported	1 yr	[27]
6	68 (39 followed-up)	Prospective clinical study	Porous polyethylene (Medpor)	Submucosal implantation into the nasal floor or lateral wall	62.9 ± 25.3 (SNOT-25); 19.3 ± 15.0 (BDI-II); 17.7 ± 12.9 (BAI)	35.5 ± 24.4 (*P* < 0.001) (SNOT-25); 8.4 ± 10.1 (*P* < 0.001) (BDI-II); 10.5 ± 11.5 (*P* < 0.001) (BAI)	Not evaluated	Not improved nasal crusting and facial pain/pressure	6 mo	[28]
7	54 (46-38 followed-up)	Prospective case series in a tertiary medical center	Porous polyethylene (Medpor)	Submucosal implantation into the nasal floor or lateral wall	64.9 ± 24.7 (SNOT-25); 20.7 ± 15.3 (BDI-II); 19.8 ± 13.3(BAI)	32.9 ± 20.7 (3 mo); 30.2 ± 22.9 (6 mo); 29.1 ± 23.8 (12 mo); (*P* < 0.01) (SNOT-25); 8.2 ± 11.2 (3 mo); 8.0 ± 9.8 (6 mo); 7.8 ± 10.7 (12 mo); (*P* < 0.01) (BDI-II); 8.5 ± 9.8 (3 mo); 9.6 ± 11.0 (6 mo); 8.7 ± 10.1 (12 mo); (*P* < 0.01) (BAI)	Not evaluated	Not reported	3-12 mo	[14]
8	1	Case report	Hydroxyapatite cement	Implantation into subperiosteal tunnel on the lateral nasal wall	Not evaluated	Not evaluated	Not evaluated	Not reported	1 yr	[60]

9	3	Clinical study	Hyaluronic acid	Submucosal injections into the inferior nasal concha and under the mucous membrane of the septum	Not evaluated	Not evaluated	Subjective symptoms improvement, improved acoustic rhinometry results (3-6 mo)	After 1 yr the implant was absorbed in 2 patients, symptoms restored	1 yr	[61]
10	5	Prospective observational study in a tertiary medical center	β-tricalcium phosphate	Implantation into submucoperiosteal pocket along the lateral nasal wall, at the site of the former inferior turbinate head between the nasal floor and the ostium of the nasolacrimal duct	90 (90-95) (NOSE); 18.7 (18.7-43.7) (RhinoQoL frequency); 30 (20-43.3) (RhinoQoL bothersomeness); 62.5 (57.6-68.7) (RhinoQoL impact)	5 (5-25) (*P* = 0.01) (NOSE); 81.2 (75-81.2) (*P* = 0.01) (RhinoQoL frequency); 81 (76.7-90) (*P* = 0.05) (RhinoQoL bothersomeness); 8.3 (5.5-11) (*P* = 0.01) (RhinoQoL impact)	Median nasolacrimal duct aperture (DNLI) 4.2 mm (1.8-6.6) (CT)	One case of partial implant extrusion 6 d after surgery	13.5 mo (8.2-21)	[20]
11	12	Prospective randomized blind clinical study	Silastic sheet	Implantation into submucoperichondrial and/or submucoperiosteal pockets fashioned along the septum, nasal floor, and lateral nasal wall	61.4 ± 16.3 (SNOT-25)	33.6 ± 17.1 (SNOT-25)	Decreased crustling (anterior rhinoscopy and endoscopic examination)	Partial implant extrusion in 4 patients	9-24 mo	[32]
12	14	Clinical study	Carboxymethylcellulose/glycerin gel (Prolaryn)	Submucosal injection into the inferior meatuses	20.8 ± 4.9 (ENS6Q); 50.3 ± 15.2 (SNOT-22); 8.6 (GAD-7)11.6 (PHQ-9)	**ENS6Q:** 10.5 (1 wk; *P* < 0.0001); 13.7 (1 mo, *P* = 0.002); 15.5 (3 mo; *P* > 0.05); **SNOT-22:** 29.3 (1 wk; *P* = 0.01); 35.5 (1 mo, *P* = 0.04); 39.3 (3 mo, *P* > 0.05); **GAD-7:** 5.4 (1 wk, *P* > 0.05); 4.9 (1 mo, *P* = 0.02); 5 (3 mo, *P* = 0.02); **PHQ-9:** 6.6 (1 wk; *P* = 0.01); 7 (1 mo, *P* = 0.004); 7.4 (3 mo, *P* > 0.05)	Not evaluated	Not reported	1 wk – 3 mo	[62]
13	3	Observational prospective cohort study	Porcine small intestine submucosal xenograft	Inferior turbinate reconstruction, implantation into submucosal pocket in the lateral nasal wall	77.6 (SNOT-25)	65 (1 wk); 57 (4 wk, *P* < 0.01); 55 (12 wk, *P* < 0.01) (SNOT-25)	Not evaluated	Mild partial implant reabsorption	1-12 wk	[63]
14	12	Retrospective clinical chart review	Autologous septal or conchal cartilage; nautologous or homologous costal cartilage	Submucosal implantation into lateral nasal wall	Not evaluated	Not evaluated	75% were satisfied with outcome; significantly decreased excessive airflow, nasal obstruction, and nasal or facial pain (*P* < 0.05) (VAS)	Under correction in 3 patients	11.8 mo (6-27 mo)	[64]
15	17	Case series with chart review	Conchal cartilage (autologous)	Submucoperiosteal implantation into lateral nasal wall	54.0 (27.0-57.8) (SNOT-25)	35.9 (24.0-51.5) (*P* = 0.007) (SNOT-25)	Good mucosal healing (endoscopy)	Not reported	6-12 mo	[30]
16	14	Case series with chart review	Costal cartilage (autologous or homologous)	Submucoperiosteal implantation into lateral nasal wall	46.6 (45-67.5) (SNOT-25)	21.9 (9.0-40.8) (*P* = 0.002) (SNOT-25)	Good mucosal healing (endoscopy)	Not reported	6-12 mo	[30]
17	7	Prospective cohort study in a tertiary	Autologous bone graft from septal bone	Submucosal implantation into nasal floor	19 (BAI); 24.4 (BDI-II)	6.8 (BAI); 6.25 (BDI-II)	Mucosa covering the graft was smooth and intact on the lateral nasal wall	Not reported	1 yr	[26]

		medical center					(endoscopy)			
18	1	Case report	Acellular dermis (Alloderm) + Cymetra (injectable acellular dermis)	Submucoperiosteal implantation	Not evaluated	Not evaluated	40% better (subjective)	Not reported	3 mo	[31]
19	8	Case series	Acellular dermis (Alloderm)	Submucoperichondrial and submucoperiosteal implantation into the former inferior turbinate; or into nasal septum and/or floor	58.3 (SNOT-20)	38.3 ($P \leq 0.02$) (SNOT-20)	Subjective improvement in smell threshold	The initial graft shrinks, and then the graft appears to maintain stable size for years	6 mo – 4 yr	[5]
20	12	Prospective randomized blind clinical study	Acellular dermis (Alloderm)	Implantation into submucoperichondrialand/or submucoperiosteal pockets fashioned along the septum, nasal floor, and lateral nasal wall	63.7 ± 15.4 (SNOT-25)	34.2 ± 15.2 (SNOT-25)	Decreased crustling (anterior rhinoscopy and endoscopic examination)	Partial implant extrusion in 3 patients during the first 2 wk, but all healed with no sequelae. Partial graft shrinkage in 2 cases 2 mo postoperatively	9-24 mo	[32]
21	9	Prospective observational clinical study	Autologous stromal vascular fraction (SVF)	Injection into medial surface of inferior turbinates	70.1 ± 24.7 (SNOT-25)	62.4 ± 35.8 ($P > 0.05$) (SNOT-25)	Decreased IL-1β and IL-8 ($P < 0.005$) (ELISA)	Seroma in 1 patient	6 mo	[47]
22	30	Clinical study	Autologous ADSCs combined with autologous fat granules	Injections into the areas of mucosal damage (every 10 d, 3 in total)	Not evaluated	Not evaluated	Inflammation significantly reduced, collagenous fibers became aligned, fewer deposits observed, and the mucosal proteins increased 1 mo post-op (H&E, Masson's, and AB-PAS stainings). Nasal resistance, nasal volume, minimum crosssectional area, and mucociliary clearance improved (acoustic rhinometry)	Not reported	3-9 mo	[49]

disadvantages of this type of implant[5,32].

Single studies showed application of hydroxyapatite cement, hyaluronic acid, β-tricalcium phosphate, silastic sheet, carboxymethylcellulose/glycerin gel (Prolaryn), autologous bone graft from septal bone, porcine small intestine submucosal xenograft for turbinate restoring in ENS patients (Table 1). Small number of enrolled patients and drawbacks detected impose restrictions on the use of these implants for ENS treatment. Despite meta-analysis[21] showing that implantation of autograft/allograft is more effective than foreign graft materials, searching for optimal implant biomaterials with good biocompatibility, stability and host tissue integration is still an actual task for regenerative medicine in line of ENS treatment. Moreover, none of presented surgical or conventional treatments solves all the key issues which underlie ENS pathogenesis that results in their low effectivity and/or not sustainable effect, not

enough to significantly improve ENS patient's symptoms. We postulate that new developing cell technologies could overcome the existing treatment limitations and lead to the significant and stable improvement in quality of life of ENS patients.

Adipose tissue derived extracellular matrix and methylcellulose hydrogels are new alternative biomaterials that could be potently used for the inferior turbinate reconsctruction. Strong limitation of fat transplantation despite of excellent biocompatibility is its high resorption up to 90% during 5 mo[33]. Decellularized adipose derived extracellular matrix (ECM) is a new alternative to fat transplantation. It was shown that combination of ECM and methylcellulose hydrogels are promising material for injection laryngoplasty for stable vocal fold augmentation. ECM/MC hydrogel did not cause inflammation or fibrosis in injection site, but number of collagen fibers and fatty granules increased[34]. Other study demonstrated that ECM/MC hydrogels are excellent scaffold for injectable stem cell delivery. Transplantation of ADSCs in ECM/MC hydrogels in cutaneous would led to rapid re-epithelialization, neovasculation and minimal scar formation[35]. The main advantages of ECM/MC hydrogel composition are high biocompatibility, thermosensitivity that allow to inject material with minimal traumatization for patients, stability and possibility to combine with stem cells.

PERSPECTIVES OF STEM CELL-BASED THERAPY

Stem cells-based therapy belonging to advances therapy medical products is an innovative treatment strategy that could give chance for those diseases, where conventional therapies are inefficient. Stem cells application show great potential for replacing or regenerating damaged cells, tissues and organs. Different types of stem cells, including adult stem cells, derivatives of embryonic and induced pluripotent stem cells undergo clinical trials for evaluation of their safety and effectivity[36]. Anti-aging therapy, wound healing, neurodegenerative diseases, metabolic disorders, musculoskeletal system disorders, autoimmune diseases are all targets for stem cells application. Taking into consideration ethical, legal and political concerns, tissue-specific adults stem cells are preferable source for use in regenerative medicine. Due to a multilineage differentiation potential, immunomodulatory properties, production of large number of soluble or vesicle-bound growth factors, cytokines, and microRNAs, multipotent mesenchymal stromal cells (MSCs) become the key player in rapidly growing field of cell-based therapy during last 30 years[37]. More than 950 clinical trial based on using MSCs were registered worldwide during 2011-2018[37,38]. Significant therapeutic effect of MSCs has been shown in treating graft *vs* host diseases, complex perianal fistulas in Crohn's disease, osteoarthritis, type II diabetes, and wound healing [39-41]. Bone marrow, adipose tissue, umbilical cord and placenta are the most abundant sources for MSCs isolation and expansion with the next application in regenerative medicine taking into consideration ethical problems, cell source availability and quantity of MSCs output[37]. All MSCs types isolated from different tissues are characterized by common features: adhesion to plastic, fibroblast-like morphology, positive for CD73, CD90, CD105 and negative for CD34, CD45, CD14 or CD11b, CD79-α or CD19, HLA-DR cell surface markers, ability multilineage differentiation into adipocytes, chondrocytes and osteocytes *in vitro*[37,42]. The broad-range of MSCs therapeutic potential could be explained by their biological properties[43]. High proliferation potential – MSCs proliferate rapidly *in vitro* allowing biotechnologists to expand cells to the appropriate amount for application. (1) Multipotency – ability to differentiate into multiple cell types under the appropriate stimuli and replace damaged cells; (2) Trophic function – MSCs secrete numerous grows factors (Ang-1, EGF, FGF, GDFN, BDNF, HGF, IGF-1, PDGF, SDF-1, VEGF) to support the local tissue regeneration[44]; (3) Homing/migration – MSCs are able to migrate to damaged tissues after systemic application; they express adhesion molecules (CD44 and integrins), chemokines receptors (CCR2, CCR7, CCR10, CXCR4, CXCR5, CXCR6), and metalloproteinases (MMP-1, MMP-9); and (4) Immunomodulation function – MSCs suppress the proliferation and activity of CD4+ T helper cells, CD8+ cytotoxic T cells, proinflammatory macrophages, neutrophils, nature killer cells and B cells. At the same time, MSCs stimulate regulatory T and B cells, anti-inflammation macrophages and immature dendritic cells. Immunomodulation properties of MSCs are realized be secretion of TGF-β, IL-6, IL-10, prostaglandin E2 (PGE2), LIF, TSG-6, inducible nitric oxide synthase (iNOS) and indoleamine 2,3-dioxygenase (IDO), *etc.*[45].

Stem cells, including MSCs, use different mechanisms to repair injured tissues. These are direct cell replenishment by MSCs differentiation, paracrine effect by growth

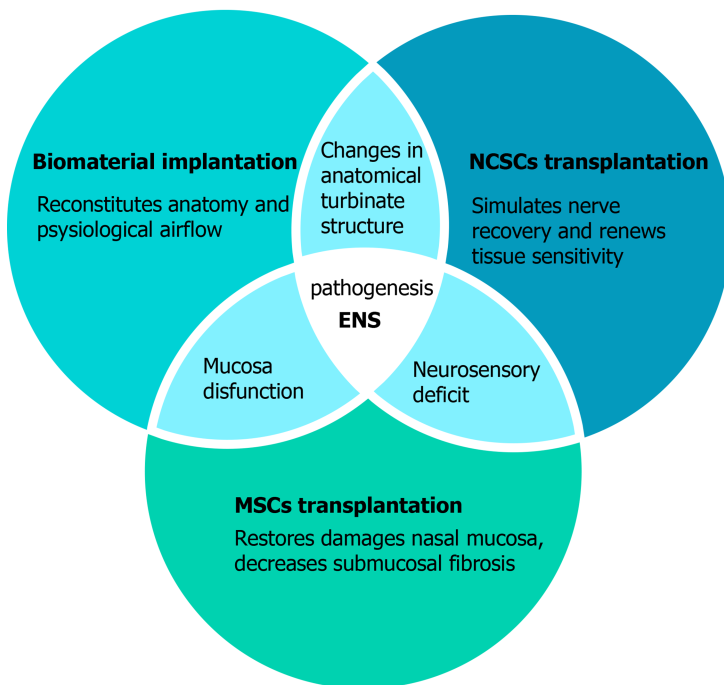

Figure 2 Conception of complex approach in treatment of empty nose syndrome using cell-based technologies and tissue engineering. The empty nose syndrome treatment should be complex and solving three main issues: Changes in turbinate anatomy, mucosa tissue dysfunction and nerve sensing disruption. Biomaterial implantation may be performed alone or together with encapsulated stem cells to restore turbinate structure and physiological airflow. The main function of mesenchymal stem cells injection is directed to decrease inflammation and regenerate nasal mucosa, while neural crest-derived stem cells should stimulate neurosensory system sensitivity. ENS: Empty nose syndrome; MSCs: Mesenchymal stem cells; NCSCs: Neural crest-derived stem cells.

factors, cytokines and hormones secretion, and protein/peptide and miRNA transfer *via* MSCs-derived extracellular vesicles[46].

Published data about MSCs application for ENS treatment are limited. Kim *et al*[47] reported that injection of the autologous stromal vascular fraction (SVF) from adipose tissue partially improved ENS symptoms. Two of nine enrolled patients felt improvement, but totally there was no statistically significant differences in SNOT-25 score. However, the level of inflammatory cytokines IL-1β and IL-8 in nasal secretions decreased after SVF injection[48]. The low effectivity of SVF for ENS treatment could be explained by the fact that SVF is a non-cultivated mixture of differentiated and progenitor cells with very low percent of real stem cells (up to 3%)[48] which vary between the patients. It was shown that the combination of fat particles with adipose tissue-derived MSCs led to significantly improved symptoms in ENS patients[49]. The potential of MSCs to recover nasal mucosa was confirmed by Friji *et al*[50] when lipoaspirate in combination with PRP was injected to five patients with primary atrophy rhinitis. This resulted in mucosal regeneration and improvement of mucosa appearance from atrophied mucosa with crusting to normal glistening mucosa[50]. We suppose that using more homogeneous and enriched population of MSCs isolated from umbilical cord or adipose tissue for ENS management will have more prominent regenerative effect (Figures 2 and 3), than lipoaspirate or SVF application.

As mentioned previously, reduction of nasal surface area after turbinate resection leads to different level of nerve damage and reduction of TRPM8 thermoreceptors number[2] that results in abnormal patient's sensation. Role of nerve signaling in pathophysiology of ENS to find approach for nerve recovery and tissue sensitivity renewal. Injection of cell-based products enriched with trophic factors is one of possible solutions. Besides already known PRP, SVF, and MSCs we purpose to use another stem cell type with high neuroprotective properties and ability to neural differentiation that could force nerve regeneration in ENS patients. Adult neural-crest derived stem cells (NCSCs) are unique rare population of stem cells found in the hair follicle, skin dermis, adipose tissue, bone marrow, palate, nasal mucosa, dental pulp *etc*[51]. Numerous studies demonstrate that NCSCs have wider range of differentiation potential compared to MSCs, precisely in neuronal and glial cell lineages[52]. Moreover, NCSCs produce rich spectrum of cytokines and growth factors like IL-2Ra, IL-3, IL-5, IL-8, IL-16, MCP-1, SDF1a, GM-CSF, M-CSF, VEGF, HGF, bFGF, as well as specific neurotrophic factors NGF, NTF3, NTF4, BDNF, GDNF[53]. The trans-

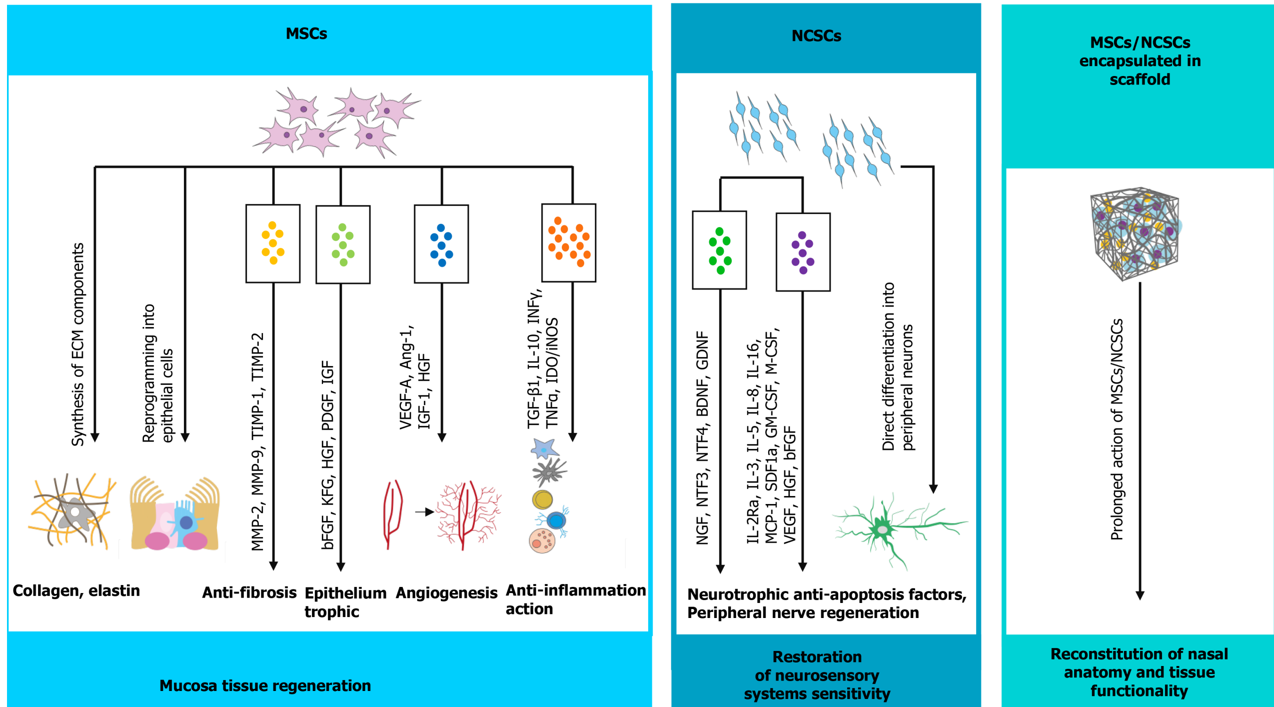

Figure 3 Realization of stem cells therapeutic potential in management of empty nose syndrome (possible mechanisms). The therapeutic effect of stem cell transplantation could be realized by two main ways. The first one is direct differentiation of transplanted stem cells under the impact of specific environmental factors, such as hypoxia and inflammation. Mesenchymal stem cells (MSCs) have the ability to transdifferentiate in mucosa epithelial cells, while neural crest-derived stem cells (NCSCs) can form peripheral neurons. However, very small amount, around 1-3%, of transplanted stem cells can differentiate. The 95% of stem cells therapeutic potential are implemented in indirect way *via* secreting plethora of paracrine factors and extracellular vesicles. MSCs-derived secretory factors promote neovascularization, immunomodulatory anti-inflammatory effect, anti-apoptotic and anti-fibrotic effect, and reduce oxidative stress that create favorable environment for mucosa regeneration. NCSCs, in particular, have strong neuroprotective properties, thus local NCSCs injection in empty nose syndrome patients could also stimulate nerve recovery by trophic support or direct reintegration in damaged tissue. MSCs: Mesenchymal stem cells; NCSCs: Neural crest-derived stem cells.

plantation of cultured adults NCSCs resulted in the restoration of bone defects[54], damaged peripheral nerves[55] and spinal cord[56] in rats. The NCSCs transplantation in an experimental model of glaucoma was effective and caused reduction of edema and restoration of the cytoarchitectonics of the layers[57]. Application of the NCSCs after the experimental orbital trauma stimulated the oculomotor muscles and retrobulbar fat tissue recovery[58]. All abovementioned biological properties of NCSCs, successful pre-clinical and clinical results of their transplantation together with several accessible sources in adult organism make NCSCs attractive candidates for regenerative medicine use, especially in field of neurology. NCSCs local injection to patients with ENS could lead not only to regeneration of nasal mucosa, but also stimulate nerve recovery by trophic support or direct reintegration in damaged tissue (Figures 2 and 3).

Efficiency of stem cell-based product application for ENS patients management may depend on numerous factors: severity of symptoms, autologous or donor stem cells, source of stem cells, their amount and quality *etc*. Based on our own experimental and clinical experiences in stem cells-based treatment of neurodegenerative diseases and ENS patients (not published), we suggest using transplantation of NCSCs alone or in combination with MSCs to archive more valuable therapeutic results depending on the case severity (Figure 2). The local injection of MSCs alone could induce regeneration of nasal mucosa epithelium and decrease inflammation, but that is not efficient for neurosensory system recovery (Figures 2 and 3). Taking into account the intrinsic neuroprotective properties of NCSCs, the following application of high quality cultured NCSCs may reconstitute nerve structure and functioning leading to normal sensation in ENS patients (Figure 3). Thus, the therapy based on the combination of NCSCs and MSCs might improve the proper functioning of epithelial cells of the nasal mucosa, reduce inflammation and, consequently, mucosal edema, recover neurosensory systems, which will contribute to the regression of ENS symptoms.

There are some ways to improve stem cell-based therapy. One option is tissue engineering approach, thus application of stem cells encapsulated in bioactive materials whose chemical and physical properties would support cell survival and proliferation. Biomaterials may increase therapeutic potential of stem cells due to imitation of physiological niche of the cells allowing to cell interaction with each other and with artificial "extracellular matrix" in 3D dimension. Such approach results in reducing cells' stress after transplantation, increased stem cells survival, their secretome enrichment, *etc.*[59]. Thus, depending on ENS severity, MSCs and NCSCs encapsulated in biomaterial could be used both to reconstitute the anatomical nasal structure and restore mucosa functionality, specifically inhibit inflammation after surgical manipulation, reduce edema, stimulate mucosa epithelium regeneration and improve local neurosensation. In case of tissue-engineering methods use we expect to achieve lifetime positive effect. Such a complex approach may help patients obtain long-awaited relief as well as reduce their time and costs.

CONCLUSION

With the advancement of stem cell technologies, the use of this regenerative medicine instrument for reconstruction of damaged nasal cavity may greatly improve conventional methods of ENS treatment, gaining not only anatomical structure but returning lost function. In recent years, stem cell-based therapies were extensively studied, and promising results were acquired for various stem cell types combined with different grafts and biomaterials. However, there remain some challenges to be solved, such as the low survival rate of transplanted cells and the difficulty of restoring the proper volume and normal architecture of the organ tissue.

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MINIREVIEWS

Lipid droplets as metabolic determinants for stemness and chemoresistance in cancer

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Abstract

Previously regarded as simple fat storage particles, new evidence suggests that lipid droplets (LDs) are dynamic and functional organelles involved in key cellular processes such as membrane biosynthesis, lipid metabolism, cell signalling and inflammation. Indeed, an increased LD content is one of the most apparent features resulting from lipid metabolism reprogramming necessary to support the basic functions of cancer cells. LDs have been associated to different cellular processes involved in cancer progression and aggressiveness, such as tumorigenicity, invasion and metastasis, as well as chemoresistance. Interestingly, all of these processes are controlled by a subpopulation of highly aggressive tumoral cells named cancer stem cells (CSCs), suggesting that LDs may be fundamental elements for stemness in cancer. Considering the key role of CSCs on chemoresistance and disease relapse, main factors of therapy failure, the design of novel therapeutic approaches targeting these cells may be the only chance for long-term survival in cancer patients. In this sense, their biology and functional properties render LDs excellent candidates for target discovery and design of combined therapeutic strategies. In this review, we summarise the current knowledge identifying LDs and CSCs as main contributors to cancer aggressiveness, metastasis and chemoresistance.

Key Words: Lipids; Lipid droplets; Lipid metabolism; Stemness; Cancer stem cells; Chemoresistance

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Core Tip: Increasing evidence suggests that lipid droplets (LDs) support cancer stem cells (CSCs) functionality at different levels. Indeed, an increased LD content has been linked to tumorigenicity, metastatic spread and chemoresistance in different cancer types, highlighting their value as prognostic and treatment response predictive

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biomarker. A deeper understanding of the molecular mechanisms by which LDs control these processes would expedite the discovery of novel potentially druggable targets and the design of more efficient therapeutic strategies aimed at eliminating highly tumorigenic CSCs.

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INTRODUCTION

Cancer stem cells

The cancer stem cell concept: Consistent evidence supports that most of the heterogeneity found in both liquid and solid cancers might be originated in the context of hierarchical organisation of the tumours. Indeed, a subset of cells with self-renewal capacity and tumour-initiating properties called cancer stem cells (CSCs) undergo asymmetrical and symmetrical divisions in order to originate bulk differentiated tumour cells and identical CSCs to perpetuate its lineage. Cancer hierarchy at cellular level was first described in acute myeloid leukaemia[1], representing a huge milestone in the understanding of cancer emergence. The CSC theory has been supported since then by increasing evidence in other malignancies such as breast cancer[2], brain tumours[3] colon and colorectal cancers[4,5], as well as pancreatic cancer[6,7], among others.

The current approach to cancer therapy has been both clarified and challenged by the existence of CSCs. On the one hand, the increasing evidence of their existence and contribution to tumorigenesis and metastasis has allowed researchers and clinicians to acquire a better understanding of cancer origin and evolution. On the other hand, proof of the implication of CSCs in treatment failure due to their intrinsic chemoresistance abilities has demonstrated that specific therapeutic strategies against this tumoral subpopulation are still urgently needed.

The origin of CSCs remains unclear, since it might vary between malignancies. One hypothesis derives from the observed similarities between CSCs and their normal homologous SCs, suggesting that local SCs may suffer a malignant transformation[8]. Other theories involve the acquisition of stemness features by differentiated cells. On the one hand, it has been suggested that differentiated cancer cells undergoing epithelial-to-mesenchymal transition acquire stem-like properties under the regulation of Notch signalling[9,10]. On the other hand, microenvironmental signals from stromal cells might facilitate non-CSCs dedifferentiation. For instance, Wnt signalling conferred self-renewal and tumorigenic abilities to colorectal cancer cells[11]. Furthermore, FGF5 and collagen production induced by Hedgehog promoted triple negative breast cancer chemoresistance by acquiring self-renewal capacity[12]. In any case, a dual scenario in which both local SCs and differentiated tumour cells originate new CSCs may be present in chemoresistant pancreatic[13] and lung[14] cancer cells.

CSC metabolism

Microenvironmental selective pressure forces CSCs to adapt continuously in order to survive and progress. For instance, as the tumour grows, glucose and oxygen levels diminish, the pH becomes acidic and reactive oxygen species (ROS) and inflammatory mediators accumulate in the tumour microenvironment. Since most differentiated tumour cells are fully glycolytic in order to cope with their enhanced proliferative rates (*e.g.* Warburg effect), resource scarcity forces CSCs to become metabolically and functionally plastic in order to survive and detoxify their microenvironment. Theoretically, an active mitochondrial metabolism would provide CSCs with an increased plasticity since a larger array of substrates could be feeding the tricarboxylic acid cycle. However, depending on the tumour type and model systems studied, CSCs use either mitochondrial oxidative phosphorylation (OXPHOS) or glycolysis[15,16] preferentially, with varying degrees of plasticity to switch from, even within the same tumour. Indeed, although the majority of pancreatic CSCs relies on OXPHOS and is very

sensitive to mitochondrial inhibition, a small portion of CSCs shows a plastic phenotype, activating glycolysis when its mitochondria are inhibited[17]. However, full metabolic plasticity comes at the expense of self-renewal capacity[17].

Importantly, OXPHOS-dependent CSCs and therapy-resistant tumour cells from different cancer types bear higher levels of the master regulator of mitochondrial biogenesis peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)[17-19], which supports OXPHOS metabolism and provides resistance to oxidative stress and chemotherapy[18-20]. Considering that PGC-1α is a transcriptional coactivator of the peroxisome proliferator-activated superfamily of receptors (PPARs) which controls the balance between glucose and lipid metabolism[21,22], we can hypothesise that PGC-1α enables CSCs to control a complex metabolic programme associating stemness to mitochondrial metabolism, including lipid and fatty acid (FA) oxidation (FAO). In fact, different studies have demonstrated that lipid metabolism is required to maintain the CSC pools in several tumour types[23-26].

LIPID METABOLISM AND LIPID DROPLETS

Cancer and lipid metabolism

Cancer cells have metabolic reprogramming abilities to sustain high proliferation rates as well as energy production, not only through high glycolysis (Warburg effect), but also through reprogrammed lipid metabolism[27-29]. Indeed, they enhance *de novo* lipid synthesis, lipogenesis and FAO, being FA synthesis one of the most important aberrations of cancer cell metabolism[30]. FAs are involved in many different aspects of tumorigenesis and tumour progression and sustain three requirements of cancer cells and CSCs: Cell membrane formation, signalling molecules and lipid-derived messengers, and energy production[31-33]. Importantly, an increased FA metabolism has been associated to poor prognosis in different types of cancer, such as pancreatic cancer or melanoma[34]. In pancreatic cancer, it is generally associated to a high expression of key regulatory enzymes like the FA synthase and sterol regulatory element-binding protein[35,36].

Cancer cells accumulate more lipids in their cytoplasm than normal cells[37]. Novikoff was the first to demonstrate the presence of cytoplasmic inclusions in the rat liver tumour cells and to identify the lipid nature of these droplets[38]. Although regarded as simple fat storage particles for long, lipid droplets (LDs) are currently considered conserved, dynamic and functional organelles involved in membrane biosynthesis, lipid metabolism, cell signalling and inflammation[39]. Indeed, they have been associated with an increased tumour aggressiveness and resistance to chemotherapy[40], considerably raising attention within the cancer biology community.

LDs

LDs, also known as lipid bodies or liposomes, are cellular organelles ranging from 20-40 nm to 100 mm, with key functions for lipid and energy homeostasis[41,42]. The quantity, size, composition and intracellular localisation differ significantly between or within cells, mainly due to their type, function and metabolic state[43]. Indeed, LDs are highly dynamic organelles which alternate periods of growth and consumption, depending on cell energy and nutritional status[39,41].

However, all LDs have a similar structure consisting of a hydrophobic core of neutral lipids, such as cholesteryl esters (CE), retinyl esters and triglycerides (TAGs) [44], separated from the aqueous cytoplasm by a monolayer of phospholipids, mainly phosphatidylcholine[45]. Additionally, LDs are coated with integral and peripheral proteins[46] derived from the cytosol or the endoplasmic reticulum (ER)[47]. These proteins can be classified into four groups: (1) Resident/structural proteins, such as members of the perilipin (PLIN)-ADRP-TIP47 family or the cell death-inducing DFF45-like effector (CIDE) family[48-50] (Figure 1); (2) Lipid metabolism enzymes, such as diacylglycerol acyltransferases 1 and 2 (DGAT1 and DGAT2), adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL); (3) Membrane trafficking proteins, including a variety of Ras related protein (Rab) GTPases, as well as soluble NSF binding protein receptor proteins; and (4) Cell signalling proteins such as mitogen-activated protein kinases and protein kinase C. Other types of proteins can be associated to the ribosome and cytoskeleton, or processes such as protein degradation [51,52].

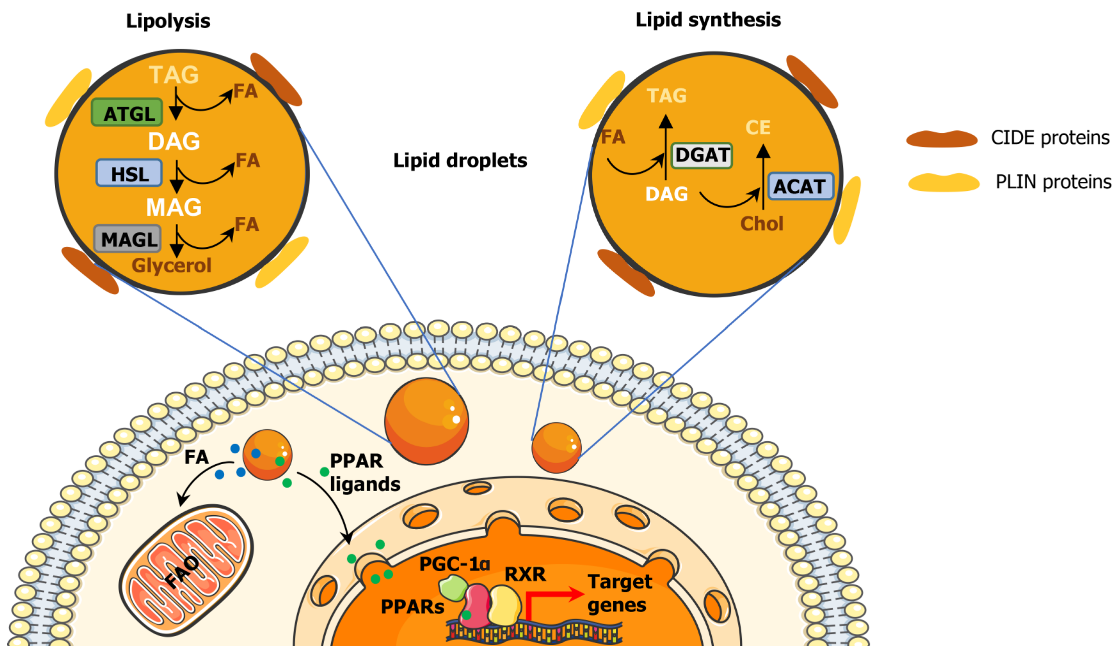

Figure 1 Structure and cellular functions of lipid droplets. Lipid droplets (LDs) have a hydrophobic core of neutral lipids, mainly cholesteryl esters and triglycerides surrounded by a monolayer of phospholipids. LDs are coated with peripheral and integral proteins such as cell death-inducing DFF45-like effector and perilipins proteins, and lipid metabolism enzymes implicated in lipid synthesis and lipolysis: diacylglycerol acyltransferases 1 and 2 (DGAT1 and DGAT2), acyl-CoA cholesterol acyltransferases 1 and 2 (ACAT1 and ACAT2), adipose triglyceride lipase, hormone-sensitive lipase and monoacylglycerol lipase. LDs play roles in energy supply, *via* fatty acid oxidation, and signalling, by producing lipid intermediates that include pro- and anti-inflammatory signalling molecules and peroxisome proliferator-activated (PPAR) ligands. Upon activation, PPARs together with the coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha form a nuclear complex with RXR that binds to the DNA to activate the transcription of target genes. TAG: Triglycerides; CIDE: Cell death-inducing DFF45-like effector; PLIN: Perilipins; ATGL: Adipose triglyceride lipase; HSL: Hormone-sensitive lipase; MAGL: Monoacylglycerol lipase; FA: Fatty acid; CE: Cholesteryl esters; PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; DGAT: Diacylglycerol acyltransferases; ACAT: Acyl-CoA cholesterol acyltransferases; PPARs: Peroxisome proliferator-activated receptors.

LD biogenesis can be described as an evolutionary model consisting of three main steps: (1) Lipid synthesis; (2) LD formation; and (3) LD growth. In step 1, TAG and CE synthesis enzymes, such as DGAT1, DGAT2 and acyl-CoA cholesterol acyltransferases 1 and 2 (ACAT1 and ACAT2), deposit neutral lipids between the sheets of the ER bilayer[53,54]. During step 2, the lipid quantity increases and, when it reaches a certain concentration, the LD detaches from the ER[55]. Thereafter, a variety of proteins such as perilipins, are recruited to the lens structure and facilitate the growth of the nascent LD[56]. Finally, step 3 only occurs in some mammalian cells, where LDs can grow by local lipid synthesis, by transporting lipids to LDs or by fusing with other LDs[57].

LDs can be broken down for energy supply and membrane synthesis through lipolysis or lipophagy (Figure 1). The lipolysis enables the release of FAs from TAGs through the consecutive action of ATGL, HSL and monoacylglycerol lipase[58,59]. Through lipophagy, LDs are enclosed in autophagosomes, fused with lysosomes and degraded by hydrolytic enzymes[60,61].

LDs are mainly found in the cytoplasm, but also in the nucleus of some cell types [62]. Their intracellular location is determined by interacting with other organelles to promote lipid exchange, metabolic dynamics and stress adaptation[63]. LDs come into contact with the ER early in their biogenesis, as well as with the lysosome in the lipophagy process[56,61]. LDs also connect with mitochondria to enable the direct flow of FAs into the mitochondrial matrix to fulfil the cell energy requirements[64]. Their interaction with peroxisomes also allows the transport of FAs, phospholipids and TAGs[65]. Moreover, there is direct and indirect contact with nucleus and Golgi organelles[66].

Besides energy supply and membrane synthesis, LDs play additional roles to ensure proper cell functionality under stress. Prolonged nutrient deprivation upregulates autophagy, causing breakdown of proteins and membranous organelles, which release amino acids and lipids potentially toxic for the cell. In this sense, LDs store neutral lipids, inert within its structure[67]. Additionally, LDs serve as extra source of lipids for FAO under nutrient stress[31,68,69] and hypoxic stress[68]. LDs also ensure the maintenance of redox homeostasis, proper mitochondrial function and membrane and organelle homeostasis[64,70]. In addition, they protect against ER stress; that is,

against imbalances in ER protein folding capacity, calcium uptake and lipid composition[41,71]. Finally, LDs produce lipid intermediates that include pro- and anti-inflammatory signalling molecules[72].

LDs IN CANCER STEMNESS AND CHEMORESISTANCE

Considering LDs regulate different cellular processes, it is not surprising that they have been strongly associated to cancer progression and aggressiveness in recent years [69,73-75]. In fact, LDs facilitate not only tumour growth, but also metastasis, chemoresistance and disease relapse in multiple types of cancers[68,74,76], all processes intimately related to CSCs.

Indeed, a direct relation between LD content and stemness has been demonstrated in different types of cancers such as pancreatic, colorectal, ovarian and breast cancer [25,77-79]: On the one hand, the isolation of cells with high LD content led to an enrichment of CSCs; on the other hand, isolated $CD133^+$ CSCs show higher LD content than differentiated $CD133^-$ cancer cells. Interestingly, tumour-initiating pancreatic cells resistant to KRAS ablation showed an LD accumulation coupled with macrolipophagy, corresponding to the fusion of LD with autophagosomes. Correlated with a high catabolism rate of endogenous lipids and FAs, Viale *et al*[80] determined that these KRAS ablation-resistant cells used autophagy/macrolipophagy to maintain their energy balance. Indeed, the inhibition of either autophagy or entry of FAs in the mitochondria (using bafilomycin or etomoxir, respectively) dramatically reduced cellular oxygen consumption rate. This metabolic stress was associated with a strong decrease of survival and sphere formation capacity[80]. Functionally, Tirinato *et al*[81] demonstrated that sorted colorectal CSC with high or low LD content were able to form tumours after subcutaneous injection in immunocompromised mice, although cells with low LD content generated delayed small tumours less frequently. These results suggested that cells with high LD content increase tumorigenic potential, while cells with low LD content represented a more differentiated and less tumorigenic population. Thereby, LD content seems directly linked to tumorigenicity and is suggested as a marker of CSCs, in addition to molecular markers[81]. Moreover, LD-related proteins from the PLINs and CIDE families can be associated to tumorigenicity in several cancer types[82]. Nevertheless, Cao *et al*[82] highlighted that an increased expression of PLIN2 was associated with a better survival rate in clear cell renal cell carcinoma (ccRCC), decreased with a higher tumour grade. Indeed, PLIN2 knockdown enhanced proliferation, migration and invasion of ccRCC cells. These findings underpin that more studies are needed to clearly identify the specific roles of LD-associated proteins in tumorigenesis or tumour progression, which may be cell or context-specific.

LDs seem to be necessary for CSCs functionality[40], not only to sustain energy demands and biomass production but also to regulate several important oncogenic signalling pathways such as Wnt/β-catenin and Hippo/Yes-associated protein 1 pathways[79] (Figure 2). In this sense, the PPARs superfamily directly associates signalling with LDs, since most lipid-derived second messengers produced in LDs act mainly through these nuclear receptors. Recently, Kuramoto *et al*[77] demonstrated that PPARα was activated in CSCs that accumulated LDs from pancreatic and colorectal cancer. At the same time, PPARα induced the expression of lipolytic factors like ATGL, leading to the release of FAs that supported stemness characteristics in a positive feedback loop. Indeed, a decreased PPARα activity, by using inhibitors or siRNAs, reduced sphere formation as well as pluripotency-related genes expression (*SOX2*, *OCT4* and *NANOG*) in pancreatic cells *in vitro*[77]. These results suggest that pharmacological agents modulating PPARs activity could represent interesting compounds in order to target CSCs.

Several studies have demonstrated the importance of LDs and the associated lipase HSL in invasion and metastasis regulation, with special relevance in pancreatic cancer [83]. For instance, oncogenic KRAS down-regulates HSL to control lipid storage and utilisation, leading to LD accumulation and tumour invasion[84,85]. Disruption of the KRAS-HSL axis or overexpression of HSL reduces lipid storage and suppresses invasive migration *in vitro* and metastasis *in vivo*[83,84]. Interestingly, Mitra *et al*[86] demonstrated by Raman spectrometry that circulating tumour cells isolated from the peripheral blood of patients with metastatic prostate cancer, accumulated LDs[86], further strengthening the relation between metastasis and LD accumulation.

Increasing evidence links lipid metabolism with chemoresistance in different cancer types[74]. For instance, FAO-derived adenosine triphosphate has been shown to drive

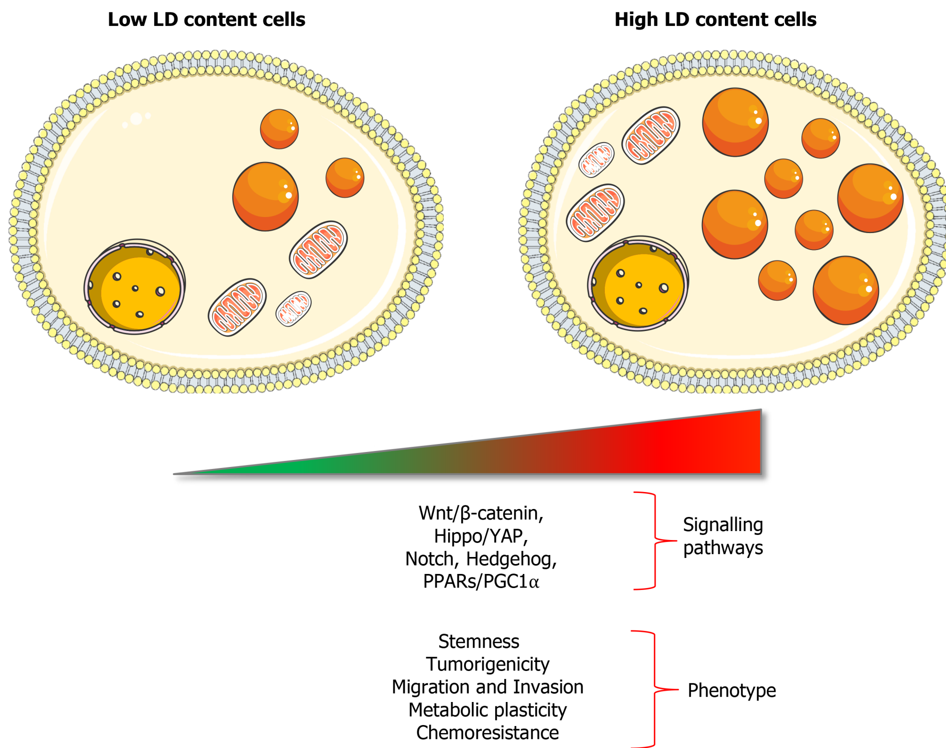

Figure 2 Features of cells with high content of lipid droplets. Cells with high lipid droplets (LD) content show activation of different signalling pathways such as Wnt/β-catenin, Hippo/Yes-associated protein, Notch, Hedgehog and PPARs/PGC-1α. Increased LD content has been linked to aggressive phenotypes in tumour cells, such as stemness, tumorigenicity, migration and invasion, metabolic plasticity and chemoresistance. LD: Lipid droplets; PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPARs: Peroxisome proliferator-activated receptors; YAP: Yes-associated protein.

chemoresistance in breast cancer and leukemic stem cells[87,88]. In addition, Incio *et al* [89] showed that 5-Fluorouracil (5-FU) uptake and efficacy in pancreatic cancer cells decreased significantly in an obese context, indicating that large obesity-caused accumulation of LDs resulting from obesity can reduce drug delivery and chemotherapy efficiency.

The contribution of LDs to chemoresistance is twofold: On the one hand, intrinsic presence of LDs has been widely reported to be a characteristic of chemoresistant cancer cell lines[68,69,74,76]. For instance, prostate cancer cells survive androgen deprivation therapy by metabolising lipids present in LDs[90]. On the other hand, chemotherapy treatments may induce *de novo* LD biogenesis. For example, doxorubicin and 5-FU induced TAG biosynthesis, accumulated in LDs in human colon carcinoma cells[74,91]. Moreover, direct or indirect pharmacological inhibition of FAO or OXPHOS is sufficient to drive LD formation in cancer cells[74]. Indeed, treatment with the c-MYC/Max inhibitor 10058-F4 induced LD accumulation resulting from mitochondrial dysfunction[92]. Interestingly, a combination of both LD presence and accumulation has been described in colorectal cancer cells. For instance, high LD content identified cancer cell lines with increased chemoresistance to 5-FU and oxaliplatin. These cells further accumulated LDs in response to chemotherapy in a process facilitated by lysophosphatidyl-choline acyltransferase 2 (LPCAT2), an LD-associated enzyme essential for phosphatidylcholine synthesis[93]. An elevated expression of LPCAT2 prevented chemotherapy-induced ER stress, further highlighting the protective role of LDs against cellular stresses[74,93]. Importantly, it has been recently reported that LDs can also act as a sink to sequester hydrophobic compounds impairing drug-induced apoptosis, resulting in chemoresistance of cancer cells[68,69].

CONCLUSION

Even if our knowledge about the mechanisms by which LDs support cancer stemness is still very limited, it seems clear now that high levels of LDs are strongly associated with cancer aggressiveness and chemotherapy resistance in different tumour types.

Considering this, measurement of LD accumulation could be potentially used as a prognostic biomarker, also with predictive value in terms of treatment response to conventional therapies. A deeper understanding of the molecular mechanisms dictating their implication in essential processes of the CSC biology, such as tumorigenicity, metastatic spread and chemoresistance, should pave the way to discover novel LD-related targets and therapeutic approaches for more effective cancer treatment.

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MINIREVIEWS

Mesenchymal stem cells and COVID-19: What they do and what they can do

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Abstract

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or coronavirus disease 2019 (COVID-19) pandemic has exhausted the health systems in many countries with thousands cases diagnosed daily. The currently used treatment guideline is to manage the common symptoms like fever and cough, but doesn't target the virus itself or halts serious complications arising from this viral infection. Currently, SARS-CoV-2 exhibits many genetic modulations which have been associated with the appearance of highly contagious strains. The number of critical cases of COVID-19 increases markedly, and many of the infected people die as a result of respiratory failure and multiple organ dysfun-

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ction. The regenerative potential of mesenchymal stem cells (MSCs) has been extensively studied and confirmed. The impressive immunomodulation and anti-inflammatory activity of MSCs have been recognized as a golden opportunity for the treatment of COVID-19 and its associated complications. Moreover, MSCs regenerative and repairing abilities have been corroborated by many studies with positive outcomes and high recovery rates. Based on that, MSCs infusion could be an effective mechanism in managing and stemming the serious complications and multiple organ failure associated with COVID-19. In the present review, we discuss the commonly reported complications of COVID-19 viral infection and the established and anticipated role of MSCs in managing these complications.

Key Words: SARS-CoV-2; COVID-19; Mesenchymal stem cells; Inflammation; Acute respiratory distress syndrome; Pulmonary fibrosis; Pneumonia; Renal injury; Cardio-vascular diseases; Regeneration; Anti-inflammatory

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Core Tip: Coronavirus disease 2019 (COVID-19) pandemic continues its rampant spread as more vengeant strains emerged in many countries. Severe cases of COVID-19 have been complicated by respiratory failure and multiple organ dysfunction with high mortality rate. Mesenchymal stem cells regenerative and anti-inflammatory abilities can be an innovative approach in repairing the damaged organs and improve the survival rate for critically ill COVID-19 patients.

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INTRODUCTION

The 2020 severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), coronavirus disease 2019 (COVID-19) pandemic catastrophe continues to strike many countries severely with million cases and thousands of death reported daily by the World Health Organization. This disease urged many clinicians and researchers to sprint to find effective treatments so as to control and manage the rampant spread of COVID-19 virus worldwide. Despite the fact that COVID-19 is primarily a pulmonary disease, it can hit other organs leading to hematological, hepatic, neurological, cardiac and renal complications[1]. There are no approved remedy to treat COVID-19 and its associated complications. There are many treatment recommendations that are considered as a "leap-of-faith" approach to save the life of many COVID-19 patients[2]. The most commonly used medications to treat symptomatic COVID-19 patients are oxygen, corticosteroids, remdesivir, chloroquine, hydroxychloroquine, lopinavir/ritonavir, nitazoxanide, vitamin super B-complex, zinc and vitamin D[2]. All these suggested medications have many limitations, including: (1) The lack of information regarding the initiation and duration of treatment regimen; (2) Many studies that have been conducted to evaluate the efficacy of these treatments recruited young patients who had relatively asymptomatic disease; (3) The long-run side effects have not been addressed yet; and (4) There is a dearth of data regarding the effect of these treatments in preventing post-infection complications which can be serious and life-threatening[2,3]. Recently, mesenchymal stem cells (MSCs) have been used as one of the promising therapeutic strategies for COVID-19 symptoms and complications[4]. MSCs are one of the most trustworthy stem cells in regenerating and repairing the damages of many organs. MSCs can be isolated and transplanted even in unrelated donor-recipient due to their hypoimmunogenic characteristics[5]. MSCs can repair and restore the function of many injured tissues and improve the quality of life for patients with chronic disorders[5]. MSCs exert their therapeutic effects *via* different mechanisms[5]. MSCs have the ability to subdue severe inflammation by releasing anti-inflammatory factors

such as interleukin-10 (IL-10), IL-4, Indoleamine 2, 3-dioxygenase (IDO), prostaglandin E2 (PGE2), and Heme oxygenase-1 (HO-1)[6]. Furthermore, MSCs release many anti-fibrotic factors, including Transforming growth factor beta-1 (TGF-β1), matrix metallo-protein (MMP) 1, 2 and 9, tissue inhibitor of metalloproteinases-1 (TIMP1) and Collagen Type III Alpha 1 Chain (COL3A1)[7]. MSCs can also promote the angio-genesis and proliferation of cells mediated by releasing many growth factors such as vascular endothelial growth factor (VEGF), Insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF), and platelet-derived growth factor (PDGF)[8]. As MSCs are multipotent stem cells, they can differentiate into many cell types, including cardiomyocytes-like cells, adipocytes, chondrocytes, Clara cells or alveolar cells, endothelial and epithelial cells, which can restore the normal function of impaired organs[9]. The encouraging therapeutic outcomes of MSCs transplantation are expected to be effective in treating COVID-19 complications and decreasing the death rate even in seriously ill patients. Numerous preclinical and clinical trials have been carried out to study the role of MSCs in alleviating COVID-19 symptoms and complic-ations, including acute respiratory distress syndrome (ARDS), pneumonia, pulmonary fibrosis, systemic inflammation, hypercoagulability, and cardiovascular complications. The search for effective COVID-19 management strategies continues to evolve, therefore, in the current review article, we discussed the research outcomes and therapeutic potential of MSCs for COVID-19 patients to provide an updated reference for many clinicians and scientific researchers.

COVID-19 INFLAMMATORY CASCADE AND THE ANTI-INFLAMMATORY ROLE OF MSCS

Current understanding of SARS-CoV-2 mechanisms suggests a central role for exaggerated activation of the innate immune system as an important contributor to COVID-19 adverse outcomes. COVID-19 progression can be divided into three distinct phases, including: (1) Early infection phase, wherein the virus infiltrates host cells in the lung parenchyma; (2) Pulmonary phase, in which viral propagation causes lung tissue injury as the host immune response is activated; and (3) The inflammatory cascade which is triggered by pathogen-associated molecular patterns (*i.e.*, viral RNA) and damage-associated molecular patterns (DAMPs, *i.e.*, cellular debris is released during pyroptosis) are exposed during active viral replication and release[10]. The inflammatory response plays a crucial role in the clinical manifestations and subsequent complications of COVID-19. Post SARS-CoV-2 entry, host factors trigger an immune response against the virus, which, if it is left uncontrolled, may result in pulmonary tissue damage, pulmonary functional impairment, and reduced lung capacity[11]. In addition to respiratory failure, other complicated feature among patients with severe COVID-19 infection is a sudden decline of the patient's health status approximately two weeks after onset. Continuous infiltration of monocytes and macrophages accompanied by extremely high levels of inflammatory response leads to atrophy of the spleen and lymph nodes, along with reduced lymphocytes in lymphoid organs, hypercoagulability, thrombosis, and multiple organ damage[12]. Cytokine storm syndrome (CSS) has been proposed as underlying the etiology of respiratory failure and multiple organ impairment in patients with COVID-19[13]. CSS is delineated as a significant pro-inflammatory cytokines gush that leads to inflam-matory cells recruitment and widespread tissue damage. High levels of the cytokines have been reported, IL-2, IL-7, IL-10, granulocyte colony-stimulating factor, interferon γ-induced protein 10, monocyte chemoattractant protein 1 (MCP1)[13]. The dispropor-tionately high levels of these cytokines were found to be associated with more severe forms of COVID-19 and increased need for intensive care units (ICU) admission.

MSCs remarkable immunomodulatory capacity is one of the most important therapeutic mechanism elicited by MSCs[14]. MSCs immunomodulation abilities can be effective through controlling both innate and adaptive immune systems[14]. Therefore, MSCs have the aptitude to "turn on and turn off" the immune system based on the body needs. Numerous studies on the immune regulation potential of MSCs reported a potent ability of MSCs to control and regulate the functions of different types of immune cells, including B lymphocytes, natural killer (NK) cells, dendritic cells, macrophages, and T lymphocytes by secreting many immunomodulatory factors, primarily TGF-β, HGF, IL-10, IDO, and PGE2[15-17]. Many studies verified the usefulness of MSCs to treat autoimmune diseases and inflammation. There is a lot of preclinical and clinical evidence for the anti-inflammatory effect of MSCs in mitigating virally engendered lung injury and mortality in mice. Several studies have illustrated

that MSCs are capable of significantly reducing acute lung injury (ALI) supervening the infection of H9N2 and H5N1 viruses by decreasing the levels of pro-inflammatory cytokines and chemokines, as well as lessening the recruitment of inflammatory cells into the lungs[18]. Applying MSCs to interfere in endotoxin liposaccharide (LPS)-induced ALI mouse model proved that MSCs can remarkably lead to reduction of inflammatory cell infiltration in lung tissue, alleviate inflammation, and regenerate the damaged lung tissues[18]. To date, there is limited published literature regarding the potential efficacy of MSCs in COVID-19 disease. In February 2020, a clinical study from China has been published in which they evaluated a single dose infusion of bone marrow (BM) MSCs (obtained commercially) in seven COVID-19 patients aged 45 to 65[17]. The recruited patients, who were either in critical condition or had mild disease, did not improve with the treatment protocol. At 14 d post-infusion, all critically ill patients were weaned off mechanical ventilation. These positive outcomes regarding the clinical efficacy of infused MSCs were also accompanied by marked decrease in the levels of C-reactive protein, NK cells, T-cells, and tumor necrosis factor-α (TNF-α). Over-activated cytokine-secreting immune cells, CXCR3+CD4+ T cells, CXCR3+CD8+ T cells, and CXCR3+ NK cells were not existent after 3-6 Dy post-infusion[17]. In May 2020, the results of a clinical trial conducted in Liaocheng People's Hospital in China, using human umbilical cord (UC) Wharton's jelly-derived MSCs was published[19]. In the trial, a single female patient was involved who tested positive for COVID-19. With standard treatment, the patient vital physical signs improved substantially, but after that the patient took a turn for the worse. Considering the severe organ injury caused by an inflammatory response, human UC Wharton's jelly-derived MSCs from a healthy donor was intravenously transfused to the patient. Two days after the infusion, the pulmonary function and symptoms of the patient with COVID-19 pneumonia significantly improved, and chest computerized tomography (CT) imaging showed a great amelioration. The patient recovered rapidly and was discharged 7 days after treatment[19]. The results showed that the frequency of CD3+ T, CD4+ T and CD8+ T cells increased, and serum C-reactive protein, IL-6 and TNF-α levels were reduced. Six days after MSC treatment, the patient became negative for SARS-CoV-2. Therefore, therapy based on the use of Wharton's jelly-derived MSCs may be effective in the treatment of patients with COVID-19[19].

Liang *et al*[20] and colleagues reported that transplantation of human UC-derived MSCs could modulate the immune response and promote the functional recovery in a 65-year-old female patient with critically ill COVID-19 and severe complications such as respiratory failure and multiple organ failure. The patient received three doses of allogeneic UC-MSCs intravenously at day 9 of infection which were followed by two infusions with an interval of three days. Following the second dose, the vital signs were improved, and she did not require the ventilator. Two days after the third dose, she was transferred out of the ICU[20]. After the administration of UC-MSCs, many clinical indexes and symptoms of the patient were improved. The counts of CD3+ T cell, CD4+ T cell, and CD8+ T cell remarkably increased to the normal level indicating the reversal of lymphopenia, which is a common feature of the COVID-19 patients and is associated with disease severity and mortality. The patient showed a significant decreased level of C-reaction protein, white blood, neutrophil, and alanine aminotransferase/aspartate aminotransferase along with increased level of total lymphocyte count. The study also suggested that the infusion of thymosin α1 will greatly enhance the immunomodulation potential of MSCs.

The safety of human UC-derived MSCs infusions for the treatment of patients with moderate and severe COVID-19 pulmonary disease was evaluated. A total of 18 patients were enrolled in clinical trial, nine of whom (five with moderate disease and four with severe disease) received three cycles of UC-derived MSCs treatment. The patients in the MSCs group displayed a reduction of serum IL-6. Moreover, this trial demonstrated that transplantation of MSCs in patients with COVID-19 was safe, and no serious adverse events were reported[21]. The data show that intravenous UC-derived MSCs infusion in patients with moderate and severe COVID-19 is safe and well tolerated.

The published clinical study and case report have demonstrated that intravenous transplantation of MSCs was safe and effective in treating COVID-19, and remarkably, MSCs may be resistant to virus infection, whereas more clinical trials with larger samples are warranted for more convincing evidence.

COVID-19 RESPIRATORY COMPLICATIONS AND MSCS

COVID-19 pneumonia and MSCs

The most common manifestations of COVID-19 primarily target the respiratory system. According to a large cohort study from China, 81% of COVID-19 patients showed mild to moderate symptoms, including dry cough and moderate pneumonia, 14% had severe presentation, such as dyspnea, hypoxia or more than 50% lung involvement on imaging, and 5% were in critical condition manifested by having respiratory failure, shock, or multi-organ system dysfunction[22]. Besides being the first target for SARS-CoV-2 virus, the respiratory system is also involved in the transmission of SARS-CoV-2 virus, which occurs through respiratory droplets emitted *via* sneezing and coughing. SARS-CoV-2 virus was found to be stable in aerosols and different surface materials, and was detected in stool, tears and conjunctival secretions [23]. These can add to certain extent to its transmission. To enter the host organism, the SARS-CoV-2 virus passes through the mucous membranes of the nose and larynx, gaining access to the respiratory tract and eventually resides in the lungs. The lung alveolar epithelial cells are considered the site where the spike proteins of the virus bind to the cell surface angiotensin converting enzyme 2 (ACE2) receptors[23]. This leads to the activation of various proteases, which cleave the spike proteins at a site close to the S1/S2 subunit boundary, inducing viral fusion and subsequent internalization by endocytosis[24]. Some patients with COVID-19 pneumonia rapidly progress to critical illness and ARDS[25]. ARDS is the most severe form of ALI which is a form of diffuse alveolar injury. ARDS is defined as "an acute condition characterized by bilateral pulmonary infiltrates and severe hypoxemia (PaO_2/FiO_2 ratio < 200) in the absence of evidence for cardiogenic pulmonary edema"[26].

COVID-19 ARDS shares mechanistic and pathophysiologic properties with influenza A virus-induced pneumonia. The presence of viral RNA in the cytoplasm of host epithelial cells stimulates antiviral pathways which then activate a number of molecular and cellular mechanisms of immunity[27]. This includes elevated serum cytokines (especially IL-1β, IL-6 and TNF-α), impaired interferon I and III responses, cytokines storm and peripheral lymphopenia[28,29]. Although these immune processes eliminate the virus, they also cause prolonged inflammation and destruction of the host alveolar epithelial cells, which result in severe lung injury and ARDS.

Compared to ARDS caused by other pathologies, the ARDS induced by SARS-CoV-2 seems to be caused by an injury to the alveolar epithelial cells with almost no effect is exerted on endothelial cells. This produces less exudation and, hence, dry cough. Clinical symptoms are often unpredictable and inconsistent with the severity of laboratory and imaging findings. The time of the onset of ARDS in COVID-19 cases is 8-12 d, which is longer compared to other respiratory conditions (up to 7 d)[25]. Mortality associated with COVID-19 is attributable to the development of pneumonia-induced ARDS, consequently, it would be of paramount importance to find effective prevention and treatment strategies for pneumonia-induced ARDS complication in COVID-19 patients[27]. MSCs are known to be an excellent candidate for cell therapy that targets lung diseases. MSCs had been successfully tested in the setting of different lung disease where they regulated the immune response by suppressing infiltrated cells and decreasing edema[30]. Plenty of studies have investigated the effects of MSCs in animal models of infection-induced ALI/ARDS. A recently published meta-analysis found that MSCs can reduce lung injury score of ALI, improve animal's survival, decrease lung edema and improve lung compliance. In addition, MSCs reduce levels of pro-inflammatory factors such as IL-1β, IL-6 and TNF-α, while increasing the level of anti-inflammatory IL-10[31]. Comparable results were observed in human clinical trials. In an open-label study in 2013, administration of allogeneic menstrual-blood-derived MSCs lowered the death rate in patients with influenza H7N9-induced ARDS, with no significant undesirable effects were reported in the treated patients over a five-year follow-up period[32]. Other clinical trials[33-35] failed to prove the therapeutic effectiveness of MSCs in ARDS due to the limited number of recruited patients or inappropriate dose of MSCs. However, they confirmed the safety of such therapy; as no MSC-related hemodynamic, respiratory adverse events, or infusion toxicities were reported. Also, there were no significant differences in the overall number of adverse events between the treatment and control groups.

Due to the lack of effective therapies against COVID-19 virus complications, and based on the promising results of many preclinical and clinical studies of MSCs in the setting of ARDS, a number of patients with severe COVID-19 were empirically treated with MSCs and their cases were reported and published[19,20,36-41]. The majority of these cases involved male patients older than 50 and were reported in China. The source of MSCs injected included Wharton's jelly, UC blood, and BM. The route of

MSCs administration was intravenous infusions, and in one of the cases having brain manifestations, MSCs were delivered intrathecally[36]. Indeed, IV infusion is the preferred route of administration of UC-MSCs since most of the infused cells will be trapped in the lungs, the organ most affected by COVID-19[4]. All patients received other recommended lines of therapy, such as antivirals, antibiotics and/or corticosteroids. There was one patient who was treated with both convalescent plasma and MSCs[39]. In all reported cases, MSCs therapy caused no adverse effects, and patients showed complete resolving of COVID-19 symptoms.

The database clinicaltrials.gov lists 71 clinical trials of MSCs in COVID-19, which are summarized in Table 1. Eight of these trials were completed and their results disclosed. The outcome of one clinical trial has been reported on the same website[42], while the results of a few others have been published. Meng and colleagues, for example, reported the findings of a non-randomized phase 1 clinical trial which enrolled 18 patients with moderate to severe COVID-19. The patients were divided into a control and an UC-MSCs treatment group. No serious adverse events were reported, although two patients receiving UC-MSCs developed transient facial flushing and fever, and one developed transient hypoxia. Treatment with UC-MSCs decreased the need for mechanical ventilation, repaired lung pathological changes and improved pulmonary functions[21]. Lanzoni *et al*[43] and colleagues conducted a double-blind, phase 1/2a, controlled trial that recruited 24 patients with COVID-19-induced ARDS. The study was randomized to either the UC-MSCs treatment or the control group. UC-MSCs infusions were found to be safe and remarkably improved the patients' survival and shortened the recovery time[43]. An open-label, individually randomized, controlled trial was carried out by Shu *et al*[44] which included 12 patients in the treatment group (UC-MSCs) and 29 in the control group. The treatment group had significantly shorter recovery time, rapid symptomatic improvement, faster alleviation of lung inflammation, and without any safety issues. In Spain, 13 patients with severe COVID-19 on mechanical ventilation were treated with adipose tissue-derived MSCs (AD-MSCs). The therapy was associated with clinical, radiological and ventilation improvements[45]. Leng *et al*[17] studied the therapeutic effects of injecting ACE2- MSCs in 7 patients with severe COVID-19 pneumonia. The patients exhibited significant clinical improvements without observed adverse effects.

Other studies have addressed the therapeutic use of MSCs-derived components, such as exosomes, in COVID-19 patients. Sengupta and colleagues run a non-randomized open-label cohort study to assess the safety and efficacy of exosomes derived from allogeneic BM-MSCs for the treatment of severe COVID-19 in 24 patients. No adverse effects related to infusion were reported. Treatment was associated with better clinical status and oxygenation, improvement in laboratory absolute neutrophils count, and a decline in acute phase reactants[46]. The above-mentioned results indicate that MSCs may play a pivotal role in the treatment of COVID-19 related respiratory complications due to their excellent safety profile and a wide range of therapeutic outcomes as improvement in the clinical, radiological and immunological status of COVID-19 concomitant pneumonia.

COVID-19-ARDS and MSCs

COVID-19, that is concomitant with ARDS, emerged as a primary concern all over the world. ARDS is a life-threatening severe lung condition that causes damage to the pulmonary capillary endothelium and alveolar epithelium. The ARDS, which is caused by infection and other mechanical stimulation, allows fluid to leak into the lungs. As a result, breathing becomes difficult as the lungs turn non-functional and cannot supply the body's vital organs with enough oxygen. Most people who get ARDS are already at the hospital for trauma or illness[47]. The Spike proteins (S proteins) of SARS-CoV-bind the ACE2 in the alveolar cells which is catalyzed by a transmembrane protease serine 2 (TMPRSS2) protease in type II alveolar epithelial cells[48,49]. The high binding affinity of SARS-CoV-2 Spike proteins for ACE2 is the main determinant of the replication rate of SARS-CoV-2 and the cause of the severity of COVID-19 disease[50].

Various animal studies and early Phase I/II clinical trials have espoused the tremendous potential of MSCs therapy in treating the ARDS. SARS-CoV-2 infects type II alveolar epithelial cells or other target cells that express ACE2. The keratinocyte growth factor (KGF), which is secreted by MSCs, can decrease injury and improve the proliferation and regeneration of alveolar epithelial cells by rising surface-active substances, including MMP-9, IL-1Ra, and granulocyte-macrophage colony-stimulating factor (GM-CSF)[51]. In addition, it has been reported that MSCs can improve gas exchange and reduce the levels of bronchoalveolar lavage fluid' chemokines and cytokines, including GM-CSF, MIG, IL-1α, IFN-γ, IL-6, and TNF-α

Table 1 Summary of clinical trials listed in *clinicaltrials.org* involving mesenchymal stem cells in the treatment of coronavirus disease 2019

	NCT number	Title	Phases	Study designs	Cells/products	No. of cells	No. of infusions	Delivery route	No. of patients	Status	Location
1	NCT04366271	Clinical trial of allogeneic mesenchymal cells from umbilical cord tissue (UC) in patients with COVID-19	Phase 2	Randomized/parallel assignment/open label	UC-MSCs	Not reported	1	Not reported	106	Recruiting	Spain
2	NCT04444271	Mesenchymal stem cell infusion for COVID-19 infection	Phase 2	Randomized/parallel assignment/open label	BM-MSCs	2×10^6/kg	1 or 2	IV	20	Recruiting	Pakistan
3	NCT04416139	Mesenchymal stem cell for acute respiratory distress syndrome (ARDS) due for COVID-19	Phase 2	Non-randomized/parallel assignment/open label	UC-MSCs	1×10^6/kg	1	IV	10	Recruiting	Mexico
4	NCT04713878	Mesenchymal stem cells therapy in patients with COVID-19 pneumonia	Not applicable	Randomized/parallel assignment/open label	MSCs	1×10^6/kg	3	IV	21	Completed	Turkey
5	NCT04352803	Adipose mesenchymal cells for abatement of SARS-COV-2 respiratory compromise in COVID-19 disease	Phase 1	Non-randomized/sequential assignment/open label	Autologous AD-MSCs	5×10^5/kg	1	IV	20	Not yet recruiting	United States
6	NCT04565665	Cord blood-derived mesenchymal stem cells (MSCs) for the treatment of COVID-19 related ARDS	Phase 1	Randomized/parallel assignment/open label	UC-MSCs	Not reported	1 or 2	IV	70	Recruiting	United States
7	NCT04429763	Safety and efficacy of MSCs in the management of severe COVID-19 pneumonia	Phase 2	Randomized/parallel assignment/masking: Triple	UC-MSCs	1×10^6/kg	1	IV	30	Not yet recruiting	Colombia
8	NCT04456361	Use of MSCs in ARDS caused by COVID-19	Early Phase 1	Single group assignment/open label	WJ-MSCs	1×10^8	1	IV	9	Active, not recruiting	Mexico
9	NCT04315987	NestaCell® MSC to treat patients with severe COVID-19 pneumonia	Phase 2	Randomized/parallel assignment/quadruple masking	NestaCell®	2×10^7/kg	4	IV	90	Not yet recruiting	Brazil
10	NCT04366323	Clinical trial to assess the safety and efficacy of intravenous administration of allogeneic adult MSCs of expanded adipose tissue in patients with severe pneumonia due to COVID-19	Phase 1/phase 2	Randomized/parallel assignment/open label	AD-MSCs	8×10^7	2	IV	26	Active, not recruiting	Spain
11	NCT04611256	MSCs in patients diagnosed with COVID-19	Phase 1	Randomized/parallel assignment/open label	AD-MSCs	1×10^6/kg	2	IV	20	Recruiting	Mexico
12	NCT04625738	Efficacy of infusions of MSC from Wharton jelly in the SARS-Cov-2 (COVID-19) related ARDS	Phase 2	Randomized/parallel assignment/masking: Quadruple	WJ-MSCs	2 × 10/kg	3	IV	30	Not yet recruiting	France
13	NCT04252118	MSC treatment for pneumonia patients infected with COVID-19	Phase 1	Non-randomized/parallel assignment/open label	MSCs	3×10^7/kg	3	IV	20	Recruiting	China
14	NCT04346368	Bone marrow-derived MSC treatment for severe patients with COVID-19	Phase 1/phase 2	Randomized/parallel assignment/masking: single (participant)	BM-MSCs	1×10^6/kg	1	IV	20	Not yet recruiting	China
15	NCT04313322	Treatment of COVID-19 patients using Wharton's jelly-MSCs	Phase 1	Single group assignment/open label	WJ-MSCs	1×10^6/kg	3	IV	5	Recruiting	Jordan

16	NCT04288102	Treatment with human UC-derived MSCs for severe COVID-19	Phase 2	Randomized/parallel assignment/double-blind/placebo controlled	UC-MSCs	4×10^7/kg	3	IV	100	Completed	China
17	NCT04629105	Regenerative medicine for COVID-19 and flu-elicited ARDS using longeveron MSCs (LMSCs) (recover)	Phase 1	Randomized/parallel assignment/double-blind	LMSCs	1×10^8	3	IV	70	Recruiting	United States
18	NCT04382547	Treatment of COVID-19 associated pneumonia with allogenic pooled olfactory mucosa-derived MSCs	Phase 1/phase 2	Non-randomized/parallel assignment/open label	Allogenic pooled olfactory mucosa-derived MSCs	Not reported	Not reported	IV	40	Enrolling by invitation	Belarus
19	NCT04336254	Safety and efficacy study of allogeneic human dental pulp MSCs to treat severe COVID-19 patients	Phase 1/phase 2	Randomized/parallel assignment/masking: Triple	Allogeneic human dental pulp stem cells	3×10^7/kg	3	IV	20	Recruiting	China
20	NCT04527224	Study to evaluate the efficacy and safety of AstroStem-V in treatment of COVID-19 pneumonia	Phase 1/phase 2	Single group assignment/open label	AstroStem-V (AD-MSCs)	Not reported	Not reported	Not reported	10	Not yet recruiting	NA
21	NCT04273646	Study of human UC MSCs in the treatment of severe COVID-19	Not applicable	Randomized/parallel assignment/open label	UC-MSCs	0.5×10^6/kg	4	IV	48	Not yet recruiting	China
22	NCT04302519	Novel coronavirus induced severe pneumonia treated by dental pulp MSCs	Early phase 1	Single group assignment/open label	Dental pulp MSCs	1×10^6/kg	3	IV	24	Not yet recruiting	China
23	NCT04728698	Study of intravenous administration of allogeneic adipose-derived MSCs for COVID-19-induced acute respiratory distress	Phase 2	Randomized/parallel assignment/masking: Double	COVI-MSC (AD-MSCs)	1-1.5 $\times 10^6$/kg	1	IV	100	Not yet recruiting	United States
24	NCT04457609	Administration of allogenic UC-MSCs as adjuvant therapy for critically-ill COVID-19 Patients	Phase 1	Randomized/parallel assignment/masking: Triple	UC-MSCs	1×10^6/kg	1	IV	40	Recruiting	Indonesia
25	NCT04348435	A randomized, double-blind, placebo-controlled clinical trial to determine the safety and efficacy of Hope Biosciences allogeneic adipose-derived mesenchymal stem cell therapy (HB-adMSCs) to provide protection against COVID-19	Phase 2	Randomized/parallel assignment/masking: Quadruple	AD-MSCs	G_1: 2×10^8; G_2: 1×10^8; G_3: 5×10^7	5	IV	100	Enrolling by invitation	United States
26	NCT04349631	A clinical trial to determine the safety and efficacy of HB-adMSCs to provide protection against COVID-19	Phase 2	Single group assignment/open label/primary purpose: Prevention	AD-MSCs	Not reported	5	IV	56	Active, not recruiting	United States
27	NCT04366063	MSC therapy for SARS-CoV-2-related ARDS	Phase 2/phase 3	Randomized/parallel assignment/open label	MSCs and EVs from MSCs	G1: 1×10^8 MSC; G2: 1×10^8 MSCs + EVs	2	IV	60	Recruiting	Iran
28	NCT04339660	Clinical research of human MSCs in the treatment of COVID-19 pneumonia	Phase 1/phase 2	Randomized/parallel assignment/masking: Triple	UC-MSCs	1×10^6/kg	1	IV	30	Recruiting	China
29	NCT04428801	Autologous adipose-derived stem cells (AdMSCs) for COVID-19	Phase 2	Randomized/parallel assignment/masking: Double	Autologous AD-MSCs	2×10^8	3	IV	200	Not yet recruiting	United States
30	NCT04573270	MSCs for the treatment of COVID-19	Phase 1	Randomized/single group assignment/masking: triple	PrimePro (UC-MSCs)	Not reported	1	IV	40	Completed	United States
31	NCT04490486	UC derived MSCs versus placebo to treat acute	Phase 1	Randomized/parallel	UC-MSCs	1×10^8	2	IV	21	Not yet	United

		pulmonary inflammation due to COVID-19		assignment/masking: Double						recruiting	States
32	NCT04355728	Use of UC-MSCs for COVID-19 patients	Phase 1/Phase 2	Randomized/parallel assignment/masking: triple	UC-MSCs + heparin	1×10^8	2	IV	24	Completed	United States
33	NCT04371601	Safety and effectiveness of MSCs in the treatment of pneumonia of COVID-19	Early Phase 1	Randomized/parallel assignment/open label	UC-MSCs	1×10^6/kg	4	IV	60	Active, not recruiting	China
34	NCT04522986	An exploratory study of adr-001 in patients with severe pneumonia caused by SARS-CoV-2 Infection	Phase 1	Single group assignment/open label	AD-MSCs (ADR-001)	1×10^8	4	IV	6	Not yet recruiting	Japan
35	NCT04390152	Safety and efficacy of intravenous Wharton's jelly derived MSCs in ARDS due to COVID 19	Phase 1/Phase 2	Randomized/parallel assignment/masking: Quadruple	WJ-MSCs	5×10^7	2	IV	40	Recruiting	Colombia
36	NCT04461925	Treatment of coronavirus COVID-19 pneumonia (pathogen SARS-CoV-2) with cryopreserved allogeneic P_MMSCs and UC-MMSCs	Phase 1/phase 2	Non-randomized/parallel assignment/open label	Placenta-derived MSCs	1×10^6/kg	3	IV	30	Recruiting	Ukraine
37	NCT04348461	BAttLe against COVID-19 using mesenchymal stromal cells	Phase 2	Randomized/parallel assignment/masking: Quadruple	Allogeneic and expanded AD-MSCs	1.5×10^6/kg	2	IV	100	Not yet recruiting	Spain
38	NCT04535856	Therapeutic study to evaluate the safety and efficacy of DW-MSC in COVID-19 patients	Phase 1	Randomized/parallel assignment/masking: Quadruple	DW-MSCs	G1: 5×10^7; G2: 10×10^7	1	IV	9	Completed	Indonesia
39	NCT04362189	Efficacy and safety study of allogeneic HB-adMSCs for the treatment of COVID-19	Phase 2	Randomized/parallel assignment/masking: Quadruple	AD-MSCs	1×10^8	4	IV	100	Active, not recruiting	United States
40	NCT04494386	UC lining stem cells (ULSC) in patients with COVID-19 ARDS	Phase 1/Phase 2	Randomized/parallel assignment/masking: Triple	UC-MSCs	1×10^8	1 or 2	IV	60	Recruiting	United States
41	NCT04397796	Study of the safety of therapeutic tx with immunomodulatory MSC in adults with COVID-19 infection requiring mechanical ventilation	Phase 1	Randomized/parallel assignment/masking: Quadruple	BM-MSCs	Not reported	Not reported	Not reported	45	Recruiting	United States
42	NCT04345601	Mesenchymal stromal cells for the treatment of SARS-CoV-2 induced acute respiratory failure (COVID-19 Disease)	Phase 1/Phase 2	Randomized/parallel assignment/open label	MSCs	1×10^8/kg	Up to 2	IV	30	Recruiting	United States
43	NCT04452097	Use of hUC-MSC product (BX-U001) for the treatment of COVID-19 with ARDS	Phase 1/phase 2	Non-randomized/sequential assignment/open label	UC-MSCs	G_1: 0.5×10^6; G_2: 1×10^6; G_3: 1.5×10^6	1	IV	39	Not yet recruiting	United States
44	NCT04492501	Investigational treatments for COVID-19 in tertiary care hospital of Pakistan	Not applicable	Non-randomized/factorial assignment/open label	BM-MSCs (+ TPE and convalescent plasma)	2×10^6/kg	1	IV	600	Completed	Pakistan
45	NCT04377334	MSCs in inflammation-resolution programs of COVID-19 induced ARDS	Phase 2	Randomized/parallel assignment/open label	BM-MSCs	Not reported	Not reported	IV	40	Not yet recruiting	Germany
46	NCT04390139	Efficacy and safety evaluation of MSCs for the treatment of patients with respiratory distress due to COVID-19	Phase 1/phase 2	Randomized/parallel assignment/masking: Quadruple	WJ-MSCs	1×10^6/kg	2	IV	30	Recruiting	Spain

47	NCT04467047	Safety and feasibility of allogenic MSC in the treatment of COVID-19	Phase 1	Single group assignment/open label	MSCs	1 x 10^6/kg	1	IV	10	Not yet recruiting	Brazil
48	NCT04392778	Clinical use of stem cells for the treatment of COVID-19	Phase 1/phase 2	Randomized/parallel assignment/masking: Quadruple	UC-MSCs	3 × 10^6/kg	3	IV	30	Recruiting	Turkey
49	NCT04537351	The MEseNchymal COVID-19 trial: a pilot study to investigate early efficacy of MSCs in adults with COVID-19	Phase 1/phase 2	Randomized/parallel assignment/open label	CYP-001 (Cymerus® MSC)	up to 2 × 10^6/kg	2	IV	24	Recruiting	Australia
50	NCT04361942	Treatment of severe COVID-19 pneumonia with allogeneic mesenchymal stromal cells (COVID_MSV)	Phase 2	Randomized/parallel assignment/masking: Triple	MSCs	1 × 10^6/kg	1	IV	24	Recruiting	Spain
51	NCT04398303	ACT-20 in patients with severe COVID-19 pneumonia	Phase 1/phase 2	Randomized/Parallel Assignment/Double-blind	UC-MSCs and conditioned UC-MSCs	G1: 1 × 10^6 G2: 100 mL conditioned media (MD)	1	IV	70	Not yet recruiting	United States
52	NCT03042143	Repair of ARDS by stromal cell administration (realist) (COVID-19)	Phase 1/phase 2	Randomized/parallel assignment/masking: Quadruple	hUC-derived CD362 enriched MSCs	400 × 10^6	1	IV	75	Recruiting	United Kingdom
53	NCT04269525	UC-derived MSCs treatment for the 2019-novel coronavirus (nCOV) pneumonia	Phase 2	Single group assignment/open label/primary purpose: Prevention	UC-MSCs	9.9 × 10^7	4	IV	16	Recruiting	China
54	NCT04602442	Safety and efficiency of method of exosome inhalation in COVID-19 associated pneumonia	Phase 2	Randomized/parallel assignment/masking: Double	EXO-1 and EXO-2 (exosomes from MSC)	0.5-2 × 10^{10} nanoparticles	20 (2/d for 20 d)	Aerosol inhalation	90	Enrolling by invitation	Russia
55	NCT04437823	Efficacy of intravenous infusions of stem cells in the treatment of COVID-19 patients	Phase 2	Randomized/parallel assignment/open label	UC-MSCs	5 × 10^5/kg	3	IV	20	Recruiting	Pakistan
56	NCT04371393	MSCs in COVID-19 ARDS	Phase 3	Randomized/parallel assignment/masking: Triple	Remestemcel-L	2 × 10^6/kg	2	IV	223	Active, not recruiting	United States
57	NCT04333368	Cell therapy using UC-derived mesenchymal stromal cells in SARS-CoV-2-related ARDS	Phase 1/phase 2	Randomized/parallel assignment/masking: Triple	WJ-UC-MSCs	1 × 10^6/kg	3	IV	47	Active, not recruiting	France
58	NCT04447833	Mesenchymal stromal cell therapy for the treatment of ARDS	Phase 1	Single group assignment/open label	KI-MSC-PL-205 (BM-MSCs)	1-2 × 10^6/kg	1	IV	9	Recruiting	Sweden
59	NCT04491240	Evaluation of safety and efficiency of method of exosome inhalation in SARS-CoV-2 associated pneumonia	Phase 1/phase 2	Randomized/parallel assignment/masking: Double	Drug: EXO-1 and EXO-(exosomes from MSCs)	0.5-2 × 10^{10} nanoparticles	20 (2/d for 20 d)	Aerosol inhalation	30	Completed	Russia
60	NCT04299152	Stem cell educator therapy treat the viral inflammation in COVID-19	Phase 2	Randomized/parallel assignment/masking: Single (care provider)	Stem Cell Educator (from UC-MSCs)	Not reported	1	IV	20	Not yet recruiting	United States
61	NCT04466098	Multiple dosing of mesenchymal stromal cells in patients with ARDS (COVID-19)	Phase 2	Randomized/parallel assignment/masking: Triple	MSCs	3 × 10^8	3	IV	30	Recruiting	United States
62	NCT04524962	Study of descartes-30 in ARDS	Phase 1/phase 2	Single group assignment/open label	Descartes 30 (MSCs RNA-engineered to	Not reported	Not reported	Not reported	30	Recruiting	United States

					secrete a combination of DNases)						
63	NCT04445220	A study of cell therapy in COVID-19 subjects with acute kidney injury who are receiving renal replacement therapy	Phase 1/phase 2	Randomized/parallel assignment/masking: Quadruple	Allogeneic MSCs (SBI-101)	G1: 2.5 × 10^8; G2: 7.5 × 10^8	Not reported	Not reported	22	Recruiting	United States
64	NCT04400032	Cellular immuno-therapy for COVID-19 ARDS vanguard	Phase 1	Non-randomized/sequential assignment/open label	BM-MSCs	G1: 2.5 × 10^7; G2: 5 × 10^7; G3: 9 × 10^7	3	IV	9	Recruiting	Canada
65	NCT04615429	Clinical trial to assess the efficacy of MSC in patients with ARDS due to COVID-19	Phase 2	Randomized/Parallel Assignment/Double-blind	MSCs	1 × 10^6/kg	1	IV	20	Recruiting	Spain
66	NCT04525378	MSC-based therapy in COVID-19-associated ARDS	Phase 1	Randomized/parallel assignment/open label	MSCs	G1: 2.5 × 10^7 G2: 5 × 10^7 G3: 10 × 10^7	G1 and G2: 2 G3: 1	IV	20	Recruiting	Brazil
67	NCT04399889	hCT-MSCs for COVID19 ARDS	Phase 1/phase 2	Randomized/single group assignment/masking: Quadruple	Human cord tissue MSCs	1 × 10^6/kg	3	IV	30	Recruiting	United States
68	NCT04445454	Mesenchymal stromal cell therapy for severe COVID-19 infection	Phase 1/phase 2	Single group assignment/open label	BM-MSCs	1.5-3.0 × 10^6/kg	3	IV	20	Recruiting	Belgium
69	NCT04276987	A pilot clinical study on inhalation of MSCs exosomes treating severe novel coronavirus pneumonia	Phase 1	Single group assignment/open label	AD-MSCs-derived exosomes	2 × 10^8 nanovesicles	5	Aerosol inhalation	24	Completed	China
70	NCT04482699	RAPA-501-Allo Off-the-shelf therapy of COVID-19	Phase 1/phase 2	Randomized/sequential assignment/masking: Double	RAPA-501-ALLO	G1: 4 × 10^7 G2: 1.6 × 10^8	Not reported	Not reported	88	Recruiting	United States
71	NCT04614025	Open-label multicenter study to evaluate the efficacy of PLX-PAD for the treatment of COVID-19	Phase 2	Randomized/parallel assignment/open label	PLX-PAD (placental MSC-like)	2 × 10^7	15	IM	40	Recruiting	Israel

COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; MSCs: Mesenchymal stem cells; UC-MSCs: Umbilical cord mesenchymal stem cells; BM-MSCs: Bone marrow mesenchymal stem cells; AD-MSCs: Adipose tissue mesenchymal stem cells; WJ-MSCs: Wharton's jelly mesenchymal stem cells; TPE: Total plasma exchange; NA: Not available.

[18]. Researching experts elucidated that MSCs treatment expresses vital anti-inflammatory cytokines like IL-1RA, IL-8, and IL-10 and an array of bioactive molecules that stimulate local tissue regeneration by buffering the cytokine and chemokine storm provoked by COVID-19 virus[52]. A published clinical study conducted in Beijing Youan Hospital in China, showed that the transplantation of MSCs improved the clinical outcomes in all recruited COVID-19 elderly patients at day 4 post-MSCs injection without serious adverse effects[17]. Cytokine-secreting immune cells, including CXCR3+CD4+ T cells, CXCR3+CD8+ T cells, and CXCR3+ NK cells, eradicated within 1 wk of MSCs transplantation with a significant reduction in the inflammation biomarkers[17]. MSCs treatment in COVID-19 patients having ARDS were able to subside the over-activated immune system and promote endogenous repair by improving the lung microenvironment[53]. Taking into regard the positive results that have been revealed in some published phase I clinical trials, MSCs will be probably effective in reducing the risk of cytokine storms which cause ARDS and

organs failure in patients with severe COVID-19 disease.

COVID-19 pulmonary fibrosis and MSCs

Many studies interestingly revealed that COVID-19 patients who develop ARDS can survive and might eventually be discharged. On the other hand, a large proportion of COVID-19 patients cannot survive once they develop idiopathic pulmonary fibrosis [54]. Idiopathic pulmonary fibrosis is a progressive lung disease manifested by compromised lung functions with extensive damage to the alveolar cells and capillaries. Patients with severe idiopathic pulmonary fibrosis need lung transplantation to improve pulmonary functions and save their lives[55]. Cytokine storm, which causes oxygen stress and the hyperactive inflammation presenting in the severe stages of COVID-19 infection, is probably the main cause of pulmonary fibrosis. Elevated levels of cytokines such as IL1-β, IL-7, IL-8, IL-9, IL-10, GM-CSF, IFN-γ, MCP1, TNF-α and VEGF have been reported in COVID-19 patients[56-58]. The levels of above-mentioned cytokines with high neutrophil counts are typically more pronounced in patients with severe stages of COVID-19 disease. The development of pulmonary fibrosis in severe cases of COVID-19 is due to the induction of oxidative stress, excessive production of reactive oxygen species (ROS), and the presence of high levels of fibrotic factors mainly TGF-b, FGF, and PDGF[59,60].

MSCs have been introduced as one of the therapeutic approaches in the treatment of COVID-19 pulmonary fibrosis[61]. Golchin *et al*[61] reported that using MSCs immunomodulatory and differentiation abilities can counteract the cytokine storm and prevent lung tissues death and pulmonary fibrosis by regenerating and reconstructing damaged tissues. Similarly, Yang *et al*[62] showed that MSCs transplantation in ALI mouse model significantly reduced lung permeability, attenuated lung epithelium apoptosis, facilitated vascular endothelium (VE)-cadherin recovery, and reduced lung injury. These beneficial outcomes were mediated by VEGF released from the transplanted MSCs. Moreover, Jung *et al*[63] reported that injecting MSCs in a LPS-induced ARDS mouse model resulted in the diminution of neutrophil infiltration, collagen deposition and lung fibrosis. MSCs have specific cytokines counteracting potentials to oppose the viral infection and reduce the spread of pulmonary fibrosis. MSCs secrete anti-inflammatory chemokines and cytokines such as IL-10, and PGE2 which help in preventing pulmonary fibrosis[64]. Preclinical studies on animal models with COVID-19 indicated that MSCs considerably improve the disease symptoms, including pulmonary fibrosis. MSCs can secrete a variety of growth factors, including epidermal growth factor (EGF), KGF, PDGF, fibroblast growth factor (FGF), HGF, and VEGF[65]. These factors can recuperate the inflammatory microenvironment of the lung tissues and promote the endogenous lung repair mechanisms and differentiation. Furthermore, MSCs prevent myofibroblast growth by reducing the levels of TGF-β, TNF-α, type I collagen, type III collagen, hydroxyproline, and serum ceruloplasmin in lung tissues, thereby alleviating pulmonary fibrosis[66]. Based on the findings of previous studies, the administration of MSCs in severe cases of COVID-19 appears to be beneficial in resolving and reducing the mortality rate of life-threatening pulmonary fibrosis.

COVID-19 RENAL COMPLICATIONS AND MSCs

Various renal complications have been reported in hospitalized COVID-19 patients, mainly the electrolyte disturbance and acute kidney injury (AKI)[67]. The incidence of renal complications in COVID-19 patients increases if there is an existing kidney diseases, *e.g.*, chronic kidney disease, or a risk factors for kidney complications, including diabetes mellitus[68]. The presence of AKI in COVID-19 patients is associated with worse prognosis and high death rate[69]. In Europe, the death rate in COVID-19 older than 60 years patients was found to be higher when renal complications existed[70]. In severe cases of COVID-19, the rate of continuous renal replacement therapy was significantly higher in North America and Europe compared to other regions[71]. The proposed mechanism of how COVID-19 can induce renal injury is supposed to be through ACE2[72]. It is already known that SARS-CoV-2 (COVID-19) virus attacks the host cells and binds *via* spike proteins (S-protein) to the extracellular enzymatic domain of ACE2 causing its internalization and down-regulation. Renal tissues contain ample amount of Ang (1-7) peptides that are produced by ACE2 mediated hydrolysis of AngII[73]. A significant decrease in ACE2 and Ang (1-7) has been found in many kidney diseases, including AKI[73]. Additionally, it has been reported that deficiency of ACE2 by either pharmacological

inhibition or genetic knocking out of ACE2 is correlated to the onset of diabetic nephropathy which is manifested by albuminuria and glomerulosclerosis. SARS-CoV-2 mediated down-regulation of ACE2 causes the loss of the beneficial and protective Ang (1-7) peptides and accumulates the AngII[73]. This molecular disturbance in the RAS system by SARS-CoV-2 induces many pathological changes in the kidney, including tubular necrosis and multiple inflammatory infiltrates, endothelial injury and dysfunction, and erythrocytes aggregation which obstructs the capillary lumina (microthrombus formation)[73]. In addition to direct SARS-CoV-2 mediated pathological changes in the kidneys, SARS-CoV-2 invades the alveolar cells and enkindles the activation of many immune cells. These recruited immune cells secrete massive amount of cytokines that circulate systematically inducing endothelial dysfunction and promoting the coagulation and micro thrombus formation resulting in multiple organ failure and septic shock[68]. AKI can occur in severe cases of COVID-19 as a result of this cytokines storm. Interestingly, a study examined SARS-CoV-2 viral load in 22 patients who died from COVID-19. It has been reported that 17 out of 22 autopsies showed high load of SARS-CoV-2 in the kidney indicating high mortality rate when COVID-19 was complicated with AKI[74]. Moreover, the co-expression of ACE2 and protease TMPRSS2 in the podocytes and proximal convoluted tubules increases the amplification rate of SARS-CoV-2 in the kidney cells[74]. MSCs have been corroborated by many studies as an effective treatment option for AKI, especially when they are injected as early as possible. Zhou *et al*[75] found that the transplantation of erythropoietin pretreated BM MSCs in AKI rat model significantly reduced the inflammation and improved the kidney function parameters. Similarly, Zhou *et al*[75] reported that the infusion of human UC MSC-derived exosomes (HucMSC-Ex) in a mouse model of sepsis-associated AKI was able to restore the renal function parameters and decrease inflammation *via* the inhibition of NF-κB activity. HucMSC-Ex transplantation was effective in ameliorating the apoptosis in renal tubular cells and improving survival in mice with sepsis. Cao *et al*[76] reported that the extracellular vesicles derived from MSCs were capable of stimulating mitochondrial antioxidant defense and ATP production in renal ischemia-reperfusion (I/R) mouse model resulting in recovery of kidney function *via* activating the Keap1-Nrf2 signaling pathway. Ko *et al*[77] used a hyperbaric oxygen and autologous adipose-derived MSC combined therapy in SD rat model of renal I/R injury. They found that this combination was effective in reducing the expression of inflammatory markers (MMP-9/TNF-α/NF-κB/ICAM-1) and apoptotic markers (mitochondrial-Bax/cleaved-caspase3/PARP), ameliorating oxidative damage and promoting angiogenesis in renal tubular cells which ultimately improved the kidney injury score and restored many renal function parameters, including the blood urea nitrogen and creatinine. Based on the proved potential of MSCs in alleviating AKI and renal I/R injury, we can anticipate that they might exert comparable therapeutic effects in COVID-19 patients with AKI and other renal complications[77]. Future studies should investigate the effectiveness of MSCs-based cell therapy in COVID-19 patients who developed renal complications. Such therapy could be a worthwhile approach in restoring their kidney functions and decreasing the death rate in this group of patients.

COVID-19 CARDIOVASCULAR COMPLICATIONS AND MSCS

Many patients with COVID-19 were reported to develop acute cardiac injury during the course of the illness. Concomitant presence of cardiac events and COVID-19 increases the mortality rate in COVID-19 patients. COVID-19 cardiovascular complications (CV) include acute myocardial injury, heart failure, pericardial effusion with or without tamponade, arrhythmias, sudden cardiac death, and thrombosis of small and large blood vessels. The clinical reports regarding the severity of COVID-19 were noticeable in patients with previous medical history of underlying CV diseases. Furthermore, many patients infected with COVID-19 developed severe myocardial injury[78], including myocardial dysfunction, cardiomyopathy, arrhythmias and heart failure during the course of critical illness[79-84]. Clinical reports revealed that 38% of patients testing positive for COVID-19 display high levels of cardiac damage biomarkers, such as cardiac troponin and creatinine kinase with significant electrocardiographic changes. Data acquired from multiple clinical centers showed conspicuous correlation between plasma lactate dehydrogenase and chronic kidney levels with COVID-19 severity and the rate of ICU[85].

The proposed mechanisms of the deleterious effect of COVID-19 on the heart are intriguing many clinicians and researchers. Acute myocardial injury caused by SARS-

CoV-2 infection could be due to its high affinity for ACE2 which is extensively expressed in the heart, and is associated with direct myocardial injury and remodeling [22,86]. Another proposed mechanism is linked to cytokine storm. Cytokine storm is triggered by an excessive response of type 1 and type 2 T-helper cells, sympathetic hyperactivity, anemia, and hypoxemic myocardial cells damage caused by respiratory dysfunction [type 2 myocardial infarction (MI)][86]. Myocardial oxygen supply/ demand mismatch as a result of severe pneumonia or ARDS can lead to inadequate supply of oxygen and subsequent myocardial damage[87]. The current treatment regimen, including antiviral medications, corticosteroids, and immunological agents; can also potentiate the risk of developing CV complications. The disturbance of electrolytes can occur in any critical systemic illness, including COVID-19 and can trigger life-threatening arrhythmias. There is a particular concern about the risk of hypokalemia in COVID-19 patients as a result of the interaction between SARS-CoV2 with the RAS system. Hypokalemia is a well-known electrolyte disturbance that is associated with serious arrhythmias[87]. Several studies have demonstrated the possible role of MSCs in CV diseases in non-COVID-19 patients. MSCs can offer the potential as regenerative cells for the CV system where through a paracrine mechanism, they activate endogenous repair mechanisms leading to blood vessel growth *via* angiogenesis, improve cardiomyocytes survival, and reduce cardiomyocytes reactive hypertrophy and fibrosis[88]. Treatment with MSCs may give a clinical benefit to patients due to their regenerative and reparative potential if there are significant myocardial injury and myocardial cell death.

The infusion and transplantation of MSCs have been deemed safe for treating MI patients[89,90]. A study was carried out to evaluate the safety and feasibility of intramyocardial MSCs injection in patients, shortly after AMI during short-term and 5-year follow-up, a safety analysis demonstrated one transient ischemic attack without any adverse events related to MSCs treatment[90].

Furthermore, MSCs injection successfully improves some cardiac functional measures post-MI[91,92]. MSCs were shown to restore endothelial function by increasing endothelial progenitor cells function and regain flow-mediated vasodilation (FMD which is clinically significant for heart failure patients[93].

There is study that has suggested a potential effect for in situ myocardial regeneration in ischemic heart failure by delivering allogeneic mesenchymal precursor cells called immunomodulatory progenitor cells (iMP) *via* intramyocardial route[94]. Intramyocardial implantation of iMP cells with CABG was safe with preliminary evidence of efficacy of improved myocardial contractility and perfusion of non-revascularized territories resulting in a significant reduction in left ventricular scar area at 12 mo after treatment. Clinical improvement was associated with a significant improvement in quality of life at 6 mo post-treatment in all patients[94].

A study focused on dose comparison of allogeneic MSCs in patients with ischemic cardiomyopathy demonstrated that the optimal dose of MSCs was 100 million cells, and this optimal dose increases the ejection fraction in ischemic patients[95].

A meta-analysis of 52 pre-clinical animal studies performed on different animal species such as pig, dog and sheep investigated the effect of cardiac stem cells therapy in ischemic cardiomyopathy (52 studies; n = 888 animals). This study reported that MSCs therapy is safe and associated with significant approximately 7.5% improvements in left ventricular ejection fraction (LVEF), without any increased mortality due to MSCs administration[96].

The use of MSCs to treat CV dysfunction and damage in COVID-19 patients has yet to be fully elucidated. The studies over the past decade provide good preliminary evidence for researchers and clinicians alike to further investigate the use of this MSCs therapy in COVID-19 patient cohorts.

CONCLUSION

As the number of COVID-19 cases upsurges rampantly with many patients suffering from life-threatening multiple organ failure, finding a curative way to save the life of critically-ill patients and improve the clinical outcomes is urgently needed. As mentioned above, there are no specific therapeutic medications for COVID-19 serious symptoms and complications, and the applied therapeutic guidelines in the clinical settings are non-specific and based on recommendations. At present, cell therapy is a highly sophisticated approach for treating many diseases, and a large number of studies have been carried out in recent months to treat the SARS-CoV-2 virus using stem cells, particularly MSCs. The safety and therapeutic role of MSCs for ARDS and

organ dysfunction that are caused by SARS-CoV-2 infection have been validated by many studies. MSCs can secrete a cocktail of factors that modulate the lung microenvironment, subdue the immune system over-activation, potentiate tissue repair machinery, invigorate alveolar epithelial cells, and inhibit pulmonary remodeling and fibrosis that result in massive improvement in the lung functions. MSCs can regenerate other damaged organs which are associated with COVID-19. Many issues related to the application of MSCs, including the ideal dose and optimum timing of MSCs delivery should be further explored with the objective of enhancing the clinical outcomes in COVID-19 critically-ill patients.

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MINIREVIEWS

Advanced glycation end productions and tendon stem/progenitor cells in pathogenesis of diabetic tendinopathy

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Abstract

Tendinopathy is a challenging complication observed in patients with diabetes mellitus. Tendinopathy usually leads to chronic pain, limited joint motion, and even ruptured tendons. Imaging and histological analyses have revealed pathological changes in various tendons of patients with diabetes, including disorganized arrangement of collagen fibers, microtears, calcium nodules, and advanced glycation end product (AGE) deposition. Tendon-derived stem/progenitor cells (TSPCs) were found to maintain hemostasis and to participate in the reversal of tendinopathy. We also discovered the aberrant osteochondrogenesis of TSPCs *in vitro*. However, the relationship between AGEs and TSPCs in diabetic tendinopathy and the underlying mechanism remain unclear. In this review, we summarize the current findings in this field and hypothesize that AGEs could alter the properties of tendons in patients with diabetes by regulating the proliferation and differentiation of TSPCs *in vivo*.

Key Words: Tendinopathy; Diabetes mellitus; Tendon stem/progenitor cells; Advanced glycation end products

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Core Tip: Patients with diabetic tendinopathy usually suffer from chronic pain, restricted joint motion, calcium deposition, and even tendon rupture. Advanced glycation end products (AGEs) have been shown to affect tendon biology and biomechanical properties. In addition, tendon-derived stem/progenitor cells (TSPCs) play an important role in tendon hemostasis, regeneration, and repair. However, the

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relationships between diabetic tendinopathy, AGEs, and TSPCs remain unclear. Thus, in this review, we summarize the current findings and discuss the possible relationships between AGEs and TSPCs. This might provide new guidance for the development of effective treatments for diabetic tendinopathy.

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INTRODUCTION

Tendinopathy is a common musculoskeletal complication of diabetes mellitus (DM)[1,2]. Patients with DM have a higher incidence of tendinopathy than healthy patients of the same age[3]. The Achilles tendon, patellar tendon, and rotator cuffs are the most frequently affected[4]. The classic symptoms of diabetic tendinopathy usually manifest as chronic pain, limited range of joint motion (ROM), and tendon rupture[2,4,5]. With the progression of diabetic tendinopathy, increased stiffness and thickness and decreased biomechanical properties of Achilles tendons have been reported, and these symptoms usually result in an altered gait and accelerated plantar ulcer formation in DM patients with poor glucose control[2]. Disordered arrangements of excessive collagen fibers and even calcified sites were observed by ultrasound at the entheses of the Achilles tendons[6]. In their electron microscopy study, Grant *et al*[7] also reported that the collagen fibers of the Achilles tendon presented in twisted, curved, and overlapping arrangements in DM patients.

Histological analysis has revealed prominent fibrochondral metaplasia and granulation tissue hyperplasia in DM patients with stenosing flexor tenosynovitis[8]. We also observed microtears in disorganized collagen fibers, blood vessels, and rounded changing cells in the patellar tendons of rats with experimental DM[9]. Moreover, as the characteristic products of DM, advanced glycation end products (AGEs) were discovered in DM tendons[10,11]. Once irreversible AGEs accumulate, they can modify proteins and ultimately damage tendon tissues. Among the various types of cells in tendon tissue, tendon-derived stem/progenitor cells (TSPCs) show multidifferentiation potential and exhibit the ability to maintain hemostasis and reverse tendinopathy[12-16]. Our previous study reported that the impaired functions of diabetic tendon-derived TSPCs showed abnormal osteochondrogenic differentiation *in vitro*, which might also account for the dysfunctions of DM tendons[9]. The histopathological alterations in the tendons of diabetic subjects could partially explain the weakened tension, decreased biomechanical properties, limited ROM, and even the ease of rupture in DM patients. However, the underlying pathological mechanism of diabetic tendinopathy remains unclear. In this review, we summarize the current findings in the fields of diabetic tendinopathy, AGEs, and TSPCs and hypothesize that AGEs could alter the fates of TSPCs to exacerbate tendinopathy in DM patients.

HISTOPATHOLOGICAL FEATURES OF DIABETIC TENDINOPATHY

Many efforts have been made to investigate the histopathological changes associated with diabetic tendinopathy. Ji *et al*[17] observed blood vessel hyperplasia and excessive collagen fibers in leptin-deficient mice. In some subjects, microtears were found to have red blood cell (RBC) deposition and chondrocyte-like cells surrounding the sites of the microtears[17]. In streptozotocin (STZ)-induced DM rats, we also found characteristic histopathological features in DM tendons, such as RBC deposition and microtears, by hematoxylin and eosin staining[9]. By immunohistochemical staining, the expression of vascular endothelial growth factor was found to be significantly increased in the experimental tendons of patients with diabetes, which may contribute to vascularization changes[18]. As characteristic products of DM, AGEs have been reported to be deposited in various organs and tissues[19,20]. In tendons of patients with diabetes, AGEs accumulate in the extracellular matrix (ECM) of tendon cells.

During the early stage of STZ-induced type I DM, we also found the deposition of AGEs in the ECM of rat patellar tendons. Moreover, we discovered decreased expression levels of type I collagen (Col I), tenomodulin (TNMD), and decorin (DCN) in tendons of patients with diabetes. Nevertheless, these tendon cells express higher levels of osteochondrogenesis-associated proteins [osteopontin (OPN), osteocalcin (OCN), SOX9, and collagen type II (Col II)] in the ECM[9]. These results suggested that the pathologic manifestations of chondrification and ossification observed in tendons of patients with diabetes might be ascribed to the aberrant differentiation of these autologous TSPCs in tendon tissue into chondrocytes and osteocytes. However, current studies cannot fully explain the alterations, especially heterotopic calcification and chondrogenesis, in tendons of patients with diabetes at the cellular and histological levels[1,9].

FORMATION AND ACCUMULATION OF AGES IN TENDONS OF PATIENTS WITH DIABETES

The niche of TSPCs in tendon tissue is complicated. Numerous studies have demonstrated the importance of niches in mediating the proliferation and differentiation of stem cells[21-23]. Many factors, such as ECM, biomechanical stimulation, biologically active factors, and pH, could affect the functions of TSPCs *in vivo*[21].

As a distinctive product of DM, AGEs can excessively deposit in connective tissues [10,24-27]. AGEs are derived from nonenzymatic products of the interactions of long-lived proteins with glucose[19,28]. The formation of AGEs is quite slow and spontaneous in healthy subjects[29]. In low metabolic tissues, such as tendons and ligaments, AGEs can accumulate with aging. In addition to aging, the base level of glucose can also affect the formation and accumulation of AGEs *in vivo*[11]. The main component of tendon ECM is collagen type I (Col I), whose half-life ranges from 1 to 2 years; due to this longevity, it is sensitive to the glycoxidation process, which in turn highlights the accumulation of AGEs in tendons of patients with diabetes, which further alters the qualities of tendon ECM[11,26,30,31]. AGEs are mainly deposited in the outer layer or the most distal and proximal regions of the tendons instead of in the core regions in aged tendon samples[32]. However, to date, no studies have focused on region-specific histological analysis of AGE deposition in tendons of patients with diabetes.

Among the subtypes of AGEs, AGE-2 (glyceraldehyde-derived AGEs) and AGE-3 (glycolaldehyde-derived AGEs) are the main subtypes that can be detected in the sera of diabetic patients and they exhibit toxic bioactivities in various cells[33]. In osteoarthritis patients with DM, AGE deposition could lead to increased skeletal fragility and a higher fracture risk in aged people[34,35]. The main reason for this might be the cross-links formed by AGEs between the collagen strands[36]. The formation of these cross-links could result in increased stiffness and decreased biomechanical properties of diabetic cartilage and tendons.

In addition to the cross-links among the collagen fibers in DM tendons, the expression of the receptor for AGEs (RAGE) was also evaluated. Activation of AGE-RAGE could mediate many downstream signaling pathways in many kinds of cells and lead to many functional responses[37,38]. For instance, it induces cell death[39], regulates the expression of the inflammatory response[39], and degrades the ECM[40]. The study by Yokosuka *et al*[27] demonstrated the accumulation of AGEs in the ossified spinal ligament and suggested that the interaction of AGEs with RAGE is an important factor for the progression of spinal ligament ossification. In osteoblast-like cells, AGEs can regulate the differentiation stages *via* specific receptors[41]. Moreover, the latest research revealed that AGEs inhibited the osteogenic differentiation of mouse adipose-derived stem cells (ASCs) *in vitro*[42]. These studies demonstrated that the chronic accumulation of AGEs has negative impacts on these tissues and organs. Therefore, more attention has been given to determining the influences or underlying mechanisms of AGEs on musculoskeletal systems.

AGES ALTER THE BIOMECHANICAL PROPERTIES OF TENDONS

It has been documented that tendon tissue exhibits an inherent triple helix structure [43]. Accumulated AGEs could cross-link neighboring collagen molecules within the tendons[20]. The intermolecular cross-links between neighboring collagen molecules

may connect lysine to arginine residues or lysine to lysine[44]. In DM patients, the arrangement of collagen fibers in the Achilles tendon exhibited a highly disorganized structure under electron microscopy, and these structural abnormalities might be ascribed to the deposition of AGEs[7].

Various studies have demonstrated that cross-links between collagen fibers could affect the biomechanical properties of the musculoskeletal system. Currently, few studies have investigated the biomechanical effects of AGEs on human tendon tissues. In osteoarthritis, cross-links caused by AGEs increased the stiffness of the collagen network in human articular cartilage[36].

However, the conclusions about the effects of AGEs on tendon mechanics are contradictory. Sell and Monnier[45] reported that the cross-links formed by AGEs could increase the C57BL/6 mouse tendon strain. In isolated rabbit Achilles tendons, after glycation *in vitro*, the maximum load, stress, strain, and Young's modulus of elasticity were increased compared with those of the nonglycated tendons[46]. Biochemical analysis revealed significantly increased expression of pentosidine, which is recognized as a marker of AGEs, in glycated rabbit Achilles tendons. The cross-links formed by AGEs between collagens increased the stiffness of the matrix[47]. Thus, the authors concluded that cross-links could directly affect the matrix stiffness and stimulate the biomechanical properties of tendons. In addition, AGEs have been reported to damage the biomechanical properties of tendon collagen in various species by diminishing tendon fiber sliding[11,48,49]. In rat tail tendons, Fessel *et al*[48] discovered that lateral molecular interconnectivity by AGEs could reduce the side-by-side sliding of collagen fibers, thus leading to increased collagen fiber failure resistance *in vivo*. An *in vitro* study also revealed dramatically decreased tendon fiber sliding and viscoelastic behavior by tissue glycation[11]. In bovine tail tendons, Lee and Veres[20] found that the cross-links formed by AGEs could significantly inhibit biomechanical plasticity *in vitro*. Some other researchers considered that the cross-links could affect the biomechanical properties by taking up space in the ECM[50]. In both aged tendons and glycated tendons *in vitro*, the molecular spacing was linearly increased[11], which might be ascribed to the formation of cross-links by AGEs between collagen fibers. Another argument was that AGEs primarily affect the mechanical properties at the failure regions of tendons of patients with diabetes[51,52].

RELATIONSHIPS BETWEEN AGES AND THE ECM OF TENDONS OF PATIENTS WITH DIABETES

In addition to the cross-links formed between collagen fibers, the deposited AGEs in the ECM could also interact with various kinds of cytokines and proteins, cause biological effects, and subsequently impair their material properties[44,45,48]. It has been reported that Fe^{2+} in tendons of patients with diabetes could promote the accumulation of AGEs in collagens, which in turn stimulated the glycosylation of Col I and other matrix proteins *in vivo*[53]. Once deposited in the ECM, these AGEs could suppress the function of the mitochondria of Achilles tendon-derived fibroblasts and impair their proliferation, further leading to reduced remodeling of the ECM[54]. In porcine patellar tendons, the proteoglycan level was decreased after sustained hyperglycemia caused the production of AGEs *in vitro*[55]. Nevertheless, only a few studies have focused on the interactions of AGEs and factors in the ECM of tendon cells, especially TSPCs. In other tissues or cell types, such as ligaments and fibroblasts, AGEs have been demonstrated to affect the expression levels of matrix metalloproteinases (MMPs), bone morphogenetic proteins (BMPs), and other factors. Accumulated AGEs in the ossified spinal ligament could elevate the expression levels of BMP-7, BMP-2, alkaline phosphatase (ALP), and OCN, an osteoblast-specific transcription factor 1[27]. In human fibroblasts, AGEs could decrease Col I and increase MMP-1 levels *in vitro*[56]. In osteoblast-like cells, AGEs could promote the degradation of Col I by stimulating the secretion of MMP-2 and MMP-9 *in vitro*[57] and stimulate the mRNA expression and serum levels of fibroblast growth factor 23 in chronic disease[58]. The expression of MMP-1 in human gingival fibroblasts was also significantly increased at both the mRNA and protein levels *in vitro* after treatment with AGEs[59].

AGES INDUCE CELLULAR EVENTS IN TENDON CELLS AND THE UNDERLYING MECHANISM

AGEs induce cellular effects on various kinds of cells mainly by activating the RAGE *in vivo*. Many studies have reported that the AGE-mediated events of various kinds of cells are activated through the interactions of AGE-RAGE[60,61]. RAGE is a receptor that can activate many kinds of ligands and it exists in normal tendon tissues. It is expressed at low levels under normal blood glucose levels, and its expression could be increased while AGEs accumulate under sustained hyperglycemia[21,62]. In addition to RAGE, many other molecules have been shown to act as receptors of AGEs, such as scavenger receptor class AI/AII[63], scavenger receptor class B type I[64], and CD36 [65]. In our unpublished research, we also observed the expression levels of AGEs and RAGE in the ECM of diabetic tendon cells *in vivo* and in isolated TSPCs *in vitro*. After the receptors for AGEs are activated, a variety of downstream cellular signaling pathways can be excited and they subsequently alter cell functions, such as proliferation, migration, apoptosis, and differentiation.

Proliferation

Generally, AGEs have been demonstrated to attenuate the proliferation abilities of various kinds of cells, such as bone mesenchymal stem cells (MSCs) and retinal pericytes[29,66]. In human MSCs, Kume *et al*[29] found that higher concentrations of AGE-2 and AGE-3 (1-100 μg/mL) could inhibit their proliferation ability and stimulate apoptosis *in vitro,* probably by upregulating intracellular reactive oxygen species (ROS). The generation of ROS has been reported to regulate these AGE-RAGE-induced cellular events[61,67]. Yang *et al*[68] reported that AGEs inhibited bone MSC proliferation and migration by inducing chemokine/cytokine secretion *via* the p38 pathway *in vitro*. Moreover, AGE-2 could suppress the proliferation of cultured bovine retinal pericytes through downregulation of the expression ratio of BCL-2/BAX[66]. In addition, AGEs could stimulate the proliferation abilities of several other kinds of cells. In osteoblastic cell lines, the effects of AGEs on cell proliferation were reported to depend on their stage of differentiation[69]. Low concentrations of AGEs could stimulate mesangial cell proliferation[70]. AGEs enhance vascularization in diabetic retinopathy by interacting with RAGE and promoting vascular endothelial cell proliferation[71]. However, few studies have investigated the impacts of AGEs on TSPCs, and further research is required.

Apoptosis

In addition to their influence on proliferation, AGEs also induce the apoptosis of many kinds of cells, including TSPCs, retinal pericytes, myoblastic cell lines, mononuclear cells, and endothelial progenitor cells[67,72-75]. Xu *et al*[72] reported that AGEs could induce TSPC apoptosis, and pioglitazone showed the ability to rescue AGE-induced apoptosis and other abnormal alterations both *in vitro* and *in vivo*. In bovine retinal pericytes, AGE-initiated apoptosis was reported to be ascribed to the activation of the caspase-10 pathway[67]. AGEs could induce the apoptosis of mouse myoblastic C2C12 cells and inhibit myogenic differentiation, while insulin-like growth factor-I exhibited therapeutic potential to attenuate the detrimental effects of AGEs on C2C12 cells[73]. In human mononuclear cells isolated from the peripheral blood of patients with type II DM, increased cellular apoptosis and decreased osteoblastic differentiation ability were highly correlated with RAGE expression[74]. The activation of ROS, Akt/eNOS, MAP kinases, and the FOXO1 transcription factor have all been reported to participate in AGE-induced apoptosis progression[54,75].

Differentiation

Several studies have illustrated that accumulated AGEs could affect the differentiation properties of stem cells in the musculoskeletal system. In TSPCs, AGEs have been reported to exacerbate osteogenic differentiation potential *in vitro*[72]. For other kinds of cells, AGEs could inhibit the osteogenic differentiation potential of mouse ASCs by suppressing the expression of OPN and runt-related transcription factor 2 (Runx2) through activating the Wnt/β-catenin signaling pathway[42]. In human periodontal ligament stem cells, AGEs attenuate osteogenesis *in vitro*, and the canonical Wnt/β-catenin and JNK signaling pathways might be involved[76-78]. RAGE in MSCs could be activated by AGE-2 and AGE-3; thus, the AGE-RAGE interaction was found to participate in the osteogenic and chondrogenic differentiation processes of MSCs[29]. AGE-3 was reported to inhibit the osteogenic differentiation and bone nodule formation of MSCs by activating RAGE and upregulating the expression of TGF-β *in*

vitro[29,79]. The expression levels of ALP and intracellular calcium in MSCs were upregulated by AGEs, while mineralization and bone nodule formation were both decreased *in vitro*. The chondrogenic and adipogenic differentiation potentials of the MSCs were also attenuated by AGEs *in vitro*[29].

AGEs and TSPCs

To date, only a few studies have focused on the influence of AGEs on TSPCs. Xu *et al* [72] reported that AGEs could reduce cell viability and increase apoptosis and autophagy of TSPCs *in vitro*. In that study, they found that AGEs induced senescence and enhanced the ossification of TSPCs *in vitro*. However, the researchers did not further investigate the underlying mechanisms of AGE-induced ossification of TSPCs. In MSCs, AGE-2 and AGE-3 showed the ability to enhance ALP activity and intracellular calcium content by activating RAGE *in vitro*[29]. Therefore, we speculate that the activation of RAGE in TSPCs could also lead to apoptosis, senescence, and aberrant differentiation by activating several signaling pathways, such as the Wnt/β-catenin, P38/MAPK, Notch, ROS, and Akt/eNOS pathways.

TSPCS IN DIABETIC TENDINOPATHY

The progression of diabetic tendinopathy is complicated and involves various kinds of factors and types of cells. Previously, we have summarized the current findings of diabetic tendinopathy, especially the cellular and underlying mechanisms[80]. In addition to tenocytes, there are many other types of cells inside the tendons. Bi *et al*[12] and Rui *et al*[13] proved the existence of stem/progenitor cells in the tendons of mice and rats. TSPCs exhibit self-colony ability and multidifferentiation properties *in vitro* [14-16]. In the patellar tendon of a collagenase-induced rat tendinopathy model, TSPCs presented lower proliferation capacity and higher osteogenic and chondrogenic differentiation potentials[16]. In an injury-induced rat tendinopathy model, TSPCs showed increased proliferation ability and higher type III collagen (Col III) and α-SMA expression than in collagenase-induced rats[15]. These findings indicate the involvement of TSPCs in maintaining tendon tissue homeostasis and mediating the pathological process of chronic tendinopathy[81]. During the development of diabetic tendinopathy, as tissue-specific cells are contained in tendon tissue, TSPCs are the most likely cells to participate in the early response. TSPCs are thought to differentiate into tenocytes and play key roles in maintaining, regenerating, and replacing differentiated tenocytes in tendon tissues. In rats with experimental DM, we found that the fate of TSPCs isolated from patellar tendons was altered, and these cells exhibited decreased proliferation properties and enhanced osteochondrogenic potential[9]. High glucose (11.1 mmol/L) could stimulate an inflammatory response of TSPCs in the human patellar tendon *in vitro*[82]. Our previous study found that high glucose (15 mmol/L and 25 mmol/L) could inhibit rat TSPC proliferation and induce apoptosis *in vitro*[83]. Moreover, insulin has been reported to increase ALP activity and the expression levels of osteogenesis-associated markers in TSPCs isolated from horse superficial digital flexor tendons[84]. Taken together, these studies indicate that the aberrant proliferation and differentiation of TSPCs are possible underlying mechanisms of diabetic tendinopathy. AGEs have been shown to induce apoptosis and to exacerbate the osteogenic differentiation potential of TSPCs *in vitro*[72]. However, the mediating mechanisms of AGEs on diabetic TSPC multidifferentiation potential are still unclear, and future studies are required to investigate the underlying processes.

CONCLUSION

In summary, we have described the enhanced osteochondrogenic differentiation ability of TSPCs from experimental diabetic rats cultured in induction medium[9]. Additionally, the outstanding expression of osteochondrogenic-associated markers and AGE accumulation were also noted. *In vitro* studies revealed that AGEs could affect the proliferative capacity, apoptosis, and multidifferentiation potential of TSPCs and other kinds of stem cells under certain pathological conditions. Taken together, we hypothesize that the accumulated AGEs in the ECM of diabetic TSPCs lead to aberrant differentiation fates and futures, contributing to the development of chronic tendinopathy in DM subjects (Figure 1). Understanding the relationships among

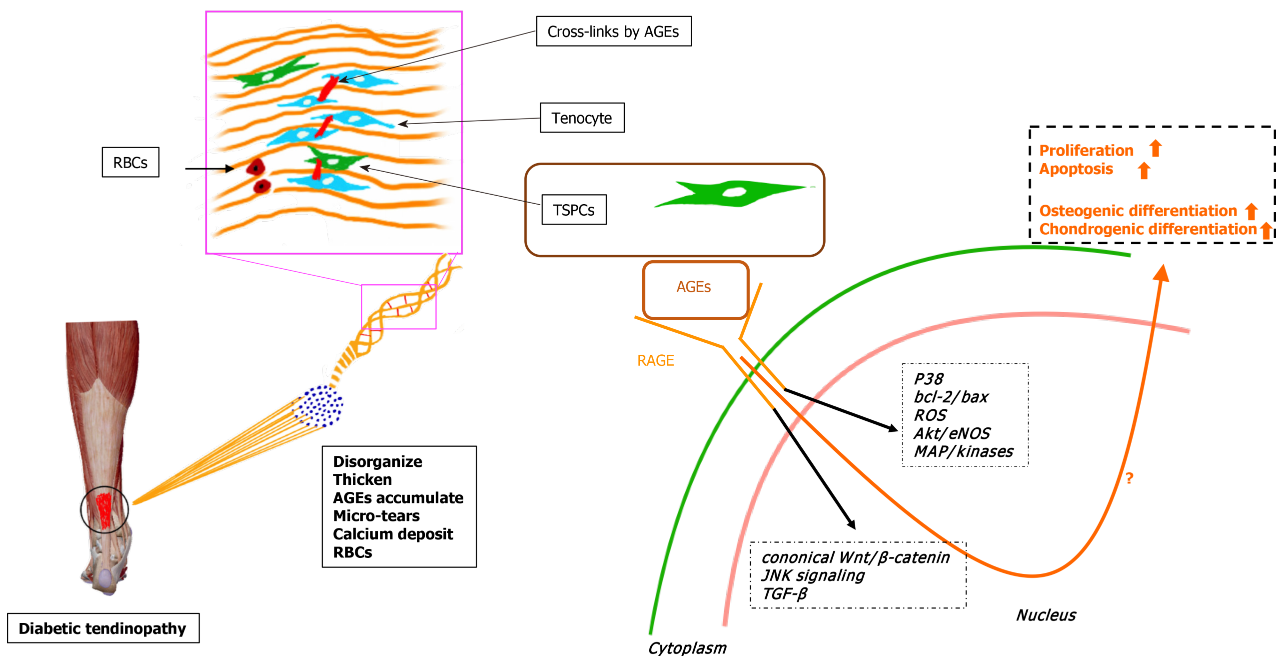

Figure 1 Hypothesis of the molecular mechanism by which advanced glycation end products regulate the fate of tendon-derived stem/progenitor cells in diabetic tendinopathy. RBCs: Red blood cells; AGEs: Advanced glycation end products; TSPCs: Tendon-derived stem/progenitor cells; RAGE: Receptor for advanced glycation end product; ROS: Reactive oxygen species; TGF-β: Transforming growth factor β.

diabetic tendinopathy, TSPCs, and AGEs will be crucial for developing new treatments for diabetic tendinopathy therapy.

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MINIREVIEWS

Current understanding of mesenchymal stem cells in liver diseases

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Abstract

Liver diseases caused by various factors have become a significant threat to public health worldwide. Liver transplantation has been considered as the only effective treatment for end-stage liver diseases; however, it is limited by the shortage of donor organs, postoperative complications, long-term immunosuppression, and high cost of treatment. Thus, it is not available for all patients. Recently, mesenchymal stem cells (MSCs) transplantation has been extensively explored for repairing hepatic injury in various liver diseases. MSCs are multipotent adult progenitor cells originated from the embryonic mesoderm, and can be found in mesenchymal tissues including the bone marrow, umbilical cord blood, adipose tissue, liver, lung, and others. Although the precise mechanisms of MSC transplantation remain mysterious, MSCs have been demonstrated to be able to prevent the progression of liver injury and improve liver function. MSCs can self-renew by dividing, migrating to injury sites and differentiating into multiple cell types including hepatocytes. Additionally, MSCs have immune-modulatory properties and release paracrine soluble factors. Indeed, the safety and effectiveness of MSC therapy for liver diseases have been demonstrated in animals. However, pre-clinical and clinical trials are largely required to confirm its safety and efficacy before large scale clinical application. In this review, we will explore the molecular mechanisms underlying therapeutic effects of MSCs on liver diseases. We also summarize clinical advances in MSC-based therapies.

Key Words: Mesenchymal stem cell; Liver disease; Clinical trial; Treatment; Safety; Efficacy

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Core Tip: Liver diseases are major threats that endanger public health globally. Mesenchymal stem cell (MSC) transplantation has been proposed as an attractive therapeutic option for liver diseases due to their differentiation potential, immune-

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modulatory properties, and paracrine release. Here, we will summarize the molecular mechanisms underlying therapeutic effects of MSCs on liver diseases and clinical trials in MSC-based therapies.

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INTRODUCTION

Mesenchymal stem cells (MSCs) can be isolated easily from a wide variety of tissues including umbilical cord blood, adipose tissue, the liver, lung, dermis, and amniotic membrane, and menstrual blood[1]. Notably, MSCs play important roles in tissue repair and regeneration because of their high potential for multipotent differentiation, capacity for self-renewal, and low immunogenicity[2]. In recent years, application of MSCs in liver diseases has attracted considerable attention. First, MSCs can self-renew and differentiate into various types of cells, including hepatocyte-like cells (HLCs), which possess similar functions of normal hepatocytes[3]. Second, MSCs have low immunogenicity and low expression of major histocompatibility complex class II and costimulatory molecules, which provides a possibility for allogeneic transplantation [4]. Third, MSCs can secrete a series of cytokines and signaling molecules, which favor injury repair and regeneration[5]. Indeed, accumulating evidence has supported that MSC transplantation is effective for the treatment of various liver diseases. Here, we will discuss the molecular mechanisms of MSCs in the treatment of liver diseases and summarize potential therapeutic efficacy of MSCs in both animal models and clinical trials.

MECHANISMS OF MSC THERAPY FOR LIVER DISEASES

Differentiation capability of MSCs

MSCs can self-renew and differentiate into various progenitors, including hepatic progenitor cells. Indeed, a variety of studies have demonstrated that MSCs could differentiate into HLCs both *in vitro* and *in vivo*[6,7]. Under appropriate conditions, in particular with specific growth factors, such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and oncostatin M (OSM), MSCs are able to differentiate into HLCs with a liver-specific morphology and function[8,9]. In line with these findings, Zhang *et al*[3] transplanted human umbilical cord-derived MSCs (UC-MSCs) into fibrotic livers of rats and observed improvement in transaminase, synthetase, human albumin (ALB), alpha-fetoprotein, cytokeratin 18 (CK18), and CK19, suggesting that MSCs could differentiate into HLCs *in vivo*. Furthermore, MSCs might fully differentiate into hepatocytes with liver functions, such as low-density lipoprotein uptake, glucose storage, and ammonia detoxification. However, this notion is debated. For example, differentiated MSCs could not express markers of mature hepatocytes, including hepatocyte nuclear factor 4 α and hepatocyte paraffin 1[10]. Similarly, only a small fraction of MSCs (less than 3% of the total liver mass) underwent hepatocyte trans-differentiation[11]. Collectively, MSCs-mediated therapeutic effects most likely rely on other mechanisms other than fully functional complementation from direct differentiation (Figure 1).

MSC-mediated immunomodulation

MSCs may modulate effector cells of innate and adaptive immune systems[12]. MSC–immune cell interaction and paracrine release may enable successful treatment of liver diseases. MSCs can regulate immune responses mediated by macrophages, dendritic cells (DCs), T cells, regulatory T cells (Tregs), B cells, and regulatory B cells (Bregs), to establish a stable and balanced microenvironment[13] (Figure 1).

Effects of MSCs on adaptive immune response: MSCs can inhibit T cell proliferation either by directly interacting with T cells or by secreting soluble factors, including

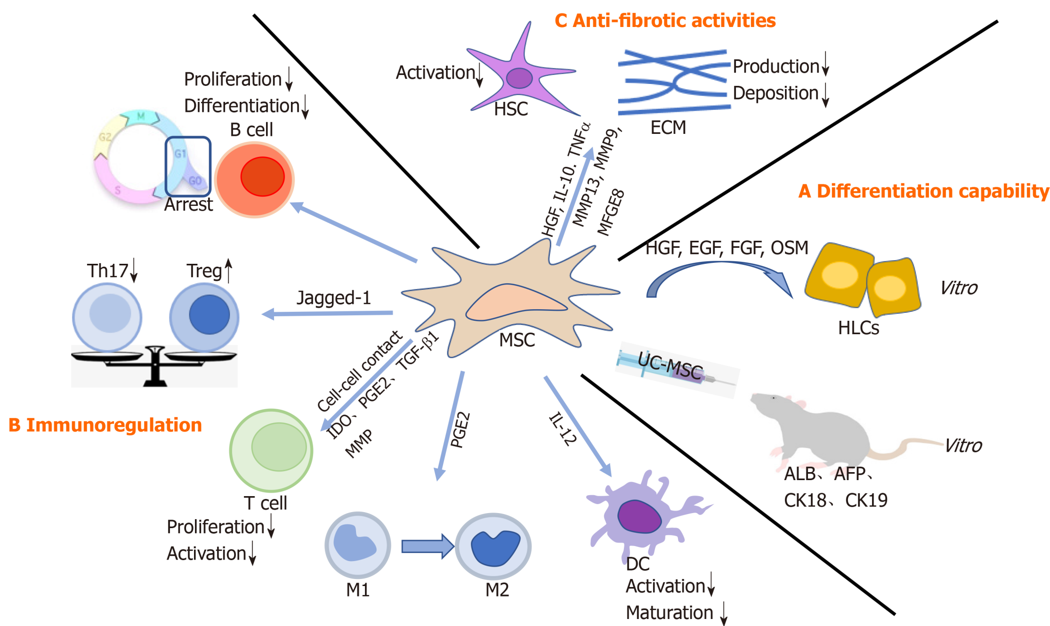

Figure 1 The mechanism of mesenchymal stem cells in liver diseases. A: Mesenchymal stem cell (MSCs) differentiate into hepatocyte-like cells both *in vitro* and *in vivo*; B: MSCs modulate effector cells of innate and adaptive immune systems; C: MSCs alleviate liver fibrosis. MSC: Mesenchymal stem cell; UC-MSC: Human umbilical cord-derived MSC; HGF: Hepatocyte growth factor; EGF: Epidermal growth factor; FGF: Fibroblast growth factor; OSM: Oncostatin M; HLCs: Hepatocyte-like cells; ALB: Albumin; AFP: Alpha-fetoprotein; CK18: Cytokeratin 18; CK19: Cytokeratin 19; DC: Dendritic cell; M1: M1 macrophage; M2: M1 macrophage; IL-12: Interleukin 12; PGE2: Prostaglandin E2; IDO: Indoleamine 2,3-dioxygenase; MMP: Matrix metalloproteinases; TGF-β1: Transforming growth factor-β1; Th17: T helper cells 17; Treg: Regulatory T cells; HSC: Hepatic stellate cell; IL-10: Interleukin 10; TNFα: Tumor necrosis factor α; MFGE8: Milk factor globule EGF 8; ECM: Extracellular matrix.

indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2)[12], and transforming growth factor-β1 (TGF-β1)[14,15]. On one hand, MSCs induced cell-cycle arrest by downregulation of cyclin D2 and upregulation of p27kip1[16]. On the other hand, in the presence of interferon-γ, MSCs upregulated IDO, which conversed tryptophan into kynurenine, consequently depleted tryptophan, and enhanced apoptosis of T cells[17,18]. Furthermore, Ding *et al*[19] demonstrated that MSCs could secrete matrix metalloproteinases (MMP), such as MMP-2 and MMP-9, to suppress T cell activation by cleaving surface CD25. Of note, imbalance between Tregs and T helper 17 (Th17) cells might be associated with a variety of liver diseases[20]. MSCs could play an immunoregulatory role by inducing Tregs and suppressing Th17 cells[21,22]. Also, Cahill *et al*[23] observed that MSCs expressed Jagged-1, which is responsible for Tregs accumulation. Consistently, there was a significant increase in Tregs and a markable decrease in Th17 cells after MSC infusion. Moreover, compared with the control group, liver function of patients in the MSC-transplantation group was improved, partially attributed to regulation of the Treg/Th17 cell balance[24]. In addition, B cells participate in the pathogenesis of liver fibrosis. MSCs could block the proliferation of B cells by inducing cell cycle arrest at G0/G1 phase. Also, the differentiation and chemotactic cytokine production of B cells were inhibited[25].

Effects of MSCs on innate immune response: Macrophages exert profound effects in the pathogenesis of chronic liver injury[26]. There are two types of macrophages: M1 inflammatory and M2 anti-inflammatory. Importantly, imbalance in M1/M2 polarization could lead to hepatocyte injury and fibrosis[27]. Intriguingly, MSCs could induce conversion of M1 into M2 tissue-resident macrophages in a PGE2-dependent manner, which was mediated by signal transducer and activator of transcription 6 and mechanistic target of rapamycin signaling[28]. Furthermore, MSCs could inhibit the activation and maturation of DCs by downregulating interleukin 12 (IL-12) production [13].

Anti-fibrotic activities of MSCs

Liver fibrosis is characterized by an imbalance between synthesis and degradation of the extracellular matrix (ECM)[29]. When the liver is damaged, pro-fibrotic factors are secreted to promote the activation and proliferation of hepatic stellate cells (HSCs), and thus contribute to ECM deposition. How can MSCs participate in fibrosis? First,

MSCs produce several molecules, such as HGF, IL-10, and tumor necrosis factor α[30], to inhibit HSC activation and collagen production. Accordingly, when MSCs were transfected with the *HGF* gene[31,32], a decrease in collagen levels and improvement in hepatocyte function were observed. Therefore, HGF-overexpressing MSCs might alleviate liver fibrosis. In addition, MSCs have the potential to reverse the fibrotic process by upregulating MMPs, such as MMP-13 and MMP-9, to degrade the ECM directly[33]. Finally, TGF-β1 is a primary mediator in liver fibrogenesis as it stimulates the synthesis but inhibits the degradation of the ECM. More importantly, TGF-β1 functions by activating drosophila mothers against decapentaplegic protein 3 (Smad3). Thus, the TGF-β/Smad signaling pathway plays a critical role in ECM accumulation and liver fibrosis progression[34]. Of note, MSC-derived milk factor globule EGF 8 (MFGE8), an anti-fibrotic protein, could reduce ECM deposition and suppress HSC activation through the TGF-β signaling pathway[35] (Figure 1).

MSC TRANSPLANTATION IN ANIMAL MODELS OF LIVER DISEASE

Recently, MSC transplantation has been applied in the treatment of acute liver injury (ALF), chronic liver disease, non-alcoholic fatty liver disease (NAFLD), and hepatocellular carcinoma (HCC). Notably, MSC transplantation can partially restore liver function, ameliorate symptoms, and increase survival rates. Major findings regarding MSC transplantation in animal models of liver diseases are summarized in Table 1.

Acute liver injury

ALF is characterized by rapid loss of function and tissue necrosis[36]. Its treatment should focus on restoration of function and prevention of disease progression. Thus, MSCs may provide functional substitution and restoration[37]. Accordingly, the therapeutic potential of MSCs in ALF has been reported in mice[38], rats[39] and monkeys[40]. For example, in a murine model of acetaminophen (APAP) induced ALF [38], intravenously transplanted human UC-MSCs significantly alleviated hepatic injury and improved survival rates. Chen *et al*[39] demonstrated that MSCs could prevent the release of liver injury biomarkers and promote the recovery of liver structure in ALF rats. Furthermore, transplantation of cocultured MSCs with hepatocytes provides better restoration of liver function, resulting in a primary decrease in aspartate aminotransferase, alanine aminotransferase (ALT), and total bilirubin (TBIL). On one hand, co-transplanted hepatocytes could provide timely support of liver functions. On the other hand, MSCs could not only reduce immune rejection of hepatocytes by the host but also improve the viability and function of hepatocytes. Similarly, in a large, non-human primate model, human UC-MSCs mitigated the progression of ALF. Guo *et al*[40] demonstrated that early peripheral infusion of human UC-MSCs could markedly improve hepatic histology, systemic homeostasis, and survival of monkeys. Mechanistically, IL-6 was critical to initiate and accelerate ALF development, while human UC-MSCs could disrupt the inflammatory cascade by inhibiting monocyte activation. Overall, in ALF, MSC transplantation might exert beneficial effects.

Chronic liver injury

Chronic liver diseases are attributed to tissue deterioration as a result of fibrosis or cirrhosis associated with persistent chronic inflammation. Therapies aim at inhibition of inflammation and restoration of tissue architecture[37]. The beneficial effects of MSC transplantation on chronic liver diseases have been well documented in animal models. For example, infusion of bone marrow-derived MSCs (BM-MSCs) could safely ameliorate liver fibrosis in a thioacetamide-induced cirrhotic rat model[41]. Interestingly, the collagen proportionate area and content of hepatic hydroxyproline were significantly decreased. BM-MSC administration could downregulate the TGF-β 1/Smad signaling pathway. Consistently, BM-MSC transplantation obviously improved liver function[42]. Moreover, liver fibrosis progression and hepatocyte necrosis were attenuated after BM-MSC administration, partially due to paracrine action of MSCs.

Non-alcoholic fatty liver disease

NAFLD is characterized by abnormal lipid accumulation in hepatocytes in the absence of alcohol abuse[43]. Of note, MSCs could relieve lipid and glucose metabolism disorders. In a rat model of type 2 diabetes mellitus, MSCs alleviated insulin resistance and improved glucose homeostasis by inducing phenotypic transition of macrophages

Table 1 Experiments using mesenchymal stem cell transplantation in animals

Disease	Treatment	Source	Animal	Main results	Mechanism	Ref.
ALF	APAP	Human UC-MSCs	Mice	Alleviate hepatic injury and improve survival rates	Mediate paracrine effects, regulate inflammatory response	Liu *et al* [38], 2014
ALF	LPS	Human UC-MSCs	Monkeys	Improve the hepatic histology, systemic homeostasis, and survival	Suppress the hepatic aggregation and maturation of circulating monocytes and their IL-6 secretion	Guo *et al* [40], 2019
LC	CCl4	Human UC-MSCs	Rats	Improve liver transaminases and synthetic function, reduce liver histopathology, and reverse hepatobiliary fibrosis	Differentiate into hepatocytes	Zhang *et al* [3], 2017
LC	CCl4	Monkey BM-MSCs	Mice	Decrease liver fibrosis, progression, and hepatocyte necrosis	Mediate paracrine effects	Fu *et al*[42], 2018
LC	TAA	Human BM-MSCs	Rats	Decrease collagen proportionate area and the content of hepatic hydroxyproline	Mediate TGF-β1/Smad signaling pathway	Jang *et al* [41], 2014
NAFLD	HFD	Mice BM-MSCs	Mice	Decrease fibrosis markers and pro-inflammatory cytokines	Regulate inflammatory process	Ezquer *et al* [46], 2011
NAFLD	HFD	Mice BM-MSCs	Mice	Decrease weight gain, expansion of subcutaneous adipose tissue, steatosis, lobular inflammation, and liver fibrosis	Suppress the proliferation of CD 4^+ T cells	Wang *et al* [47], 2018

MSCs: Mesenchymal stem cells; AFL: Acute liver failure; LC: Liver cirrhosis; NAFLD: Non-alcoholic fatty liver disease; APAP: Acetaminophen; LPS: α-amatoxin and lipopolysaccharide; TAA: Thioacetamide; HFD: High-fat diet; BM-MSCs: Bone marrow-derived MSCs; UC-MSCs: Umbilical cord-derived MSCs.

[44]. In recent years, the therapeutic potential of MSCs has been explored in NAFLD. Indeed, MSCs exhibit therapeutic effects on NAFLD by improving carbohydrate and lipid metabolism, as demonstrated by a marked decrease in glucose and lipid profile, including triglyceride, total cholesterol, and low-density lipoprotein cholesterol. Moreover, UC-MSC infusion significantly attenuated histological hepatic lesions, as evidenced by decreased lipid accumulation and hepatic steatosis. These findings were explained by upregulation of fatty acid oxidation-related genes and downregulation of lipogenesis-related genes[45]. Previously, Ezquer *et al*[46] transplanted BM-MSCs into mice that were fed a high-fat diet (HFD). Interestingly, the mice were obese, hypercholesterolemic, hyperglycemic, and insulin resistant; however, fibrosis markers and pro-inflammatory cytokines were substantially reduced. Therefore, this controversy is not related to a reversion of metabolic syndrome but to preclusion of inflammatory process. In addition, in a mouse model of HFD-induced NAFLD, MSC transplantation relieved weight gain, expansion of subcutaneous adipose tissue, steatosis, lobular inflammation, and liver fibrosis, through suppressing the proliferation of CD4+ T lymphocytes in the spleen[47]. These findings indicated that MSCs could have clinical value in NAFLD therapy *via* immune regulation. Of note, NAFLD can stem from simple steatosis, subsequently progressing to non-alcoholic steatohepatitis (NASH). NASH presents with hepatic inflammation, fibrosis, and cirrhosis, and eventually progresses to HCC[48]. It is noteworthy that insulin resistance is a hallmark for NAFLD progression to NASH[49]. Chen *et al*[50] reported that MSC therapy improved lipid metabolism in HFD-fed rats, as reflected by substantially decreased lipid droplet accumulation in hepatocytes. Also, MSCs could reduce fasting insulin level in serum. These results indicated that MSCs have potential in preventing the development of NASH. Furthermore, MSCs could remarkably improve intracellular calcium homeostasis and endoplasmic reticulum (ER) stress *in vitro*, and the latter might be involved in the pathology of NAFLD.

Hepatocellular carcinoma

MSCs can rapidly respond to “damage signals” and mobilize from bone marrow or other tissues to inflammatory or fibrotic microenvironment[51]. Specific signals mediating MSCs migration mainly include pro-inflammatory growth factors and chemokines, such as insulin growth factor, HGF, FGF, and TGF-β[52]. Furthermore, CXC motif chemokine receptor type 4 (CXCR4) could regulate MSC migration from bone marrow to the liver[53]. For example, genetically modified MSCs overexpressing CXCR4 exhibited higher migratory activity towards and functional improvement of the liver, likely relying on upregulation of stromal cell-derived factor (SDF-1) (the ligand for CXCR4) that is typically present at inflammatory sites and highly expressed

in an injured liver[54]. HCC can be caused by chronic liver diseases with varying degrees of chronic inflammatory fibrosis, which enable MSCs to migrate to HCC microenvironment. Garcia *et al*[55] reported that MSCs could migrate and home to HCC and fibrotic microenvironment. Also, HCC cells secreted autocrine motility factor could induce MSCs migration towards them[56]. Furthermore, HCC-released factors including IL-8, growth-regulated oncogene (GRO), and monocyte chemotactic protein-1 can enhance MSC migration after exposure to conditioned media (CM) from HCC [57]. Multipotent MSCs can block HCC progression by spurring apoptosis and inhibiting proliferation *in vitro*, as well as suppressing tumor growth and metastasis *in vivo*[58,59]. Qiao *et al*[60] suggested that CM from MSCs were able to inhibit HepG2 proliferation by downregulating nuclear factor-κB. Similarly[61], when severe combined immunodeficiency disease mice were injected with equal numbers of MSCs and H7402 human hepatoma cells, tumor formation was delayed and hepatoma growth inhibited. However, increasing evidence suggests that MSCs as a doble-bladed sword may promote HCC progression. For instance, soluble factors from MSCs could promote the proliferation and invasion of canine HCC cells[62]. In agreement with this finding, Gong *et al*[63] reported that BM-MSCs could promote microvascular formation in transplanted hepatoma area in nude mice.

CLINICAL TRIALS USING MSCS FOR TREATMENT OF LIVER DISEASES

Numerous clinical studies have been initiated to investigate the therapeutic potential of MSCs in the treatment of liver diseases. Main findings regarding MSC transplantation in liver diseases are summarized in Table 2.

A phase I–II clinical trial included eight patients with liver cirrhosis[64]. All patients received an injection of autologous BM-MSCs previously transdifferentiated in hepatocytes *via* the peripheral or portal vein. No severe side-effects were observed until the end of follow-up at 24 wk after transplantation, which emphasized the safety of using autologous BM-MSCs as a treatment. All patients had improved performance status and quality of life partially because of reduced volumes of ascites. Furthermore, liver function was ameliorated as verified by model for end-stage liver disease (MELD) score, prothrombin complex from international normalized ratio (INR), and serum creatinine. Four out of eight patients had significantly decreased MELD score whereas seven had normalized creatinine levels in 8 wk after treatment. In another phase I–II clinical trial, Zhang *et al*[65] randomized 45 patients with decompensated liver cirrhosis resulting from chronic hepatitis B into two groups: 30 patients received UC-MSC transfusion, and 15 received saline as controls. The patients receiving MSCs had significantly reduced volumes of ascites and levels of serum liver cirrhosis markers when compared to the control group. Importantly, UC-MSC transfusion could improve liver function, as evidenced by an increase in ALB whereas a reduction in TBIL, prothrombin time activity (PTA), or MELD-sodium (MELD-Na) score. Of note, MELD-Na score has been demonstrated as a marker for better prognosis of liver diseases. In a phase II trial, Suk *et al*[66] transplanted BM-MSCs in 48 patients with alcoholic cirrhosis. Child-Pugh scores and histologic fibrosis were improved after BM-MSC transplantation compared with 24 control patients. However, two-time injections failed to display better effects on fibrosis in comparison with one-time injection of BM-MSCs, which indicated that one-time injection of BM-MSCs might be sufficient for inducing regression of fibrosis. In general, these trials shed light on the safety and efficacy of MSCs in patients with liver cirrhosis. Similarly, several trials on end-stage liver diseases, especially acute-on-chronic liver failure (ACLF), were performed. In a phase II trial, Peng *et al*[67] transplanted autologous MSCs from iliac bone aspirates to patients with hepatitis B-related liver failure. Follow-up of patients receiving MSCs-transplantation identified a significant improvement in ALB and TBIL in 2 wk, whereas prothrombin time (PT) and MELD score in 3 wk. However, during the 192-wk follow-up, long-term outcome was not markedly improved after transplantation. Notably, no significant difference in the incidence of HCC or survival rate was observed between the cirrhosis and non-cirrhosis groups, indicating that autologous BM-MSC transplantation might be preferable for cirrhosis with regard to the development of HCC and mortality. Thus, this clinical trial proposed that BM-MSC transplantation was safe with favorable short-term efficacy in the treatment of end-stage liver diseases; however, survival rate was not markedly improved. Additionally, MSCs derived from hepatitis B patients presented impaired function as reflected by weakened proliferation, reduced activity, and fastened aging/senescence[68]. Allogeneic MSC transplantation might overcome major limitations of autologous MSC

Table 2 Clinical trials using mesenchymal stem cells to treat liver disease

Disease	Phase	No.	Stage	Source	Dose	Route of delivery	Main results	Ref.
LC	I–II	8	MELD score ≥ 10	Autologous BM-MSCs	30-50 million cells	Peripheral or the portal vein	Reduce volumes of ascites; improve MELD scores, INR, and serum creatinine	Kharaziha *et al* [64], 2009
LC	I–II	30	MELD Na score approximately 14	UC-MSCs	0.5 × 106 cells/kg	Peripheral	Reduce volumes; improve ALB, TBIL, PTA, and MELD Na scores	Zhang *et al* [65], 2012
ALC	II	48	Child-Pugh B/C	BM-MSCs	5×10^7 cells/kg	Hepatic artery	Improve Child-Pugh scores and histologic fibrosis	Suk *et al*[66], 2016
Liver failure	II	53	MELD score: CG: 29.15 ± 3.72; EG: 30.01 ± 3.99	Autologous BM-MSCs	None	Hepatic artery	Improve ALB, TBIL, PT, and MELD scores	Peng *et al*[67], 2011
ACLF	II	56	17 ≤ MELD score ≤ 30	Allogeneic BM-MSCs	$1.0\text{-}10 \times 10^5$ cells/kg	Peripheral veins	Improve ALT, ALB, TBIL, and MELD scores; decrease mortality	Lin *et al*[69], 2017
ACLF	II	24	MELD score: CG: 26.32; EG: 24.05	UC-MSCs	0.5×10^6 cells/kg	Cubital vein	Improve ALB, CHE, PTA, and MELD score; increase survival rate	Shi *et al*[70], 2012

LC: Liver cirrhosis; ALC: Alcoholic liver cirrhosis; ACLF: Acute-on-chronic liver failure; CG: Control group; EG: Experimental group; BM-MSCs: Bone marrow-derived MSCs; UC-MSCs: Umbilical cord-derived MSCs; MELD: End-stage liver disease; ALB: Albumin; ALT: Alanine aminotransferase; TBIL: Total bilirubin; PT: Prothrombin time; PTA: Prothrombin time activity; INR: International normalized ratio; CHE: Cholinesterase.

treatment. In a trial[69], allogeneic BM-MSC transplantation was employed in patients with HBV-related ACLF: 56 patients were infused weekly for 4 wk with $1\text{-}10 \times 10^5$ cells/kg allogeneic BM-MSCs while 54 patients were treated with standard medical therapy as a control group. Interestingly, allogeneic BM-MSC treatment could markedly ameliorate laboratory parameters, such as ALT, ALB, TBIL, and MELD scores. More importantly, mortality from multiple organ failure and severe infection was significantly decreased. In addition, no severe side-effects were observed until the end of follow-up at 24 wk after treatment. In another trial[70], 24 patients received 0.5×10^6 cells/kg UC-MSCs *via* the cubital vein. Those patients receiving MSC transplantation had better liver function as indicated by increased ALB and PTA levels. In particular, they exhibited a decreased MELD score and increased survival rate, inconsistent with previous finding (Peng *et al*[67]). The difference might be caused by different sources of MSCs. Compared with BM-MSCs, UC-MSCs had higher proliferation and clonality capacity[71]. Furthermore, UC-MSCs expressed lower levels of senescence markers, which made UC-MSCs more advantageous over BM-MSCs for therapy of end-stage liver diseases[72]. Based on the data, MSC therapy in the treatment of liver disease is limited by the quality of MSCs and therapeutic strategies. Although these results demonstrated that MSC transfusion is safe and may serve as a novel therapy for patients with liver diseases, some limitations remain in these studies. For example, follow-up time is not long enough and larger-scale studies are needed. Overall, there are still some problems that need to be clarified about the clinical application of MSC in the future, for example, the contraindications for MSC therapy in liver disease. Of note, in clinical trials, patients with the following conditions should be excluded, including pregnant and lactating women, severe heart or lung function failure, other important organ dysfunctions, proven other malignancies, spontaneous peritonitis or concomitant infection, active gastrointestinal bleeding, and active substance abuse.

CONCLUSION

MSCs have emerged as a promising treatment for liver diseases due to their hepatic differentiation potential, as well as anti-fibrotic activities and immunomodulatory properties. Currently, accumulating evidence has indicated the efficacy of MSC in

animals. However, many concerns remain to be addressed in clinical use of MSCs for liver diseases, including optimal timing of injection, optimal types of stem cells, the minimum number of effective cells, as well as the best route of administration. Recently, MSC-secreted exosomes have attracted attention. Exosomes are safe with controllable outcomes. Thus, this cell-free therapy may become a new therapeutic strategy for patients with liver diseases. In conclusion, using MSCs as a therapy for treating liver diseases holds great promise although requires large randomized and controlled clinical trials to confirm their safety and efficacy in the clinic.

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