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OPINION REVIEW

Stem cell-derived biofactors fight against coronavirus infection

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Abstract

Despite various treatment protocols and newly recognized therapeutics, there are no effective treatment approaches against coronavirus disease. New therapeutic strategies including the use of stem cells-derived secretome as a cell-free therapy have been recommended for patients with critical illness. The pro-regenerative, pro-angiogenic, anti-inflammatory, anti-apoptotic, immunomodulatory, and trophic properties of stem cells-derived secretome, extracellular vesicles (EVs), and bioactive factors have made them suitable candidates for respiratory tract regeneration in coronavirus disease 2019 (COVID-19) patients. EVs including microvesicles and exosomes can be applied for communication at the intercellular level due to their abilities in the long-distance transfer of biological messages such as mRNAs, growth factors, transcription factors, microRNAs, and cytokines, and therefore, simulate the specifications of the parent cell, influencing target cells upon internalization and/or binding. EVs exhibit both anti-inflammatory and

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tolerogenic immune responses by regulation of proliferation, polarization, activation, and migration of different immune cells. Due to effective immunomodulatory and high safety including a minimum risk of immunogenicity and tumorigenicity, mesenchymal stem cell (MSC)-EVs are more preferable to MSC-based therapies. Thus, as an endogenous repair and inflammation-reducing agent, MSC-EVs could be used against COVID-19 induced morbidity and mortality after further mechanistic and preclinical/clinical investigations. This review is focused on the therapeutic perspective of the secretome of stem cells in alleviating the cytokine storm and organ injury in COVID-19 patients.

Key Words: COVID-19; Secretome; Mesenchymal stem cell; Exosome; Stem cell; Biofactors

Core Tip: The world has witnessed unbelievable damage due to the coronavirus disease 2019 (COVID-19) pandemic. The rapid propagation of the disease requires emerging therapeutic strategies. The central role of the immune system during COVID-19 highlights the importance of a balanced immune response in order to prohibit overexaggerated responses and further multiorgan dysfunction. Stem cell and stem cell-derived secretome-related therapies have gained increasing momentum in the treatment of a broad range of diseases in the past decade. In particular, the immunomodulatory properties of stem cell-derived biofactors could be a new avenue in the treatment of COVID-19 patients.

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is designated as the etiology of coronavirus disease 2019 (COVID-19), which is a pandemic viral disease. The symptoms from death or acute respiratory distress syndrome (ARDS) to mild upper respiratory symptoms[1]. Excessive systemic immune activation of patients generates a cytokine storm, which is found in severely ill COVID-19 patients. Recent evidence indicates that the cytokine storm could play a key role in disease progression, resulting in the failure of various organs or even death. Hence, approaches which prevent the cytokine storm may be significant in mitigating COVID-19[2,3].

Complications such as cardiovascular system dysfunction, primarily acute myocardial injury, arrhythmia, or heart failure[4], neurological complications[5], gastrointestinal symptoms[6], and acute kidney injury[7] have been identified in a substantial proportion of COVID-19 patients. The unprecedented COVID-19 pandemic demands urgent therapies. Currently, multiple medicines involving anti-viral, anti-malarial, and anti-inflammatory agents are being investigated. Regardless of the patient's recovery and survival due to various therapeutics, lung damage in these patients does not fully recover. Recently, promising stem cell therapies and importantly secreted extracellular vesicles (EVs) have been shown to exhibit anti-inflammatory effects and attenuate COVID-19-related lung injury.

A new therapeutic approach which involves cellular therapies is promising in treating chronic and acute lung diseases due to their anti-inflammatory, immunomodulatory, regenerative, pro-angiogenic, and anti-fibrotic features. Mesenchymal stem cell (MSC)-secretome (a paracrine mechanism) composed of EVs and free soluble proteins mediate those therapeutic impacts[8]. The remarkable properties of exosomes have gained considerable attention as a probable therapeutic option in COVID-19. *In vivo* and *in vitro* studies have been conducted to determine the various therapeutic effects of MSC-secretome in tissue regeneration, heart, and lung diseases. The inflam-

mation suppressing effects of MSC-secretome are due to the prevention of monocyte differentiation into dendritic cells, prohibiting natural killer (NK) cells proliferation and cytotoxicity, stimulating macrophage polarization from the pro-inflammatory (M1) to anti-inflammatory (M2) phenotype, regulating the inflammatory characteristics of T helper cells, and inhibiting T cell proliferation. Growth factors found in the MSC-secretome regenerate the damaged lung tissue by increasing proliferation and reducing apoptosis of resident lung epithelial and endothelial cells. Additionally, antimicrobial peptides (AMPs) have been observed in MSC-secretomes and demonstrate antimicrobial properties, whereas protease inhibitors reduce extra protease function in the lung, preserving the protease/anti-protease equilibrium[9]. Exosomes extracted from MSC act as multitargeting agents. Therefore, they diminish the cytokine storm and prevent the inhibition of COVID-19-related anti-viral defenses in hosts[10]. Exosomes may hamper the cytokine storm and inflammatory process due to their reparative properties and thus induce endogenous repair. Hence, MSC-secretome might be a valuable cell-free substitute to cell-based therapies alone or in combination with pharmacological agents. In this review, the therapeutic potential of the secretome of stem cells in mitigating COVID-19-induced cytokine storm and organ damage is presented.

STEM CELL-BASED THERAPEUTICS

Evidence has shown the promising role of MSCs in COVID-19 pneumonia treatment. Human umbilical cord MSCs given to a 65-year-old female with severe COVID-19 induced substantial recovery through modulation of the immune system and regeneration of damaged tissue with high safety[11]. Every three days, MSCs were administered intravenously by clinicians three times (5×10^7 cells each time). Leng *et al* [12] demonstrated the enhancement of pulmonary function and symptom improvement in seven COVID-19 patients with pneumonia in only two days after administration of MSCs. In their study, only one administration per kilogram of weight containing 1×10^6 cells was performed. The authors proposed that the therapeutic impact mainly occurred based on the immunoregulating features of MSCs. Remarkably, MSCs are not virus infectable as they are angiotensin-converting enzyme 2 negative. Hence, for treating seriously ill COVID-19 patients under certain protocols, MSCs can be considered a potential treatment option[13]. As the severity of this viral infection is closely associated with the host's immune response, the immunomodulatory effects of MSCs can efficiently prohibit the cytokine storm and thus treat severe cases of COVID-19. Indeed the outcomes of COVID-19 patients can be enhanced by the transplantation of MSCs using various methods, including "as a result of their immunoregulatory impact, as a result of inducing regeneration and repairing tissue, and as a result of their antimicrobial, antifibrotic, and angiogenic properties".

All these methods improve lung repair and prevent multiple organs from exaggerated immune response-derived damage. The ongoing COVID-19 clinical trials based on MSCs have been reviewed recently[14]. It now seems that MSCs convey their therapeutic effects through the paracrine pathway. As these cells can discharge secretome (active biological substances), therefore they can be potentially addressed as drug stores[15].

THE SECRETOME OF STEM CELLS: A CELL-FREE ALTERNATIVE TO CELL-BASED THERAPEUTICS

The secretome is defined as a stem cell secretion composed of regulatory factors and various soluble molecules, including AMPs, angiogenic growth factors, lipid mediators, and anti-inflammatory cytokines. Evidence has shown that these molecules are packed into EVs, also known as cell-secreted vesicles[16,17].

Common secretory mechanisms are involved in the excretion of secretomes by stem cells (Figure 1). Following the administration of the secretome or the culture medium in patients, through a paracrine signaling pathway, neighboring cells assimilate them [18]. Two important secreted EVs, are exosomes and MVs, also known as microvesicles alongside the apoptotic bodies secreted by stem cells[19]. The fusing of plasma and multi-vesicular bodies facilitates exosome (30-100 nm) elicitation. However, cellular membrane budding generates MVs (100-1000 nm), which possess cellular cytoplasm. EVs are discharged into the extracellular microenvironment and act like soluble

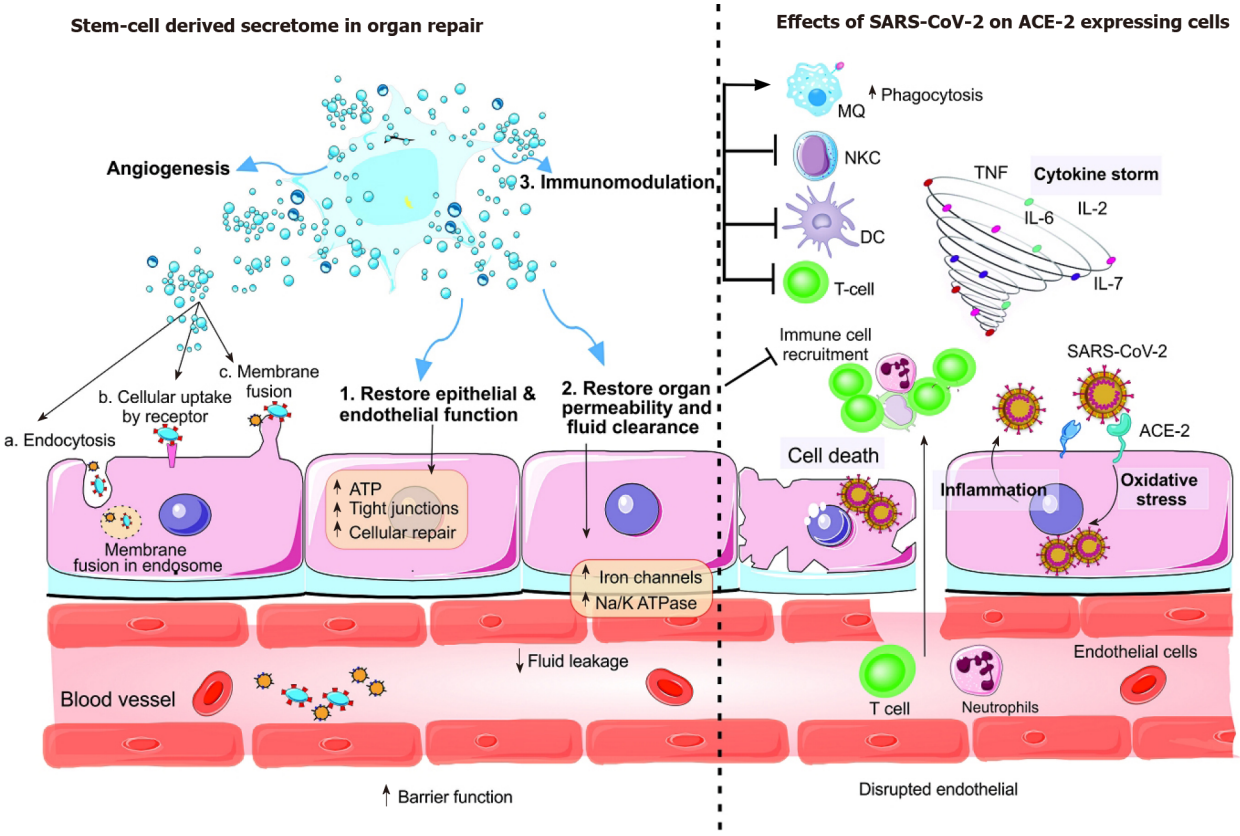

Figure 1 The stem cell secretome. After the interaction between host angiotensin-converting enzyme 2 (ACE2) receptor and the spike S protein of severe acute respiratory syndrome coronavirus 2, the membrane is fused, and the viral genome is released into the cell or the virus enters the cell by clathrin-dependent/independent endocytosis. Most of the cells in human organs including the intestine, heart, kidney, and alveolar type II (AT2) cells of the lungs express ACE2 receptors. Extracellular matrix metalloproteinase inhibitor (EMMPRIN or CD147) and two proteases transmembrane serine protease 2 (TMPRSS2) are required for virus entry into the host cell. The lungs are damaged after direct destruction of capillary endothelial cells and AT2, the renin-angiotensin system is disrupted or the immune response is diminished indirectly. Following virus-induced infection, pathogen-associated molecular patterns lead to recognition of the virus by the innate immune system, activation of nuclear factor-κappa B and IRF3 pathways, type I interferon (IFN) expression and consequently activation of the JAK/STAT pathway and finally the expression of IFN-stimulated genes (*ISG*) which have anti-viral activity. The abovementioned effective immune response is required for successful virus clearance and clinical disappearance of the disease. Nonetheless, the IFN response may be delayed due to evasion of IFN and *ISG* mediated killing by the virus which in turns leads to hyper-inflammatory neutrophils and macrophage infiltration at the pulmonary site accompanied by pro-inflammatory cytokines including granulocyte-colony stimulating factor, tumor necrosis factor, MCP1, and interleukin-1b/2/6/7/8/17[3]. This hyper-activation of T lymphocytes and the innate response is called the 'cytokine storm' and is responsible for lung disorders including acute respiratory distress syndrome, pneumonitis, viral sepsis, respiratory and organ failure. A high number of pro-inflammatory cytokines leads to hyaluronan synthase 2 induction which elevates hyaluronan production and fluid accumulation in the lungs[78]. In critical cases of coronavirus disease 2019, the virus enters the peripheral blood and translocates to various target organs including kidney, heart, and intestine and can cause multiple organ failure. ACE2: Angiotensin-converting enzyme 2; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; TNF: Tumor necrosis factor; IL: Interleukin; DC: Dendritic cell.

components and through endocrine and paracrine methods, they express their biological effects. In a more general definition, MSC-secretome contains all the secreted bioactive factors of MSCs, with both extravesicular and the soluble elements [20,21].

MSC-DERIVED EVS

Upon secretion, proteins, and EVs, through ligand-receptor interactions or internalization, engage with the target cells and regulate cellular responses. The secretome stimulates endogenous stem cells and progenitor cells, prevents apoptosis, attenuates the inflammatory response, triggers extracellular matrix remodeling and angiogenesis, prevents fibrosis, and regulates chemoattraction. It was revealed that following the MSCs' functional mitochondria or mitochondrial DNA transfer to target cells, they protect cellular aerobic respiration with non-healthy mitochondria or modulate T cell functions[21]. By resembling their parent cells, EVs from MSCs have characteristics such as immunoregulatory, anti-oxidative, progenerative, pro-metabolic, anti-apoptotic, and anti-inflammatory, in the microenvironment of damaged tissue. In

MSC-based therapy, extracted EVs from MSCs are considered a substantial alternative to cell-based treatments[22]. Their efficacy is presently under *in vitro* examination for lung damage treatment utilizing MSC-derived EVs in various preclinical experiments [22]. EVs extracted from MSCs showed efficacy in lung injury due to influenza in a pig model. Studies have revealed that extracted EVs from MSCs are available 12 h after virus infection and diminish the levels of pro-inflammatory cytokines as well as viral replication[23]. Ang-1 mRNA (an angiogenic trophic factor) is found in EVs from MSCs. Due to this factor's role in limiting leukocytes and vessel endothelial cells' interaction and sustaining the vascular barrier integrity, Ang-1 mRNA is considered substantial in endothelial cell stabilization during the injury process. The impact of EVs extracted from MSCs on lipopolysaccharide-induced acute lung damage in an experimental mouse model sheds light on the contributions of Ang-1 mRNA transfer by EVs to restore pulmonary capillary permeability[24]. Furthermore, EVs extracted from MSCs influence inflammation by inhibiting the expression of tumor necrosis factor alpha (TNF-α) and stimulating interleukin (IL)-10 secretion. Upon Ang-1 mRNA transfer and the internalization of EVs extracted from MSCs into injured endothelium cells, within the damaged lung microvascular endothelium, Ang-1 mRNA partially preserves protein permeability[25]. EVs are increasingly regarded as a substantial alternative to cell-based therapy.

Secretome administration is associated with multiple advantages in comparison with complete MSCs therapy. The secretome lacks self-replication and is not involved in endogenous tumor development, as it has low immunogenicity, and upon intravenous injection, it contributes to low emboli formation. Therefore, the secretome is deemed more advantageous than cells[26]. The MSC-secretome can be altered and preserved more conveniently than cells with fewer costs regarding technological advances. It also suits emergency interventions as the product is ready-to-use[27]. With regard to monoclonal antibody therapy, the costs of the MSC-secretome are lower, which is vital in the management of a pandemic. Nonetheless, many concerns regarding EVs should be resolved before clinical application, and the delivery route (intravenous or inhalation), purification, bio-distribution, production, and characterization should be determined.

EXOSOMES

Exosomes are nanoparticles (40-150 nm) which have various bioactive components, including proteins, growth factors, lipids, microRNAs (miRNA), long noncoding RNAs, and transfer RNAs. The lipid contents of exosomes provide the platform for their infusion with neighboring cells and plasma membrane[28]. Following internalization of the secretome components, neighboring cells alter several downstream pathways, such as fibrosis inhibition, immunoregulation, damaged tissue remodeling, and apoptosis suppression[8].

With regard to exosome isolation and production, MSCs discharge exosomes under circumstances such as cytokine treatment, serum starvation, or hypoxia[29]. Purification and the introduction of exosomes into the body can then take place. It has been shown that exosomes derived from MSCs generate an impact resembling that of MSCs[30]. The multiple proteins, miRNAs, and mRNAs transported from secretory cells to the exosomes' target cells exhibit anti-inflammatory traits[31]. Exosomes can stimulate regulatory cytokines, decrease the production of inflammatory cytokines, and prevent inflammation[32]. Impeding NK cells, CD4+ and CD8+ T cells can occur with MSC-exosomes[33]. They induce T cell IL-7 expression and stimulate the expression of IL-10 by regulatory cells, which are implicated in the suppression of inflammation. Furthermore, MSC-exosomes, by secreting transforming growth factor β (TGF-β) prevent CD4+ and CD8+ T cell differentiation and suppress inflammation *in vivo*[34]. MSC-exosomes treatment inhibits the activation and proliferation of NK cells [35]. MSC-exosomes engage in prohibiting pro-inflammatory states by shifting M1 macrophages to M2 phenotypes[36]. Moreover, MSC-exosomes prevent the secretion of pro-inflammatory factors including IL-17, interferon (IFN)-γ, IL-1β, TNF-α, and IL-6 [37], and stimulate the secretion of anti-inflammatory factors including TGF-β, IL-4, and IL-10[38]. Also, their function decreases the serum chemokine level[39]. The immunoregulatory features of MSC-exosomes are associated with their anti-inflammatory components, including PD-L1, HLA-G, Galectin-1, and IDO[40-42]. Furthermore, MSC-exosomes, by escalating ATP levels in alveolar type II cells, increase their survivability[43]. In addition, exosomes possess adhesion molecules which accurately guide them to the damaged site. The exosome components then cross the

blood-brain barrier. They are low-cost and do not undergo independent self-renewal processes. Thus, they impede serious consequences involving tumor development and other adversities.

THE ADVANTAGEOUS THERAPEUTIC IMPACT OF STEM CELL-DERIVED SECRETOME

The therapeutic effects of MSC-derived EVs on lung and heart injuries have been demonstrated. There are also studies on the impact of MSC-EVs on hemagglutination of swine, avian, and human influenza viruses[23]. In addition, MSC-exosomes reduced mortality in H7N9 patients with no concomitant toxic complications during the follow-up period[44]. One study revealed that S proteins within exosomes can be considered a novel vaccine for countering SARS-CoV infections[45]. In a test of S-containing exosomes immunogenicity in mice, the results showed amplified titers of neutralizing antibody. Moreover, with regard to economics, MSC-exosomes therapy was a less expensive treatment than maintaining and extending individualized clonal cell populations[46]. In the following section, we provide a general review of the present findings on stem-cell extracted secretomes in preclinical studies for lung and heart injuries (the organs most damaged by SARS-CoV-2).

STEM CELL-DERIVED SECRETOME IN THE PATHOGENESIS OF ORGANS

EVs extracted from MSCs have been shown to have an impressive impact on ARDS and acute lung injury. This is the result of the immunoregulatory and anti-inflammatory features of MSC-EVs[24], which induces shrinkage of the permeability of the endothelium and epithelium of alveoli[25], enhancing alveolar fluid clearance[43], macrophage phagocytosis improvement[47], and direct mitochondrial transfer with host cells promoting tissue repair.

Secretome in the blood has impressive stability, subsequent to MSC intravenous administration, and reaches the lungs *via* blood flow. It is then distributed in the tissues and promotes bacterial clearance, resolution of inflammation, enhances immune regulation, and preserves capillary barrier function[19]. The soluble components of MSCs inhibit inflammation, and EVs induce tissue repair. EVs secreted from MSCs, in particular, lung injuries, provide metabolites, DNA, miRNA, mRNA, and proteins to cells thus enhancing lung repair, and restoring and regenerating lung function[48].

Lung accumulation of MSCs occurs after systematic administration. Following secretion of their components, they enhance the pulmonary microenvironment, preserve the epithelial cells of alveoli, inhibit pulmonary fibrosis, and strengthen lung function[13]. Furthermore, distant affected organs (for example, the cardiovascular system) can take advantage of MSCs due to the secretory characteristics of these cells. Various studies have focused on the circulation of the cellular cargo, and preclinical trials revealed their capacity to manipulate diverse pathways to promote cellular communication. miRNA (a composition of exosomes) has been demonstrated to have a significant role in physiological functions, including immune modulation, development, epigenetic modifications, and so on[49]. The manufacture and isolation of EVs could be a beneficial therapy in pulmonary injuries[50].

An experimental mouse model of neonatal hyperoxia showed that MSCs from human bone marrow and Wharton's jelly inhibited lung fibrosis, enhanced pulmonary vascular remodeling, and stimulated lung development in bronchopulmonary dysplasia. MSC-derived exosomes exhibited anti-inflammatory activity and altered the pro-inflammatory M1 pulmonary macrophages to anti-inflammatory M2 macrophages followed by inhibition of lung inflammation and the immune response facilitating organ development[51]. Exosomes derived from MSCs have demonstrated mitigating effects in an asthma and ARDS model of lung damage[52]. The potential role of exosomes in alveoli fluid clearance was identified during an *ex vivo* experiment involving human donor lung (not suitable for transplantation) perfusion. This was assisted by exosome CD-44 which was involved in the internalization mechanism in damaged host cells[53]. In addition, exosomes extracted from MSCs have been indicated in the direct inhibition of viral multiplication.

Overall, exosomes extracted from MSCs show promising effects on decreasing pulmonary edema and protein permeability, reversing lung inflammation, the prolif-

eration of lung epithelium, and the polarization of lung macrophages[54]. Additionally, MSC-derived exosomes are effective in the treatment of cardiovascular [55] and renal disease[56].

The therapeutic impact of MSC-EVs on acute myocardial infarction has been reported. This has been shown to involve the following underlying mechanisms: Reduction of the inflammatory response[29], reduction of cardiac fibrosis, mitigation of cardiomyocyte apoptosis[57], induction of angiogenesis[58] and promotion of cardiomyocyte autophagy[59]. It was also shown that, fibroblast growth factor, composed of MSC-secretome (derived from adipose tissue), inhibited viral replication processes[60].

STEM CELL-DERIVED EXOSOMES; A NANO-PLATFORM FOR COMBATING COVID-19

COVID-19 patients may develop multiorgan damage. In the initial phases of infection, mainly pneumocyte type II cells are infected, and other target cells may be bronchial cells, monocytes, macrophages, and enteric cells. Moreover, the principal SARS-CoV-2 cardiovascular complication is acute myocardial injury[61]. Heart tissue biopsies from COVID-19 patients revealed mononuclear inflammatory infiltration, more commonly found in cardiomyocyte necrosis sites[62]. Applying stem cell-derived secretome to organs damaged by COVID-19 is possible, according to extracted data. Also, the survival rate of septic mice increased following MSC-derived exosome treatment[63]. With regard to MSC-derived exosomes as supportive therapy in the current pandemic, this can be beneficial in inhibiting the effects of COVID-19 [42] and healing organ damage.

Experimental studies on the biological activity of MSC secretomes have demonstrated the possibility of applying MSC-derived secretomes for seriously ill COVID-19 patients as a cell-free therapy. EVs and proteins contained in MSCs affect endogenous stem and progenitor lung cells. They promote cell differentiation and proliferation, inhibit the inflammatory response, prevent apoptosis, reduce fibrosis, and recover capillary barrier function. Due to their similarity to parental MSCs, they are also effective in the management of chronic and acute lung injuries[64]. Exosomes (ExoFlo™) derived from MSCs of allogeneic bone marrow have been proposed as a treatment for seriously ill COVID-19 patients according to the first prospective nonrandomized open-cohort study conducted. ExoFlo is considered to be a hopeful therapeutic candidate for COVID-19 due to its capacity to restore oxygenation, immunity reconstitution, safety traits, and downregulation of the cytokine storm[10]. This study included twenty-four patients suffering from ARDS with severe and moderate-to-severe symptoms. Exoflo was administered intravenously in a single dose, with no harmful effects identified 72 h after administration. The study showed an 83% survival rate, cytokine storm downregulation, substantial hypoxia recovery, and immune restoration. Exosome-involved COVID-19 clinical trials are ongoing in the United States, China, Turkey, and Russia (NCT04276987, NCT04493242, NCT04491240, ChiCTR2000030484, ChiCTR2000030261, NCT04384445, NCT04389385).

The advantage of MSC-derived secretome therapy is its two forms (inhalable and injectable formulation) for potential clinical applications[9]. Both forms exist as freeze-dried powder and can be used in patients with a severe COVID-19 lung infection. An examination of the inhalable secretome form for COVID-19 pneumonia was conducted in a clinical trial (NCT04276987) in China, and its tolerance was examined in healthy individuals (NCT04313647)[8]. Assessment of its therapeutic efficacy demands further randomized controlled trials with comprehensive delivery of the exosomes. Moreover, in addition to MSC-derived secretomes, the secretome of oral tissue stem cells is also considered to have a therapeutic impact in infected cases due to their immunoregulatory and anti-inflammatory characteristics. Non-invasive therapy is superior to invasive therapy in prophylaxis and results in minimum risk of the treatment process, and prevents COVID-19, offering a novel immunoregulatory pathway for COVID-19 therapy[65].

The potential role of exosomes in treating COVID-19 can be classified into three general divisions. First, instead of cell therapy, the exosomes derived from multiple MSCs are utilized. Second, particular mRNAs and miRNAs are incorporated into the exosomes. Third, exosomes could be used as drug carriers in the treatment of COVID-19[66]. The efficacy of stem cell-derived secretome therapies is the main focus of continuing clinical trials. Nonetheless, the effectiveness, safety, and long-term consequences of these therapies require further study.

CHALLENGES IN TREATING COVID-19 USING STEM CELL-DERIVED SECRETOME

Administering stem-cell EVs as a possible treatment for COVID-19 is supported by initial examinations. However, for the sake of scientific rationale, further understanding and justification of MSC-EVs and other EV treatment effects on COVID-19 are required. MSCs have demonstrated promising effects on COVID-19 pathogenesis. However, their resemblance to exosomes is uncertain. Regulation of the immune response is the main impact of MSC-EVs, rather than impeding it (Figure 2). By regulating the response, they moderate it. They also strengthen tolerance and improve homeostasis[67]. Although stimulating tolerance in graft-*vs*-host and other non-infectious diseases is effective, it sometimes has an adverse effect on replicating pathogens. Albeit in selected models, *Escherichia coli* and influenza infection did not escalate but other replicating bacteria and viruses can experience augmented uncontrollable infection due to tolerance stimulation[68].

Prior to MSC-EVs application in COVID-19 patients, particular concerns must be resolved. The EV isolation method, purification, and characterization must be determined. These have a meaningful impact on the examination results, and in clinical trials can generate obscure conclusions. These parameters involve the origin of MSC-EVs. MSCs (as a heterogeneous cell essence) are derivable from various tissues. Even derivatives of the same tissue vary in inter-individual and clone-specific functions[69]. In fact, comparing four MSC-EV samples harvested from separate donor-derived bone marrow placed in conditioned media revealed substantial cytokine component differences[70]. Moreover, in one study MSCs from young individuals (suffering from acute lung injury), but not elderly individuals, attenuated lipopolysaccharide-induced acute lung injury[71].

The problem of EV heterogeneity from dissimilar resources, preparations, and other issues can be solved by manufacturing immortal MSC colonies that can be deliberately examined for potency and production of EVs[72]. Besides the problem of derived MSC-EVs from different origins, another consideration is their various responses to different disease conditions. Therefore, it is unclear whether the divergent immune response regulation of exosomes is due to tissue specificity. Regardless of the immunoregulatory properties, MSC-EVs seem to influence additional biological mechanisms with therapeutic functions[73] and other probable unanticipated effects. Recently conducted studies demonstrated that adipose-derived MSC-EVs had higher thrombogenic traits than bone marrow-derived MSC-EVs[74]. Accordingly, the origin of parental cells can potentially result in a higher thrombosis risk. The complement pathway stimulation accompanied by the procoagulant condition in a fraction of serious COVID-19 cases can result in the devastating microvascular injury syndrome [49], and the application of MSC-EVs may be ineffective.

A further hurdle is sustaining their stability and productivity with time[75]. Exosomes, from MSCs at -80°C, are viable for an extended period. Nevertheless, exosome clustering appears after storage due to freeze-thaw cycles. Moreover, preservation at low temperature during transportation and handling contributes to translational application impediments[76].

The manufacture of EVs requires living cells that are cultured under GMP-compliant (good manufacturing practice-compliant) processes to conserve safety and quality standards criteria. Hence, EV manufacture resembles the ethical and scientific guidelines used for MSCs. Also, basing any therapy on EVs extracted from MSCs requires the approval of national regulatory agencies to ensure their safety and productivity. The International Society for Cellular and Gene Therapies and the International Society for Extracellular Vesicles decrease the risk of critical side effects by deliberately weighing the probable benefits and risks of MSC-EVs for COVID-19 and have already provided preclinical data in connection with animal models and relevant MSC clinical trial-derived data. Additionally, they urge deliberate EV use evaluation by rational clinical trial design, applying well-characterized EV preparations generated according to strict GMP conditions and under proper regulatory oversight[77].

CONCLUSION

MSC-derived secretome demonstrates beneficial results as a cell-free therapy for acute and chronic lung diseases. It exhibits immunoregulatory, pro-angiogenic, anti-inflammatory, regenerative, and anti-protease characteristics. Due to the prominent role of

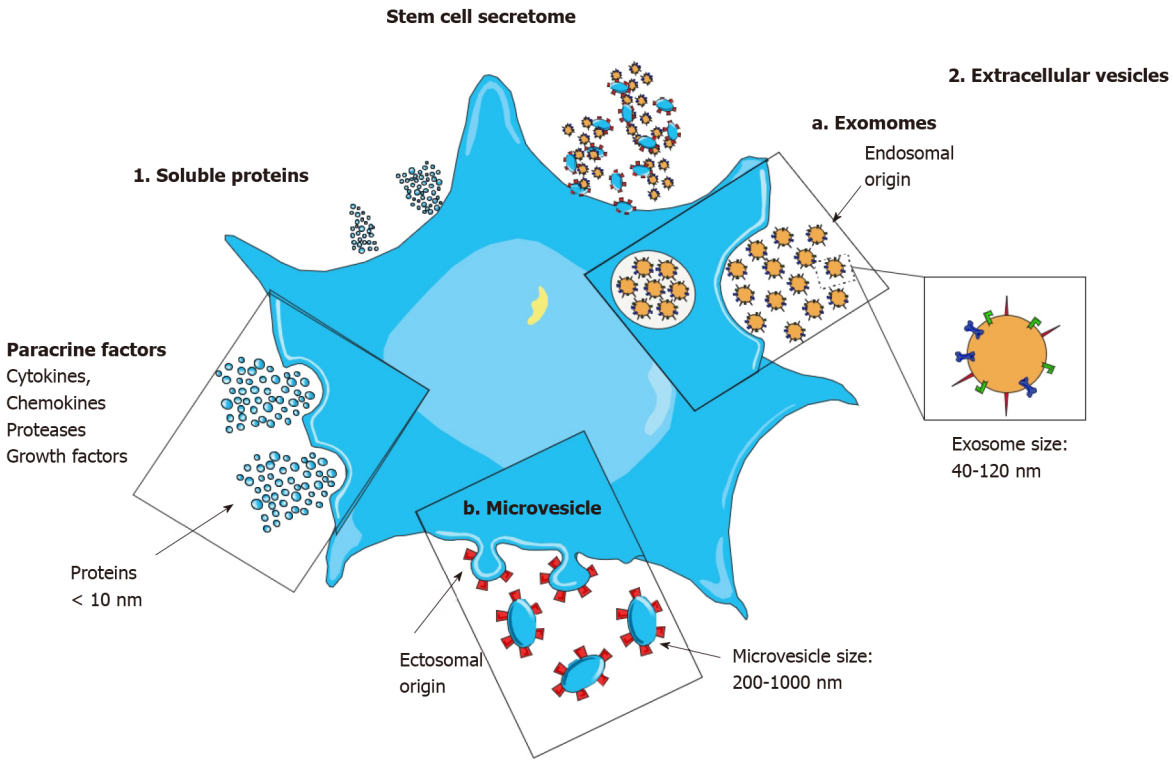

Figure 2 Immunomodulation by the stem cell secretome. The behavior of immune cells is altered by the secretion of various immunomodulatory factors. Mesenchymal stem cells (MSCs) secrete SOD3 which inhibits neutrophil activation and infiltration of leukocytes. Also, MSCs secrete PGE2, SDF-1, and interleukin (IL)-10 and lead to macrophage polarization to the M2 phenotype. Furthermore, MSCs induce HLAG5 secretion (shift to CD4β CD25β T reg population), Il-4 secretion (shift to Th2 population), constitutive expression of transforming growth factor β1 (TGF-β1), HGF, COX2, IL-10, IDO, and PGE2 which in turn inhibit T-cell proliferation. The secretion of IL-2 prevents inactivated NK cell proliferation, and secretion of HLAG5, TGF-β1, IDO, and PGE2 prevent cytokine secretion. In addition, MSCs cause B cells arrest in the G0/G1 phase and reduce the level of circulating immunoglobulins and secretion of CXCR4, CXCR5, CXCR7 by B cells. Due to the high amount of ISG gene expression, MSCs induce an antiviral response in the lungs. After secretion of immunomodulatory, anti-inflammatory, and microRNAs mediators and their extracellular vesicles-mediated transfer, MSCs lead to regulatory lymphocyte and M2 macrophage production. Differentiation of MSCs to various lung epithelial cells or differentiation of host tissue-resident stem cells lead to the secretion of numerous growth factors and angiogenic factors to stimulate revascularization, and consequently repair structural injury. Recovery of alveolar cell functions, their ATP stores, and metabolic capacity is feasible by direct transfer of functional mitochondria. Anti-fibrotic cytokines in high amounts reduce collagen fibers and subsequently hyper-inflammation and oxidative stress. A combination of anti-viral drugs with the immunomodulatory cargo of MSCs exosomes is a promising intervention tool in disease treatment[54]. Remdesivir is the best example of drug loading on exosomes[79]. Nevertheless, more studies are needed to clarify the exact mechanism of the specificity, targeted delivery and safety of exosomes.

MSC-derived exosomes in inhibiting the inflammatory response and in injured tissue regeneration, they may be a valuable therapeutic option in COVID-19-related pneumonia. Additionally, exosomes are considered potential nanocarriers, biomarkers, and vaccines for COVID-19 treatment. Regarding the concerns on their outcome, it is important to assess the risk when utilizing MSC-exosomes for COVID-19 by determining applicable preclinical findings *in vivo* models.

Upon reaching the lungs, the inhalable administered secretome encounters three main hurdles (anatomical, pathological, and immunological) and exerts their therapeutic effects. Although, the advantages of cell-free therapy are significant, it is considered a novel approach and requires secretome optimization and standardization *via* a comprehensive investigation of its components, dosing conditions, quality control, formulation and preparation process, and long-term storage strategies. Further data on the therapeutic mechanism, new formulation strategies, scalable and GMP-compliant isolation processes, and the capability to convey EVs and soluble proteins through non-invasive pathways of administration are required. These challenges will be groundbreaking, and will provide impressive clinical outcomes in the treatment of acute and chronic lung diseases.

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REVIEW

Application of mesenchymal stem cells derived from human pluripotent stem cells in regenerative medicine

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Abstract

Mesenchymal stem cells (MSCs) represent the most clinically used stem cells in regenerative medicine. However, due to the disadvantages with primary MSCs, such as limited cell proliferative capacity and rarity in the tissues leading to limited MSCs, gradual loss of differentiation during *in vitro* expansion reducing the efficacy of MSC application, and variation among donors increasing the uncertainty of MSC efficacy, the clinical application of MSCs has been greatly hampered. MSCs derived from human pluripotent stem cells (hPSC-MSCs) can circumvent these problems associated with primary MSCs. Due to the infinite self-renewal of hPSCs and their differentiation potential towards MSCs, hPSC-MSCs are emerging as an attractive alternative for regenerative medicine. This review summarizes the progress on derivation of MSCs from human pluripotent stem cells, disease modelling and drug screening using hPSC-MSCs, and various applications of hPSC-MSCs in regenerative medicine. In the end, the challenges and concerns with hPSC-MSC applications are also discussed.

Key Words: Human pluripotent stem cells; Differentiation; Mesenchymal stem cells; Regenerative medicine; Disease modelling; Drug screening

Core Tip: Mesenchymal stem cells (MSCs) exhibit great potential in regenerative medicine. However, the clinical application of primary MSCs has been greatly hampered by the limitations of primary MSCs. MSCs derived from human pluripotent stem cells (hPSC-MSCs) are an attractive source of cells to overcome such problems with primary MSCs. This review summarizes the various derivation approaches and applications of hPSC-MSCs in regenerative medicine. Lastly, the challenges with the use of hPSC-MSCs are also discussed, which indicate that more efforts are needed for

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INTRODUCTION

Mesenchymal stem cells (MSCs) are adult stem cells with fibroblast-like morphology and plastic adherence. They express MSC surface antigens such as CD73, CD90, and CD105 but lack hematopoietic markers such as CD11b, CD19, CD34, and CD45[1]. More importantly, MSCs can give rise to multiple mesenchymal lineages, including bone, cartilage, and fat cells[1-3]. Friedenstain and colleagues first described an adherent subpopulation in bone marrow termed as marrow stromal cells[4-7]. The term of MSCs was later introduced in 1991 to refer to these cells[8]. MSCs reside in nearly all tissues, including bone marrow and adipose tissues, among others. Due to their expandability, multipotency, immunosuppression, and limited ethical concerns as compared to other types of stem cells, human MSCs have emerged as an attractive cell source for regenerative medicine. Moreover, MSCs exhibit low expression of major histocompatibility (MHC) antigens, thereby reducing the need for MHC match between different donors and recipients in allogeneic MSC transplant. Due to these characteristics that MSCs possess, MSC-based allogeneic transplantation is now the forefront of regenerative medicine. As a fast-growing field in regenerative medicine, MSCs represent the most clinically used stem cells with over 1000 registered clinical trials with an established safety record in patients that can efficaciously treat more than 30 diseases. However, there are several limitations of primary MSCs that greatly hamper their clinical application. They include limited cell proliferative capacity, gradual loss of differentiation potential during *in vitro* expansion, variation across donors, rarity in organs, invasive procedures required for harvesting, *etc.*

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), represent a promising solution to overcome the issues associated with primary MSCs. Due to the pluripotency of hPSCs, they exhibit unlimited proliferation ability and are able to differentiate into various types of cells, including MSCs. Therefore, hPSCs can provide unlimited and uniform MSCs as an alternative cell source to primary MSCs. This review summarizes the derivation approaches and various applications of hPSC-MSCs, and ultimately the challenges associated with safety and efficacy of hPSC-MSCs are discussed.

DERIVATION OF HPSC-MSCS

Although primary MSCs have been widely used for clinical application, the previously mentioned limitations with the use of primary MSCs significantly hamper their clinical applications. To overcome the problems with primary MSCs, substantial advancements have been made to develop a number of approaches for derivation of MSCs from hPSCs, including hESCs and iPSCs. These approaches include spontaneous differentiation *via* coculture with OP9, fetal bovine serum (FBS)-containing media, and embryonic body (EB), or directed differentiation *via* delicate control of signalling pathways. The principle of these approaches is to deprive pluripotent signals of hPSCs, thereby driving differentiation into MSCs.

During embryonic development, MSCs develop from neural crest cells (NCCs), lateral plate mesoderm, or paraxial mesoderm, which further develop into craniofacial skeleton, appendicular skeleton, and axial skeleton, respectively. The neural crest is a transient structure formed through epithelial-mesenchymal transition (EMT) with potential to differentiate into a wide range of cell types, including MSCs. It was shown that neural crest cells were derived from hPSCs[9-13], which were able to develop or differentiate into MSCs[14-16]. Morikawa *et al*[15] showed that MSCs in the adult bone marrow had at least two developmental origins, one of which was the neural crest. By lineage tracing, Takashima *et al*[16] showed that Sox1+ neuroepithelium gave rise to

MSCs in part through a neural crest intermediate stage. The combination of the glycogen synthase kinase 3 beta inhibitor and transforming growth factor-beta (TGFβ) inhibitor very efficiently induced hPSCs towards hNCCs (70%-80%), which further differentiated into MSCs with chemically defined medium[14]. The mesoderm is a major source of MSCs, and we recently reported a stepwise, serum-free, chemically defined and highly efficient protocol to generate hPSC-MSCs *via* lateral plate mesoderm. The resultant iPSC-MSCs displayed similar MSC surface antigen profile, gene expression profile, and epigenetic profile. iPSC-MSCs had three lineage differentiation. Significantly, hPSC-MSCs were able to repair cartilage defects, similar to bone marrow-MSCs (BM-MSCs)[17]. Upon differentiation, mESCs gave rise to VEGFR-2$^+$ PDGFR$^+$ population followed by VEGFR-2$^-$PDGFR$^+$ population *via* paraxial mesoderm [18]. hESC-derived KDR$^-$PDGFRa$^+$ paraxial mesoderm-like cells showed robust chondrogenic activity and generated a hyaline-like translucent cartilage particle whereas STRO1$^+$ BM-MSCs showed relatively weaker chondrogenesis and formed more fibrotic cartilage particles *in vitro*[19].

MSCs in the placenta develop from trophoblasts in the extraembryonic tissue chorion[20]. MSCs can also be derived *via* trophoblasts. hESCs cultured in serum containing medium[21] and serum free medium[22] containing BMP4 and A83-01 were able to differentiate into trophoblasts and then into MSCs. Trophoblast-derived MSCs produced less interleukin 6 (IL-6), C-X-C motif chemokine ligand 10, and C–C motif chemokine ligand 2 but more programmed death-ligand 1 in response to IFN gamma (IFNγ) treatment as compared with MSCs[21]. Compared with MSCs from serum containing medium, serum free approach took longer than serum containing approach to derive MSCs, but serum-free derived MSCs grew faster and produced less IL-6 and interleukin 8[22].

Barberi *et al*[23] first reported that MSCs were derived from hESCs by coculturing hESCs with monolayer of murine OP9 stromal cells. However, the undefined condition in this approach inevitably led to spontaneous differentiation, giving rise to an undesired type of cells. Besides MSCs, non-MSCs such as CD34 (+) primitive hematopoietic cells, were also present[24]. Vodyanik *et al*[25] showed that MSCs were derived from a common precursor of mesenchymal and endothelial cells called mesenchymoangioblast by coculturing hESCs with OP9.

Culturing hPSCs in the undefined condition of FBS-containing MSC medium is another way to derive hPSC-MSCs by providing growth factors required for differentiation towards MSCs. When hESCs or iPSCs were cultured in FBS-containing MSC medium for 4 wk to derive hPSC-MSCs, hPSC-MSCs inhibited cell proliferation and cytolytic function of natural killer (NK) cells in the same fashion that BM-MSCs did. However, they were more resistant to preactivated NK cells as compared with adult BM-MSCs[26]. A high density of hESCs on a porcine gelatin-coated dish were cultured in a medium containing 10% FBS for 7 d to outgrow the cells and then enrich hESC-MSCs by 1-2 passages[27]. Functional iPSC-MSCs were also derived on coating with gelatin, and the resultant iPSC-MSCs pre-induced into osteogenesis for 4 d formed bone in the calvaria defects confirmed by human specific nuclear antigen and mitochondrial antibodies[28]. hESC/iPSCs were seeded onto collagen coating and cultured in FBS-containing medium for 10 d to generate hESC/iPSC-MSCs[29]. Spontaneously differentiated cells (raclures) from feeder-free hESCs were cultured in FBS-containing MSC medium for 4 wk, and hESC-MSCs were enriched by following passage[30]. Chen *et al*[31] reported the derivation of hPSC-MSCs by serum-free medium containing TGFβ inhibitor and EMT inducer (SB431542) for 10 d to induce the mesoderm followed by induction of MSCs in FBS-containing MSC medium. The resultant hPSC-MSCs had robust osteogenesis and chondrogenesis but weaker adipogenesis. This approach does not require EB and feeder cell coculture.

To mimic *in vivo* development, Brown *et al*[32] derived hESC-MSCs *via* EB in MSC medium and enriched them by sorting for CD73 and CD105. EBs from iPSCs were exposed to TGFβ1-containing medium, and two types of MSCs were generated. Although early (aiMSCs) and late (tiMSCs) outgrowing cells were similar in surface antigen profile and three lineage differentiation, aiMSCs were better in osteogenesis than tiMSCs and BM-MSCs. Compared with BM-MSCs, aiMSCs were more of stemness whereas tiMSCs were more osteogenic, and *in vivo* bone formation was confirmed *via* ectopic injection[33].

The use of undefined components (such as FBS and feeder) or animal-derived components affects clinical applications of hPSC-MSCs. To overcome the problems from undefined conditions, serum-free and chemically defined protocols are desired to generate clinically compliant hPSC-MSCs. Lian *et al*[34,35] reported a clinically compliant protocol to generate hESC-MSCs and iPSC-MSCs. After 1 wk of differentiation, MSCs were enriched by FACS for CD24- CD105+ cells. The transplanted iPSC-

MSCs were superior to BM-MSCs in attenuating severe hindlimb ischemia, which may result from better *in vivo* survival and trophic factors of iPSC-MSCs, and higher proliferation of iPSC-MSCs related to increased hEAG1 potasium channel expression[36]. The use of animal products, such as gelatin for coating, compromises the application of hPSC-MSCs. To generate xeno-free MSCs, FBS was replaced with human serum, and porcine gelatin was replaced with human gelatin. Transplanted hESC-MSCs into renal capsule formed cartilage[27]. Human platelet lysate is an alternative to FBS for the generation of hPSC-MSCs. Compared with the FBS-containing medium, the hPL-supplemented medium generated significantly more MSCs[37].

COMPARISON BETWEEN PRIMARY MSCS AND HPSC-MSCS

hPSC-MSCs are similar to primary MSCs in morphology, immunophenotype, differentiation potential, gene expression profile, and epigenetic modification[17,22,38-40]. However, there are some differences observed between primary MSCs and hPSC-MSCs. hPSC-MSCs are smaller in size and proliferate faster than BM-MSCs and adipose tissue-MSCs[22,36,39-41]. hPSC-MSCs express higher levels of cell proliferation-related genes whereas BM-MSCs express higher levels of immune-related genes, therefore hPSC-MSCs had a superior proliferative ability to BM-MSCs[39,42,43]. In addition, iPSC-MSCs express higher levels of pluripotent genes and lower levels of mesodermal genes compared with original MSCs, which harbor mtDNA mutations from original MSCs as well as iPSCs. Compared with primary MSCs, iPSC-MSCs express a lower level of VCAM1, leading to lower initiating cell frequency of HSCs after long-term culture with iPSC-MSCs as feeder[44]. Compared with dental tissue-derived MSCs, re-differentiated iPSC-MSCs expressed higher levels of pluripotent genes and lower levels of mesodermal genes, but displayed lower mitochondrial respiration[45]. iPSC-MSCs also express the lowest level of the HLA-II upon stimulation with IFNγ compared with BM-MSCs and fetal-MSCs. Compared with BM-MSCs, more iPSC-MSCs survived, and less inflammatory cell accumulations and better recovery of hind limb ischemia were also observed upon transplant. These suggest that iPSC-MSCs are not sensitive to IFNγ stimulation and have a stronger immune privilege after transplantation[46]. In differentiation potential, hPSC-MSCs differentiated less effectively along the adipogenic, osteogenic, or chondrogenic lineages compared with BM-MSCs[42], especially poorer adipogenesis[31,47,48]. Both hESCs and iPSCs inefficiently formed hyaline cartilage compared with BM-MSCs[43]. In immunosuppression, iPSC-MSCs were impaired in suppressing T cell proliferation compared with primary MSCs but were rejuvenated with regard to age-related DNA methylation, and this suggests that iPSC-MSCs reacquire incomplete immunomodulatory function, and MSC-specific DNA methylation pattern associates with tissue type and aging[38] (Table 1).

DISEASE MODELLING AND DRUG SCREENING

The understanding of the pathological mechanism is critical to developing the therapeutic drugs for the treatment of various genetic diseases. *In vitro* models to mimic *in vivo* development are very useful to investigate the pathology of human genetic diseases and further develop therapeutic drugs. However, due to inaccessible human tissues and the lack of animal models, research on human genetic diseases and drug screening remains very limited. With the breakthrough in iPSC technology, it makes it possible to model human diseases and develop their therapeutic drugs *in vitro*. The iPSC-MSC platform can recapitulate the embryonic bone and cartilage development, and therefore provide new insights into pathological progression of human genetic bone and cartilage diseases for disease modelling and further the development of therapeutic drugs.

Hutchinson-Gilford progeria syndrome (HGPS) is a rare but fatal genetic disorder caused by progerin, a truncated and farnesylated form of Lamin A, which causes systemic accelerated aging in children. Zhang *et al*[49] generated iPSC-MSCs from HGPS patients and showed that HGPS-iPSC-MSCs displayed abnormalities, including increased nuclear dysmorphology, DNA damage, and accumulation of calponin-staining inclusion bodies, leading to their compromised viability under stress, especially to hypoxia. Using HGPS iPSC-MSCs platform, seven compounds were screened from 2800 small molecules, including all-trans retinoic acid and 13-cis-retinoic acid, which decreased ALP activity and progerin expression[50].

Table 1 Comparison between primary mesenchymal stem cells and mesenchymal stem cells derived from human pluripotent stem cells

Comparison	Primary MSCs	hPSC-MSCs	Ref.
Cell number	Limited	Unlimited	[17,36]
Proliferation	Slower	Faster	[36,39,42,43,48,57]
Life span	Shorter	Longer	[17]
Variation	Higher	Lower	[119]
Differentiation potential	Higher	Lower, *esp*. adipogenesis	[31,43,47,48]
Immunosuppression	Higher	Lower	[38,46]
Pluripotent genes	Lower	Higher	[45]
Mesenchymal genes	Higher	Lower	[45]
VCAM1	Higher	Lower	[44]
HLA-II	Higher	Lower	[46]

MSCs: Mesenchymal stem cells; hPSC-MSCs: Human pluripotent stem cells derived MSCs; VCAM1: Vascular cell adhesion molecule 1; HLA-II: Human leukocyte antigen gene complex class II.

Fibrodysplasia ossificans progressiva (FOP) is an inherited disease characterized by heterotopic endochondral ossification in soft tissues after birth and caused by a point mutation in ACVR1. iPSC-MSCs from FOP patients were generated, and it was found that SMAD1/5/8 and SMAD2/3 were activated and chondrogenesis was enhanced *via* MMP1 and PAI1 in FOP-iMSCs[51-53]. Hino *et al*[54] screened 6809 small molecule compounds using high-throughput screening, and mTOR signaling was identified to be a critical pathway for aberrant chondrogenesis. Further mechanism study showed that ectonucleotide pyrophosphatase/phosphodiesterase 2 linked FOP-ACVR1 to mTOR signaling, causing FOP pathogenesis.

APPLICATIONS OF HPSC-MSCS IN REGENERATIVE MEDICINE

Due to the multipotency, immunosuppression, and unlimited cell sources, hPSC-MSCs have been used for various applications in regenerative medicine (Table 2).

Bone regeneration

Like BM-MSCs, iPSC-MSCs had osteogenic potential, and therefore they could form typically calcified structure in the scaffolds[55]. iPSC-MSCs had good viability and osteogenic differentiation on the CPC scaffold[56]. iPSC-MSCs were similar to BM-MSCs in preventing bone loss and promoting bone repair for the necrosis region of the femoral head[57]. Engineered non-native peptides increased the attachment of iPSC-MSCs to the scaffolds and enhanced bone and vasculature formation *in vivo*[58]. Biofunctional agents, such as Arg-Gly-Asp (RGD), improved the proliferation and bone mineralization of iPSC-MSCs[59]. When iPSC-MSCs were treated with metformin, a widely used drug for diabetes, they showed enhanced bone formation and increased osteogenic markers and mineralized nodule formation, suggesting that metformin might be used to improve bone and periodontal regeneration[60]. Recently increasing reports have shown that MSCs exerted their pleiotropic effects by the secretion of soluble paracrine factors rather than their differentiation potential[61]. MSC-derived exosomes contain cytokines, growth factors, mRNAs, and regulatory miRNAs[62]. iPSC-MSC exosomes increased the proliferation, migration, and osteogenesis of BM-MSCs[63], significantly prevented bone loss, and promoted local angiogenesis by activating the PI3K/Akt signalling pathway in endothelial cells in a steroid-induced rat osteonecrosis model[64] (Figure 1).

Genetic modification can improve the bone formation of iPSC-MSCs. Distal-less homeobox 3 (DLX3) overexpression enhanced bone formation of iPSC-MSCs as shown by increased osteogenic genes and mineralized nodules at the expense of decreased proliferation[65]. Bone morphogenetic protein 2 overexpression enhanced bone formation on RGD-grafted calcium phosphate cement (CPC) of iPSC-MSCs[66]. Neural EGFL like 1 (NELL1) overexpression greatly improved osteogenesis of iPSC-MSCs on

Table 2 Mesenchymal stem cells and mesenchymal stem cells derived from human pluripotent stem cells

hPSC-MSCs	Disease model or application	Animal model or human	Therapeutic effects	Ref.
iPSC-MSCs	CKD	Rat	Protect the kidney against CKD injury	[85]
iPSC-MSCs	Adriamycin nephropathy	Mouse	Prevent adriamycin nephropathy	[82]
iPSC-MSCs	Obesity-associated Kidney injury	Mouse	Ameliorate endoplasmic reticulum stress	[83]
hPSC-MSCs	UUO	Mouse	Protect against kidney fibrosis in vivo and *in vitro*	[84]
hESC-MSCs	LN	Mouse	Prevent the progression of LN	[81]
iPSC-MSCs	TNBC	Mouse	Significantly decrease the incidence and burdon of metastases	[117]
iPSC-MSCs	Breast cancer	Mouse	Decrease EMT, invasion, stemness, and growth of cancer cells	[119]
iPSC-MSCs	Skin wounds, pressure ulcers, and osteoarthritis	Mouse	Have therapeutic potential in skin wounds, pressure ulcers, and osteoarthritis	[127]
hESC-MSCs	Arthritis	Mouse	Ameliorate collagen-induced arthritis by inducing IDO1	[72]
iPSC-MSCs	Osteonecrosis of the femoral head	Rat	Prevent osteonecrosis of the femoral head	[64]
iPSC-MSCs	Vascularized composite allotransplantation	Rat	Induce T cell hyporesponsiveness to prolong hind limb survival	[106]
iPSC-MSCs	Limb ischemia	Mouse	Exosomes of iPSC-MSCs attenuate limb ischemia by promoting angiogenesis	[121]
iPSC-MSCs	Limb ischemia	Mouse	Insensitivity of iPSC-MSCs to interferon γ potentiates repair efficiency of hind limb ischemia	[46]
iPSC-MSCs	Limb ischemia	Mouse	Attenuate limb ischemia	[35]
iPSC-MSCs	Periodontal defects	Rat	Aid periodontal regeneration	[68]
iPSC-MSCs	Bone defects	Mouse	Regenerate non-union bone defects more efficiently than BM-MSCs upon BMP6 overexpression	[33]
iPSC-MSCs	Calvaria defects	Mouse	Repair calvaria defects	[28]
iPSC-MSCs	Osteochondral defects	Rat	iPSC-MSCs are able to repair cartilage defects	[17]
iPSC-MSCs	FOP		FOP-iPSC-MSCs enhance chondrogenesis *via* activin A enhanced mTOR signalling	[53,54]
hESC-MSCs	Lupus and uveitis	Mouse	Increase survival of lupus-prone mice and decrease symptoms of uveitis	[40]
hESC-MSCs	EAE model of multiple sclerosis	Mouse	Improve EAE symptoms	[101]
hESC-MSCs	EAE	Monkey	Attenuate disease progression in a primate EAE model	[41]
hESC-MSCs	EAU	Mouse	Slow down the development of EAU	[103]
iPSC-MSCs	Inflammatory bowel disease models	Mouse	Promote intestinal repair *via* TSG-6	[111]
hESC-MSCs	Experimental inflammatory bowel disease	Mouse	Protect against experimental inflammatory bowel disease	[107]
iPSC-MSCs	SS	Mouse	Prevent the progression of SS	[112]
iPSC-MSCs	Allergic rhinitis		Modulate T-cell phenotypes towards Th2 suppression through inducing Treg expansion	[108]
iPSC-MSCs	Asthma Inflammation	Mouse	Alleviate asthma inflammation by CX43-mediated mitochondrial transfer	[110]
iPSC-MSCs	Corneal injury	Mouse	Exert therapeutic effects in the cornea by reducing inflammation	[99]
iPSC-MSCs	Skin wound	Rat	iPSC-MSC-Exos improve cutaneous wound healing by promoting collagen synthesis and	[120]

			angiogenesis.	
iPSC-MSCs	SR-aGvHD	Human	iPSC-MSCs are safe and well tolerated	[114]

CKD: Chronic kidney disease; UUO: Unilateral ureteral obstruction; LN: Lupus nephritis; TNBC: Triple-negative breast cancer; EMT: Epithelial–mesenchymal transition; IDO1: Indoleamine 2, 3-dioxygenase 1; FOP: Fibrodysplasia ossificans progressive; mTOR: Mammalian target of rapamycin; EAE: Experimental autoimmune encephalomyelitis; EAU: Experimental autoimmune uveitis; TSG-6: TNFα-stimulated gene-6; SS: Sjogren's syndrome; CX43: Connexin 43; Exos: Exosomes; SR-aGvHD: Acute steroid-resistant graft versus host disease.

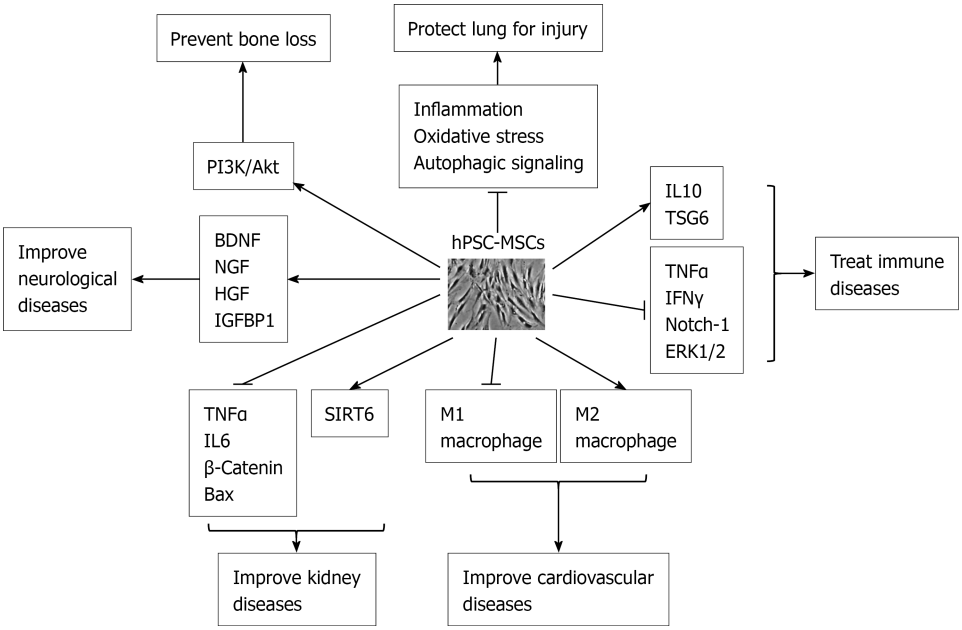

Figure 1 Signaling pathways of mesenchymal stem cells derived from human pluripotent stem cells in improving various diseases. Mesenchymal stem cells derived from human pluripotent stem cells (hPSC-MSCs) improve diseases or prevent against injury through immunosuppression or paracrine effects. hPSC-MSCs secrete a variety of soluble paracrine factors to exert their therapeutic effects on immunosuppression, proliferation, differentiation, anti-apoptosis, angiogenesis, *etc.* PI3K: Phosphoinositide 3-kinase; Akt: Protein kinase B; BDNF: Brain-derived neurotrophic factor; NGF: Nerve growth factor; HGF: Hepatocyte growth factor; IGFBP1: Insulin-like growth factor-binding protein 1; TNFα: Tumor necrosis factor; IL6: Interleukin 6; Bax: BCL2-associated X; SIRT6: Sirtuin 6; IL10: Interleukin 6; TSG6: TNFα-stimulated gene-6; IFNγ: Interferon γ; ERK1/2: Extracellular signal-regulated protein kinases 1 and 2.

RGD-CPC[67].

Due to osteogenic differentiation potential, iPSC-MSCs have the capacity for periodontal regeneration. When transplanted into periodontal defects, iPSC-MSCs formed new mineralized tissues and significantly improved regeneration, suggesting that iPSC-MSCs represent a promising stem cell source for clinical application in periodontitis[68].

Cartilage repair

Articular cartilage has limited intrinsic healing potential, leading to a loss of joint function. Like BM-MSCs, iPSC-MSCs can differentiate into chondrocytes *in vitro*[69]. In view that autologous chondrocytes and primary MSCs are limited in cell number, iPSC-MSCs are gaining attention as a new cell therapy for cartilage regeneration due to unlimited cells and chondrogenic differentiation potential. Our previous data showed that primary BM-MSCs were able to repair cartilage defects effectively[70]. Multiple injections of hESC-MSCs into knee joint of osteoarthritis (OA) rats induced by anterior cruciate ligament transection repaired cartilage better than the single dose and negative control groups in a rat OA model[71]. hESC-MSCs also ameliorated collagen-induced arthritis by inducing indoleamine 2,3-dioxygenase 1 (IDO1) in mice[72]. In addition, exosomes from hESC-MSCs prevented cartilage destruction by maintaining the chondrocyte function[73]. By our defined, step-wise and chemically defined protocol, we generated iPSC-MSCs *via* lateral plate mesoderm and have shown that iPSC-MSCs repaired osteochondral defects similar to BM-MSCs[17].

Lung repair

As an attractive candidate for cell-based therapy, MSCs are therapeutically beneficial

to improving lung disease or repairing lung damage. iPSC-MSCs protected lung cells against mitochondrial dysfunction and apoptosis induced by oxidative stress to reduce lung injury and inflammation in *in vivo* models of lung disease[74]. iPSC-MSCs reduced airway inflammation and hyperresponsiveness to protect against lung diseases induced by oxidative stress, such as chronic obstructive pulmonary disease [75]. iPSC-MSCs protected the lung against ischemia-reperfusion injury (IRI) by suppressing the inflammatory, oxidative stress, and autophagic signalling pathways [76]. Treatment with iPSC-MSCs also significantly prevented airway allergic inflammation, decreased Th2 cytokine levels, and changed long non-coding RNAs profiles [77]. iPSC-MSCs ameliorated cigarette smoke (CS)-induced apoptosis and proliferation imbalance of airway cells partly through the paracrine section of stem cell factor (SCF) [78]. Asthma is a chronic disease with inflamed airways. iPSC-MSCs were able to prevent chronic allergic airway inflammation[79]. Compared with BM-MSCs, iPSC-MSCs transferred mitochondria to bronchial epithelial cells more effectively *via* tunnelling nanotubes. Therefore, iPSC-MSCs were superior to BM-MSCs in attenuating CS-induced airspace enlargement[80].

Kidney disease

hPSC-MSCs improved both acute and chronic adriamycin nephropathy (AN) by preventing renal function loss. hESC-MSCs prevented the progression of fatal lupus nephritis in a mouse model by significantly decreasing two inflammatory cytokines associated with systemic lupus erythematosus, tumour necrosis factor α (TNFα) and IL-6[81]. iPSC-MSCs prevented the apoptosis of tubular cells by downregulating B-cell lymphoma 2 associated X (Bax) and Bax/B-cell lymphoma 2 and upregulating survivin in the short-term AN model whereas iPSC-MSCs inhibited fibrosis *via* hedgehog signalling in the long-term AN model[82]. iPSC-MSCs also ameliorated palmitic acid-induced lipotoxic kidney injury by alleviating endoplasmic reticulum (ER) stress, inflammation, and apoptosis to suppress ER stress and its downstream pro-inflammatory and pro-apoptotic effects *via* hepatocyte growth factor (HGF)/c-Met signalling[83]. Chronic kidney disease (CKD) is characterized by a gradual loss of kidney function over time due to renal fibrosis[84]. Intravenously administrated iPSC-MSCs effectively protected the kidney against CKD injury in CKD parenchyma[85]. iPSC-MSCs were also able to effectively protect kidney from acute ischemia-reperfusion injury[86]. hPSC-MSC-derived exosomes reduced the renal fibrosis, decreased inflammatory reactions, and improved renal function in unilateral ureteral obstruction mice by increasing SIRT6 and decreasing β-catenin[84] (Figure 1).

Cardiovascular diseases

MSCs have the potential to improve cardiovascular diseases. Coculture with hESC-MSCs promoted the maturation of hESC-derived cardiomyocyte microtissues[87]. iPSC-MSCs increased the level of M2 macrophages and deceased the level of M1 macrophages after cardiac arrest (Figure 1), suggesting that iPSC-MSCs play a crucial role in immunomodulation during cardiopulmonary resuscitation[88]. iPSC-MSCs improved CS-induced cardiac remodelling and dysfunction better than BM-MSCs as shown by an increase in percentage of left ventricular ejection fraction and fractional shortening. iPSC-MSCs attenuated cardiac pro-inflammatory cytokines and restored anti-inflammatory cytokines[89]. Conditioned medium from iPSC-MSCs alleviated heart failure and reduced cardiomyocyte apoptosis and fibrosis better than that from BM-MSCs, showing that iPSC-MSCs could provide cell-free therapeutic cardioprotection[90]. Extracellular vesicles (EVs) of iPSC-MSCs mitigated arterial ageing by attenuating ageing-associated vascular endothelial dysfunction, arterial stiffness, and hypertension[91]. In addition, overexpression of myocardin in iPSC-MSCs resulted in partial transdifferentiation into cardiomyocyte phenotype[92].

Neurological diseases

MSCs demonstrate significant neuroprotection and promote functional recoveries of the pathological nervous system. MSCs were shown to secret brain-derived neurotrophic factor and nerve growth factor, which supported neuronal cell survival and induced nerve regeneration (Figure 1). Conditional medium of hESC-MSCs could significantly ameliorate neurological deficits and infarct volume in middle cerebral artery occlusion (MCAO) rats[93]. hESC-MSCs differentiated into neural-like cells in standard neurogenic differentiation medium, and hESC-MSCs in sphere secreted more HGF and IGFBP1 than those in single-cell suspension[94] (Figure 1). hPSC-MSCs expressed higher levels of neural genes than BM-MSCs and rapidly differentiated into neural-like cells when differentiated into neural lineage[95]. Although ESC-MSCs

induced autophagy similar to BM-MSCs, ESC-MSCs survived better in amyloid-β (Aβ) -induced cellular models and reduced more intracellular Aβ levels compared with BM-MSCs. ESC-MSCs significantly decreased Aβ-induced cell death and promoted autophagolysosomal clearance of Aβ in a rat model of Alzheimer's disease, leading to higher memory performance. Intra-arterially transplanted ESC-MSCs were safe and free from cerebral ischemia[96]. iPSC-MSCs markedly decreased brain-infarct volume and improved neurological function mainly by inhibiting inflammation[97]. ESC-MSCs had a superior neuroprotective capacity over fetal MSCs in mouse hypoxic-ischemic brains[98].

In addition, hESC-MSC EVs also protected retinal ganglion cells and preserved retinal function in a mouse model of optic nerve injury by improving retinal ganglion cell (RGC) survival and preventing retinal nerve fiber layer degeneration. iPSC-MSCs significantly reduced corneal opacity by reducing inflammation similar to BM-MSCs [99]. Transplanted iPSC-MSCs significantly improved the survival of RGCs by effectively transferring functional mitochondria to RGCs[100].

Multiple sclerosis (MS) is a potentially disabling disease of the central nervous system caused by an attack of the protective sheath by the immune system, leading to communication problems between the brain and the rest of the body. As yet, there is no cure for MS, the most common demyelinating disease. Compared with BM-MSCs, hESC-MSCs improved efficacy in a mouse experimental autoimmune encephalitis (EAE) model of MS due to its lowered IL-6 expression. In addition, hESC-MSCs are less vulnerable than BM-MSCs in therapeutic capacity during *in vitro* culture[101]. After hESC-MSCs were intrathecally injected into the central nervous system of EAE-induced monkeys, hESC-MSCs greatly decreased the clinical symptoms, brain lesions, and neuronal demyelination in the EAE monkeys. hESC-MSCs could transdifferentiate into neural cells *in vivo* in the CNS of the treated monkeys as shown by elevated expression of genes for neuronal markers, neurotrophic factors, and neuronal myelination[41].

Immune disease

hPSC-MSCs have a strong immune regulatory effect during anti-inflammation. Microphages serve as a bridge between innate and specific immune responses. hPSC-MSCs altered macrophage polarization by suppressing the Notch-1 signalling pathway[102] (Figure 1). Due to the immunosuppression property of iPSC-MSCs, they have been used for the treatment of various immune diseases. hESC-MSCs slowed down the development of severe experimental autoimmune uveitis through systemic immune modulation[103], whereas iPSC-MSCs inhibited proliferation, shifted the secretome of peripheral blood mononuclear cells, and significantly suppressed CD8 T proliferation, activation, and differentiation[104]. iPSC-MSCs also suppressed T-cell effector cells of Th1/Th2 and increased regulatory T cell (Treg) response[105]. iPSC-MSCs prolonged hind limb survival by reducing mononuclear cell infiltration, lowering TNFα and IFNγ, increasing interleukin 10, and thus protecting against acute rejection in a rat vascularized composite allotransplantation model[106] (Figure 1). iPSC-MSCs disrupted NK cell cytolytic machinery to prevent allograft rejection by decreasing activation markers and ERK1/2 signalling, leading to impaired immunologic synapses and secreted cytotoxic granules. However, iPSC-MSCs were more resistant than BM-MSCs to pro-activate NK cells[26]. hESC-MSCs could protect against an experimental model of inflammatory bowel disease[107]. iPSC-MSCs modulated T-cell phenotypes towards Th2 suppression by inhibiting lymphocyte proliferation and promoting Treg response, suggesting that iPSC-MSCs can treat allergic airway diseases[108]. iPSC-MSCs regulate T cell responses by decreasing secreted soluble factors[109]. iPSC-MSCs also improved asthma inflammation by connexin 43-mediated mitochondrial transfer[110]. iPSC-MSCs accelerated intestinal epithelial cell proliferation to promote intestinal repair in murine colitis through tumor necrosis factor-stimulated gene-6 (TSG-6) *via* Akt-dependent interaction between the extracellular matrix HA and CD44+ cells[111]. iPSC-MSC EVs prevented the progression of Sjogren's syndrome (SS), a chronic autoimmune disease, by suppressing activation of immune cells and proinflammation factors essential for SS progression[112]. Due to intrinsic immunosuppression, MSCs significantly prolonged the survival of humanized mouse model of graft *vs* host disease (GvHD)[113]. The first iPSC-MSC clinical trial was reported in 2020. iPSC-MSCs were produced using an optimized and good manufacturing practice-compliant manufacturing process to treat steroid-resistant acute GvHD. Based on the complete response, overall response, and overall survival of participants, the higher dose level of iPSC-MSC showed better outcomes than the lower dose, and iPSC-MSCs were safe and well tolerated without serious adverse events reported[114].

Cancer treatment

Like primary MSCs, hPSC-MSCs also have therapeutic potentials in treating cancer or repairing tissue damages caused by cancers. hPSC-MSCs can overcome the limitation of drug delivery. iPSC-MSCs expressing cytosine deaminase limited tumor growth and decreased lung metastases in a mouse xenogeneic model of human breast cancer [115]. EVs from hPSC-MSCs also showed promising results to improve cancer treatment. hESC-MSC microvesicles decreased the proliferation of leukemia cells[116]. Treatment with iPSC-MSC nanovesicles showed no detectable immunogenicity and significantly decreased the incidence of metastases from triple-negative breast cancer in mouse models[117]. iPSC-MSC nanovesicles also significantly decreased tumor growth of metastatic prostate cancer[118]. These suggest that iPSC-MSC nanovesicle is a promising platform to improve the treatment of metastatic cancer. iPSC-MSCs can home to cancers with a similar efficiency as BM-MSCs. As compared with BM-MSCs, iPSC-MSCs expressed lower levels of interleukin-1 and TGFβ receptors, downstream pro-tumor factors, and hyaluronan and its cofactor TSG6, and therefore iPSC-MSCs have much less potential to promote tumours than BM-MSCs by promoting the EMT, invasion, stemness, and growth of cancer cells[119].

Other applications

hPSC-MSCs are also used for other applications. iPSC-MSC exosome improved cutaneous wound healing by promoting collagen synthesis and angiogenesis[120]. Furthermore, iPSC-MSC exosome *via* intramuscular injection could enhance microvessel density and blood perfusion by activating angiogenesis-related molecule expression and promoting HUVEC migration, proliferation, and tube formation[121]. iPSC-MSCs supported the proliferation of hematopoietic stem and progenitor cells (HPCs), and maintained a primitive immunophenotype and colony forming unit of CD34+ HPCs. Long-term culture initiating cell frequency was lower compared with primary MSCs, suggesting that iPSC-MSCs are less suitable than primary MSCs as feeder cells[44]. iPSC-MSCs also can be used as feeder cells to culture human iPSCs. Human iPSCs cultured on human iPSC-MSC feeder were slightly thinner and flatter than the other feeder system. However, iPSC-MSCs still maintain the proliferation and pluripotency of iPSCs[122]. hESC-MSCs restored the structure of the injured ovarian structure and function in premature ovarian failure *via* paracrine effect and ovarian cell survival to rescue fertility in mice[123,124]. hESC-MSC secreted trophic factors to support hepatocytes on an acute liver failure model[125]. hESC-MSC EVs ameliorated cirrhosis in thioacetamide-induced chronic liver injury[126].

DISCUSSION

Primary MSCs have drawbacks due to their limited scalability, interdonor variability, and inconsistent outcomes of clinical trials. iPSC-MSCs have the potential to overcome the fundamental limitations of conventional and donor-derived MSC production processes. The derivation of hPSC-MSCs has made substantial progress with an increasing number of reports on the use of hPSC-MSCs for regenerative medicine over the past years. However, the issues and challenges related to safety and efficacy of hPSC-MSCs remain to be understood and addressed. These include the effects of cell origins and derivation approaches on hPSC-MSCs, the understanding of difference between hPSC-MSCs and primary MSCs, MSC stemness/potency biomarkers, the differentiation potential of hPSC-MSCs, choice of autologous or allogeneic hPSC-MSC source, manufacturing of clinical grade hPSC-MSCs, *etc.*

Effects of cell origins and derivation approaches on the features of hPSC-MSCs

The use of MSCs is already in various phases of clinical applications. However, little is known about the difference in features of hPSC-MSCs from different origins, particularly in their differentiation potential, a critical feature to their clinical application. Although hPSC-MSCs derived from various approaches exhibit MSC morphology and express MSC surface antigens, their differentiation potential is not as efficient as BM-MSCs, especially in adipogenesis[31,47]. Due to epigenetic memory or incomplete reprogramming, iPSC variations exist, and iPSC-MSCs exhibit preferential differentiation into their original cell lineage. Eto *et al*[127] showed that iPSC-MSCs *via* the mesoderm and neuroepithelium had the capacity for self-renewal and multipotency as well as therapeutic potential in skin wounds, pressure ulcers, and OA in a mouse model. However, different therapeutic effects of iPSC-MSCs from different origins were also observed, suggesting that the therapeutic efficacy of hPSC-MSCs is

dependent on cell origins. In addition, hPSC-MSCs derived by differentiation approaches vary extensively in their quality and efficiency. The use of fibroblast growth factor in the differentiation medium[27,47,128] promotes MSC proliferation at the expense of its differentiation potential[129]. Therefore, the effects of cell origins and differentiation approaches on iPSC-MSCs need to be elucidated.

Mechanisms underlying difference between hPSC-MSCs and primary MSCs

Compared with primary MSCs, hPSC-MSCs have advantages of faster proliferation, longer life span, more reliable and homogeneous cell source, but somehow immature differentiation potential and impaired immunosuppression. What are intrinsic and extrinsic mechanisms underlying the difference between iPSC-MSCs and primary MSCs?

The lack of MSC stemness/potency biomarkers to identify good quality of MSCs

So far, little is known about regulators or biomarkers associated with MSC stemness/potency, and there is no critical quality attribute available for use to distinguish good MSCs from bad ones before cellular manufacturing. The mechanism underlying MSC stemness or potency remains poorly understood, which greatly hampers the clinical application of hPSC-MSCs. It was shown that kindlin-2 increased the survival, proliferation, stemness, and migration of iPSC-MSCs. Kindlin-2 knockdown increased apoptosis and differentiation response whereas kindlin-2 overexpression increased proliferation, decreased apoptosis, and slowed down trilineage differentiation. More significantly, kindlin-2 overexpression increased the migration of iPSC-MSCs in the wound-scratch assay[130]. In the future, substantial efforts are needed to explore MSC stemness/potency-related regulators or biomarkers for clinical application.

Differentiation potential of hPSC-MSCs

It is well accepted that MSCs have potential to differentiate into multiple mesenchymal lineages, such as osteoblasts, chondrocytes, and adipocytes. However, it is still controversial that MSCs can directly differentiate into other types of functional cells, such as cardiomyocytes-like cells[131], hepatocytes[132], neuron-like cells[133], and pancreatic β cells[134]. The underlying mechanism of iPSC-MSCs improving these conditions need to be elucidated.

Autologous vs allogeneic hPSC-MSCs

MSCs have anti-inflammatory and immune-modulatory properties. However, patient-derived autologous hPSC-MSCs still represent a better option for regenerative medicine as there are lesser concern regarding the immune response compared with allogeneic MSCs.

Clinical grade hPSC-MSCs

Although iPSCs are generated by integration-free methods and iPSC-MSCs are derived by a number of approaches, there are few approaches available to regenerate clinical-grade hPSC-MSCs for clinical application. Most protocols have used undefined components, such as FBS, feeder cells, and other animal-derived components, which compromise the clinical application of iPSC-MSCs. To generate clinical grade iPSC-MSCs, reliable, efficient, scalable, and clinically compliant approaches are required throughout the whole manufacturing process of iPSC-MSCs. These processes include generation and expansion of iPSCs, freezing and thawing of iPSCs, differentiation of iPSCs towards MSCs, expansion of iPSC-MSCs, freezing and thawing iPSC-MSCs, *etc.* In addition, comprehensive assays should be established to evaluate the safety, quality, or potency of hPSC-MSCs during cellular manufacturing for clinical application.

CONCLUSION

hPSC-MSCs have enormous potential for regenerative medicine, and can be used for disease modelling, drug screening, and treatment of various diseases in regenerative medicine. Although multiple approaches have been reported in deriving MSCs from hPSCs, the use of undefined and animal-derived components greatly compromises the clinical application of hPSC-MSCs. Much effort is needed to derive clinically relevant and sufficient hPSC-MSCs with good quality for clinical application, and criteria need be established to evaluate the safety and efficacy of hPSC-MSCs before clinical

application. In addition, many issues or challenges with hPSC-MSCs also need to be addressed.

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REVIEW

Strategies to improve regenerative potential of mesenchymal stem cells

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Abstract

In the last few decades, stem cell-based therapies have gained attention worldwide for various diseases and disorders. Adult stem cells, particularly mesenchymal stem cells (MSCs), are preferred due to their significant regenerative potential in cellular therapies and are currently involved in hundreds of clinical trials. Although MSCs have high self-renewal as well as differentiation potential, such abilities are compromised with "advanced age" and "disease status" of the donor. Similarly, cell-based therapies require high cell number for clinical applications that often require *in vitro* expansion of cells. It is pertinent to note that aged individuals are the main segment of population for stem cell-based therapies, however; autologous use of stem cells for such patients (aged and diseased) does not seem to give optimal results due to their compromised potential. *In vitro* expansion to obtain large numbers of cells also negatively affects the regenerative potential of MSCs. It is therefore essential to improve the regenerative potential of stem cells compromised due to "*in vitro* expansion", "donor age" and "donor disease status" for their successful autologous use. The current review has been organized to address the age and disease depleted function of resident adult stem cells, and the strategies to improve their potential. To combat the problem of decline in the regenerative potential of cells, this review focuses on the strategies that manipulate the cell environment such as hypoxia, heat shock, caloric restriction and preconditioning with different factors.

Key Words: Hypoxia; Stem cell aging; Growth factors; Heat shock; Caloric restriction

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Core Tip: Stem cell-based therapies can treat various diseases and disorders. Mesenchymal stem cells have high self-renewal as well as differentiation potential, however; their potential for cell-based therapies is severely compromised with donor age, disease status and extensive *in vitro* expansion. Thus autologous use of stem cells isolated from unhealthy, older donors does not seem to give optimal results. It is therefore essential to improve the negative effects of age and disease on resident adult stem cells before clinical use. We herein discuss the strategies such as hypoxia, heat shock, caloric restriction and preconditioning with different factors to enhance the stem cell function.

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INTRODUCTION

Stem cell-based therapies hold great promise for neurodegenerative diseases, cardiovascular diseases, immunological disorders, skin diseases and cancers. Mesenchymal stem cells (MSCs) are adult stem cells found in many adult (bone marrow, adipose tissue, dental pulp, peripheral blood, menstrual blood) and neonatal tissues (cord blood, cord tissue, Wharton's jelly, Chorionic villi), have potential for self-renewal and multi-lineage differentiation as well as the capacity to secrete many therapeutic factors with chemoattractive, immunomodulatory, angiogenic and anti-apoptotic functions[1,2]. Although MSCs originate from the mesoderm, they can differentiate not only into a variety of mesenchymal tissues (such as bone, cartilage, adipose, and haematopoietic tissue) as well as into non-mesodermal tissues (such as glial cells and neurons). MSCs have low immunogenicity, have immunomodulatory and immunoregulatory properties, are easy to isolate and culture. Due to these properties MSCs are considered ideal for replacing damaged or lost cells and tissues in the body and are currently the focus of scientists in hundreds of clinical trials (www.clinicaltrials.gov).

The regenerative potential of MSCs, however; may be compromised with advanced age and disease conditions of the cell donors. Aging is a normal physiological process in living organisms that affects the cells, tissues, and organs of the body. The age of adult resident stem cells is directly proportional to the age of the donor and therefore the functional properties of stem cells severely deteriorate with increasing age of donors. As the stem cells age, their regenerative potential declines as evidenced by the slow healing of wounds in aged individuals[3]. It is also pertinent to note that this decline in regenerative potential of stem cells plays a critical role in initiation of number of age-related diseases in old people. With advance age, the ability of stem cells to properly function is compromised leading to cell apoptosis, senescence and complete loss or at least decline in their regenerative potential[4,5]. Studies indicate that the therapeutic potential of stem cells significantly declines with an increase in stem cell age *in vitro* and *in vivo*[3,6]. Similarly, underlying disease conditions of donors also seem to upset stem cell function[7]. In addition, number of adult stem cells is very low in their adult niches while stem cell-based therapies often require large number of cells for a potential positive effect. To obtain a high cell number, cells are usually expanded *in vitro*. The *in vitro* expansion deteriorates stem cell function and does not often give desired results after transplantation[8]. Thus, the regenerative potential of cells is significantly compromised when isolated from "old", "unhealthy" persons and especially with *in vitro* expansion.

The main segment of the population who can get benefits from regenerative therapies are the aged individuals with diseases[9]. However, the autologous use of unhealthy stem cells derived from aged donors does not seem to give the desired results due to their compromised function. The solution to the problem is either to use cells isolated from young donors or rejuvenate the unhealthy cells before use. Autologous use of stem cells is preferred for cell based regenerative therapies and therefore use of stem cells from young donors for transplantation into aged people is not without problems. Autologous use of stem cells for such patients (aged and

diseased) does not seem to give the required results due to their age or disease status. This seems a major roadblock for cellular therapies and therefore it is essential to improve the regenerative potential of "aged" and "diseased" stem cells for their successful autologous use. Studies indicate that compromised stem cell function can be reversed using various strategies before clinical use. Previously, many strategies to improve the regenerative potential of stem cells were proposed and described in different studies. In the current review such strategies have been comprehensively described to address major clinical hurdles faced due to the reduced regenerative potential of compromised cells. The review will open new avenue for the stem cell based regenerative therapies for their autologous use in aged and diseased patients. In the current review, age and disease depleted function of resident adult stem cells, and the strategies to improve their potential have been described. To combat the problem of decline in the regenerative potential of cells, we aim to focus on the strategies that manipulate the cell environment such as heat shock, hypoxia, caloric restriction (CR), preconditioning with different factors.

STEM CELL FUNCTION DETERIORATES WITH ADVANCED AGE, DISEASE AND EXTENSIVE *IN-VITRO* EXPANSION

It has long been known that advanced age is linked with reduced reparative and regenerative potential (Figure 1). With increasing age, the body becomes unable to maintain tissue turnover and homeostasis. It is believed that reduced repair of organs and tissues at the organismal level is due to diminished functional capabilities of tissue resident stem cells[10]. Stem cells in the body reside in a special microenvironment called stem cell niches. Stem cells respond to the niche signals either by proliferating, differentiating or by remaining in quiescent state. Such a response ensures that tissues and organs needs are accurately met[9]. In aged individuals, this response is significantly delayed taking longer to repair and heal the damaged tissues and organs [11]. Stem cells residing in the elderly are affected by the age related changes and thus are not as affective for tissue rejuvenation as are the cells from young donors. In a past study, it was found that the function of stem cells isolated from aged mice was adversely affected[3]. Interestingly, this decreased function of stem cells from aged mice was corrected by exposing the old mice to factors present in the serum of healthy young mice. This parabiotic pairing (shared circulatory system) of old and young mice restored the diminished proliferation and differentiation potential of aged cells[10]. The general properties of stem cells *i.e.*, self-renewal and differentiation are significantly decreased with donor age making the aged stem cells less efficient to respond to signals from niches and growth factors. The yield, number of colonies, proliferation as well as differentiation potential of cells isolated from different animal and human tissues was negatively affected by donor age[12-15]. In addition, aged stem cells exhibited more senescent (p16, p21, SA-β-gal) and apoptotic (p53, annexin V, caspases) features as well as reduced SOD level, telomeres shortening, high ROS levels and diminished functional ability (wound healing, angiogenesis, migration *etc.*)[12-15]. These findings of different reports indicate that donor age has negative impact on basic stem cells characteristics and thus adversely affect the regenerative potential of stem cells.

Similar to donor age, various diseases of donors particularly the age-related diseases such as diabetes and heart failure also make the cells unhealthy and therefore limit their therapeutic potential. In healthy individuals the stem cell niche is tightly regulated by the combined action of local and systemic factors. In diseased conditions, however; an altered microenvironment changes stem cell properties that result in compromised quality of their use for regenerative therapies. It has been shown that disease conditions of cell donors negatively impact the function of endogenous progenitor cells[16]. Diabetes (type I & II) has been shown to lower the number of $CD34^+KDR^+$ EPCs[17,18]. Pérez *et al*[19] (2018) has comprehensively discussed the diseases that potentially affect stem cell behavior[19]. Diseases such as osteoporosis, cardiovascular diseases, diabetes, obesity, hypercholesterolemia, glucocorticoid imbalance, arthritis, cancer and aplastic anemia have been shown to negatively impact a variety of stem cell types[19]. Generation of oxidative stress with certain diseases and the resultant compromised stem cell proliferation, differentiation and mobilization are well documented in literature[18-20]. Diabetes, for example, negatively regulates stem cell proliferation, differentiation, paracrine activity, SOD activity, chemotactic ability, angiogenesis and heart repair[21]. Similarly, stem cells isolated from adipose tissue of obese persons show low yield, impaired migration and angiogenesis[22-24]. It has

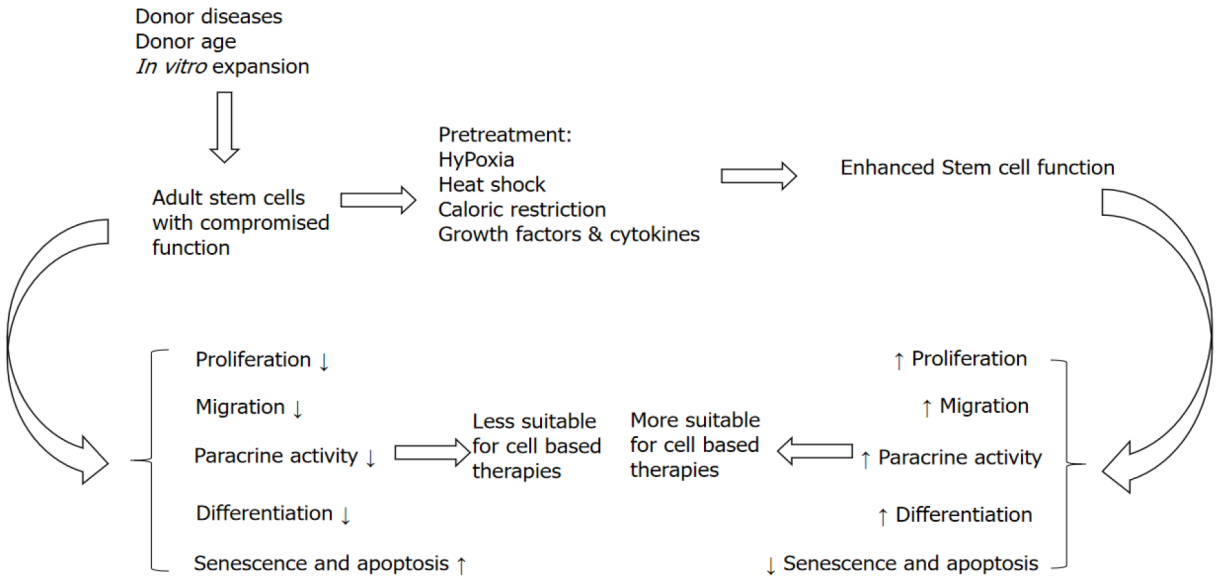

Figure 1 Increased donor age, disease conditions and *in vitro* expansion of cells reduce stem cell potential, making the cells less suitable for cell-based therapies. Stem cell function can be enhanced using strategies such as hypoxia, heat shock, caloric restriction and growth factor preconditioning. These strategies positively affect proliferation, migration, paracrine activity and differentiation potential of cells, and reduce senescence and apoptosis. Such pretreatment of cells makes the cells more suitable for cell based regenerative therapies.

been shown that the effects of disease conditions are similar to those that are portrayed by aged donors. For example, the production of ROS, telomere shortening, reduced expression of telomerase, high expression of apoptotic and senescent markers and resultant reduced repair and regenerative capability are manifested with advanced age and also with onset of certain diseases[25]. It is pertinent to mention here that the onset of diseases in aged individuals affects the regenerative potential of stem cells more adversely as compared to diseases in young donors.

High number of stem cells are needed for cell-based therapies to fully appreciate their therapeutic potential for repair of damaged tissues. However, stem cells are found in low numbers in most adult tissues and therefore *in vitro* expansion is required to obtain large number of cells. MSCs have high regenerative potential but they are also vulnerable to replicative senescence[26]. In prolonged *in vitro* cultures, stem cells become senescent and undergo deleterious changes such as reduced proliferation and multi-lineage differentiation capability, shortening of telomere length and morphological changes. Studies indicate that passaging of the cells for prolonged times negatively affected their potential applications for tissue engineering and regenerative medicine[27]. The passaging of stem cells from "old" and "unhealthy" donors is particularly risky to obtain desired results as these cells already have compromised characteristics as mentioned above.

As stem cells are the basis of tissue engineering and regenerative medicine applications, a reduced regeneration potential of stem cells due to increased donor age, disease condition or *in vitro* expansion may compromise the efficacy of autologous cell therapies. Due to medical advancements, life expectancy has been significantly increased that resulted in a substantial increase in the aged population. Similarly, due to unhealthy lifestyles the frequency of occurrence of diseases has also been increased. As a result, stem cell based therapies are becoming more and more popular in recent years. It is therefore important to use different strategies to improve the stem cell function before use in patients to obtain the desired medical improvements.

ENHANCEMENT OF COMPROMISED STEM CELL FUNCTION

With time researchers have adopted different methodologies and protocols in an attempt to enhance compromised stem cell function. These modifications include best source of stem cells, type of serum for culture, cell plating density, glucose concentration, cell delivery method, transplant method, timing and dosages, which have improved some aspects of cell therapy but not up to the optimal level. The limited improvement is due to low numbers or poor survival of the cells after transplantation

due to a harsh ischemic environment at the host site[3,28,29]. To compensate for the reduced functions of stem cells, researchers were encouraged to investigate novel strategies to improve the compromised stem cell function to maximize the therapeutic effect of stem cells. In this regard significant attention has been given to strategies that manipulate the culture conditions such as hypoxia, heat shock, CR, and preconditioning with different factors.

Hypoxic preconditioning

Oxygen concentration can be adjusted during cell culturing to optimize cell function for cell based regenerative therapies[30]. Naturally stem cells reside in niches inside the body where oxygen concentrations are significantly lower as compared to normal oxygen concentrations. Studies indicate that oxygen concentration in different tissues and organs depends on the distance from the capillaries. Oxygen tension in the lungs for example is 20% which lowers to 2% to 9% when entering other organs and tissues. Oxygen concentration in tissues that are important stem cell sources (such as adipose tissue, bone marrow, placenta, cord tissue *etc.*) is variable and is low as compared to normoxic conditions (Table 1). For example, it is 2%-10% in adipose tissue[31] and 1%-6% in bone marrow[32,33]. So, although stem cells reside in anatomical sites that are relatively oxygen deficient, conventionally they are cultured *in vitro* under normoxic conditions (20%-21%) in CO_2 incubators regardless of their source and oxygen concentration in the tissues from where they are isolated. So hypoxic physiological niches in which most type of stem cells normally reside are largely ignored which may make the cells unhappy and unhealthy.

Being an important component of the stem cell microenvironment, oxygen tension provides signals for maintenance of stem cell properties[34]. Studies indicate that the cells may grow better if the same *in vivo* oxygen concentrations are provided to them for *in vitro* culturing. Stem cell culturing under hypoxia is physiologically more relevant to their niche and thus can affect the regenerative potential of cells. Culturing the cells under hypoxic conditions may improve their regenerative potential in terms of their improved proliferation, differentiation, adhesion, angiogenesis and growth factor secretion.

There is a clear consensus on the fact that hypoxia promotes the proliferative potential of cells. It has been shown that hypoxic insult significantly improves survival, stemness and proliferation of MSCs derived from adipose tissue[35] and bone marrow derived stem cells[36,37]. Proliferative potential of MSCs was significantly higher in hypoxic culture condition as compared to normoxic conditions [38] in long term cultures. Oxygen concentrations of 1%-5% has been demonstrated to significantly increase the proliferation of MSCs while maintaining their normal morphology[36,37]. Similarly, the proliferative potential of BM-MSCs was significantly enhanced under hypoxia[39]. In this study, 1% hypoxia significantly enhanced the proliferative potential of BM-MSCs. Collectively, these studies indicate that hypoxic insult increases the self-renewal potential of stem cells. Some studies however indicate that initially hypoxia has a negative affect on cell viability and proliferation, however, reoxygenation following hypoxia promotes these processes[40].

Low oxygen concentrations also help maintenance of stemness characteristics of cells. In periodontal ligament cells[41], adipose tissue MSCs[42] and dental pulp cells [41], 2% hypoxia maintained the cell stemness for prolonged periods of time. Under 24-h hypoxic conditions mRNA expression of pluripotency markers Oct-4, Sox-2 and c-Myc upregulated significantly concomitant with increased protein expression of these markers[41].

The effect of hypoxia on differentiation of stem cells has also been investigated by number of researchers with conflicting reports and therefore the role of hypoxia in the differentiation of stem cells remain controversial. Regarding differentiation of stem cells into adipocytes, culturing the cells under hypoxic conditions seems to inhibit it [39]. Carrière *et al*[43] (2004), reported decreased adipocyte differentiation of 3T3-F442A preadipocytes in 1% hypoxia[43]. Similarly, Hung *et al*[39], 2012 observed compromised adipogenic potential of bone marrow derived MSCs when hypoxia was applied for 4 wk[39]. In another study, it has been demonstrated that hypoxia negatively regulates the differentiation of ASCs. The authors demonstrated that hypoxia reversibly arrested ASCs in an undifferentiated state and maintains the expression of pre-adipocyte factor 1 (Pref-1) that has been shown to negatively regulate adipogenic differentiation[44]. Contrary to these findings an extreme hypoxia (0.2%) induced more adipogenic differentiation that resulted in more lipid droplets accumulation and upregulation of adipocyte specific genes such as LPL, CFD, PGAR and HIG2[45]. Under severe hypoxia, significantly lower adipogenic differentiation was observed as compared to differentiation of BM-MSCs in normoxic conditions[46].

Table 1 Oxygen concentrations in various stem cells niches

Tissue/Organ	Oxygen concentration	Ref.
Adipose tissue (source: ASCs)	2%-10%	[31]
Bone marrow (source: MSCs)	1%-6%	[32,33]
Eye (retina, corpus vitreous) (Source: Limbal stem cells)	1%-5%	[119,120]
Brain (source: Neural stem cells)	0.5%-8%	[121,122]
Heart (source: Cardiac progenitor cells)	4%-14%	[123]
Kidney (source: Renal stem/progenitor cells)	4%-14%	[124]
Liver (source: Liver stem cells)	4%-14%	[125]
Umbilical veins and arteries	2.4%-3.8%	[126]

MSCs: Mesenchymal stem cells.

However, as indicated in another report, hypoxic preconditioning (2% oxygen) of adipose tissue derived MSCs induces more adipocyte differentiation[47].

Hypoxia however favors differentiation of MSCs into osteocytes. Studies indicate that hypoxia promotes osteogenic differentiation of MSCs[39]. In another report hypoxia positively regulated osteogenesis of MSCs derived from rat bone marrow. In this study, hypoxic preconditioned rat derived MSCs produced more bone when implanted into rats[48]. Moreover, Tsai *et al*[49], (2011) demonstrated that culturing of cells under hypoxic conditions significantly promoted their osteogenesis and chondrogenesis *in vitro* and their bone repair ability *in vivo*[49]. Similarly, in a number of studies 1% to 5% oxygen enhanced the chondrogenic differentiation of ASCs[50-53]. Interestingly, Jurgens *et al*[53], 2012 found that hypoxia can promote differentiation of cells into chondrocytes to the same extent as transforming growth factor-b1[53] and enhance the expression of hypoxia inducible transcription factor-2a, SOX5, SOX6, and SOX9, and that of aggrecan, versican, and collagens II, IX, X, and XI[54]. Contrary to these results D'Ippolito *et al*[55] (2006) reported reduced osteogenic commitment of human bone marrow derived MSCs when cultured and differentiated under hypoxic conditions[55]. These interesting findings indicate that hypoxic effect may be cell source and species specific. Chen *et al*[56], 2015 set the hypoxic conditions at 0.2% and found that this extreme hypoxia can impair the osteogenic differentiation as indicated by the attenuation of alkaline phosphatase (ALP) activity and the reduced expression of osteogenic markers osteocalcin and osteopontin[56].

The key regulators that alter the cellular and molecular functions of stem cells during hypoxia are reactive oxygen species, HIF-1a and micro RNAs. The electron-transport chain within the mitochondria is the major source of ROS production in the cells. Although accumulation of high ROS levels in the cells may cause adverse effects in terms of genetic and physiological dysfunction, and induction of senescence and apoptosis[57-59], low ROS levels function as signaling molecule and positively affect cell characteristics by serving as second messengers, triggering the phosphorylation of signaling molecules[60,61] such as tyrosine kinase. Activation of tyrosine kinases leads to the activation of the PI3K/Akt and MAPK signaling pathways that also can alter stem cells characteristics. Different microRNAs such as miR-210 have been found to consistently induced during hypoxia. miRNA-210 is regulated by HIF-1a and ROS-related pathways during hypoxia[62]. HIF-1a is a master transcription factor that regulates many genes involved in the differentiation of cells. It becomes activated during hypoxia and directly binds with the HIF-responsive element (HRE) to alter stem cell functions.

In conclusion, hypoxia has a profound impact on the biological and functional properties of stem cells and could be used as a strategy to improve their regenerative potential before clinical use (Figure 1). Hypoxia not only enhances the self-renewal potential of cells but also their differentiation into multiple cell types. However, it must be noted that the inconsistent or controversial reports in the literature are probably due to the use of different hypoxia levels, variable durations of exposures and a variety of cell types. The question is not if hypoxia alters stem cell function but rather the use of the correct hypoxic preconditioning for different cell types for an accurate period of time that is most important. In addition, it is important to note that previous studies have often been performed using H_2O_2 for short time periods.

However, development of sophisticated trigas CO_2 incubators now provide a more refined way of culturing the cells under hypoxia for long periods of time (Figure 2).

Heat shock

Hormesis is a phenomenon in which low doses of a harmful stressor produce a cascade of beneficial biological effects. Temperature is one such stressor that has recently been used to manipulate the cell functionality. The hormetic effect of high and low temperature for a short period of time has been shown to effect *in vivo*-as well as *in vitro*-age-related dysfunction in cells. Temperatures below and above the standard culture temperature (32 ℃ and 41 ℃) have been shown to prevent or reverse aging and age related impairment, and significantly impact the regenerative potential of cells. Adult stem cells exhibit therapeutic potential for regenerative medicine and tissue engineering applications. However, age related changes may make these cells less effective for medical use to treat various diseases and disorders (Figure 1). Similarly, *in vitro* expansion of adult cells negatively affects the regenerative potential of cells as indicated by a decline in adipogenic, osteogenic, chondrogenic and myogenic differentiation potential of MSCs with *in vitro* passaging (Figure 1). Adult stem cells are found in low numbers in their niche but are required in large number for clinical use and therefore many promising tissue engineering and regenerative medicine applications require expansion to obtain large numbers of cells. The expansion of cells results in increased senescence and apoptosis, and reduced regenerative potential representing a severe limitation for their use. Expansion of cell at high or low temperatures can significantly enhance the regenerative potential of stem cells and thus could be used as a strategy to enhance their potential.

The anti-aging effect of heat shock treatment has been well documented in a series of studies with interesting results. Heat shock treatment has been found to maintain the long, spindle shaped morphology of MSCs by preventing or reducing age-related alterations such as the irregularly enlarged and flattened shape of cells[63,64]. Similar results were obtained by Choudhery *et al*[65], (2015) in a study in which the stressed cells (HS at 41 ℃) exhibited more thin, long and spindle shaped morphology of MSCs as compared to control cells that had more flattened morphology (a typical age-related alteration)[65]. Heat shock also enhanced viability of cells at different passages during expansion of cells. There were significantly more viable cells at passage 5 and passage 8 when a mild heat shock was applied as compared to non-treated cells[65]. In this study, the percentage viability as determined by the trypan blue exclusion assay as well as flow cytometry using 7-AAD/Annexin V was significantly higher at different passages[65].

A significant increase in the proliferative potential of cells was observed when cells were treated with mild heat shock. The number of cumulative population doublings were increased 10% to 15% as a result of heat shock treatment for a short period of time[64]. In another study, the maximum population doublings were higher for cells that underwent heat shock at 41 ℃ for 60 min once in a week. The cells that were treated with heat shock achieved 36.0 ± 3.4 doublings while the cells in control group achieved only 26.2 ± 1.1 doublings. The doubling time was also shorter for heat shocked MSCs (2.1 ± 0.2 d) as compared to those that were not treated with heat shock (3.2 ± 0.2 d)[65]. Self-renewal is a complex regulatory process under the control of various transcription factors such as Nanog, Oct4, Sox2, STAT3 and others[66]. These transcription factors work in collaboration to regulate self-renewal of cells. Interestingly, the heat-shock proteins expressed as a result of stress (*e.g.* heat shock stress) interact with these transcription factors to regulate normal cell development and functioning[67]. HSP90, HSP70 and HSP27 are also particularly involved in cell self-renewal[68].

The anti-aging effect of repeated mild heat stress on cell growth and other cellular and biochemical characteristics has been well documented[63]. In another study, heat shock alleviated apoptosis in BMSCs and improved survival[69]. The protective effects of heat shock in this study were attributed to elevated levels of heat shock proteins HSP70 and HSP90 along with attenuation of autophagy. Heat shock has been shown to enhance the survival of transplanted cells concomitant with reduced apoptosis and senescence[65,70]. After heat shock treatment, the expression of senescent associated markers such as β-galactosidase, P16 and P21 were significantly downregulated in cultures of cells that were subjected to heat shock[65]. Feng *et al*[71], (2010) explored the cytoprotective effects of HSP90 on rat MSCs. In this study apoptosis was induced with hypoxia and serum deprivation, and heat shock improved viability, paracrine effect and elevated Bcl-2/Bax and Bcl-xL/Bax expression in MSCs[71].

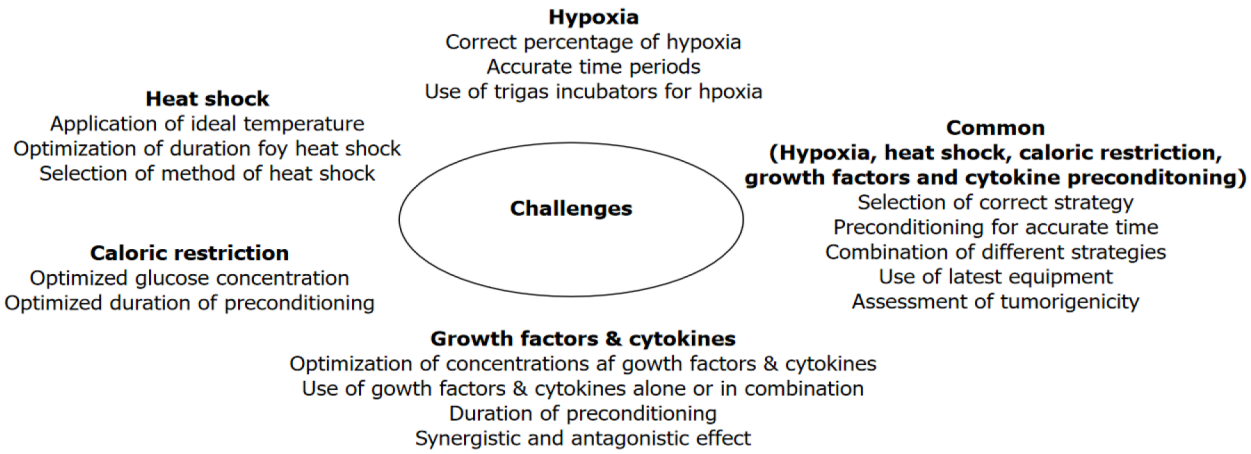

Figure 2 Challenges and limitations in using preconditioning strategies such as hypoxia, heat shock, caloric restriction and growth factor & cytokine. Certain challenges are common for all these preconditioning strategies. Selection of correct strategy for correct duration for preconditioning of mesenchymal stem cells (MSCs) isolated from different sources is important. Assessment of the use of more than one strategies at the same time, use of sophisticated equipment for application of these strategies and evaluation of tumorigenicity after use of preconditioned strategies is required. The figure insets further describe the specific challenges in using specific strategies for preconditioning of MSCs.

It is pertinent to note that differentiation of MSCs into various lineages was also elevated after heat shock treatment. MSCs, under exposure to heat shock produced more extracellular matrix (that stained black with von Kossa staining) as compared to non-heat-shocked MSCs. The expression of lineage-specific osteogenic genes such as ALP, osterix, ostepontin, bone morphogenetic protein 2 (BMP2) and osteocalcin as assessed with RT-PCR was also upregulated in heat-shocked MSCs[56,65]. Adipogenic induced MSCs cultures that were exposed to repeat heat shock showed more oil red O uptake and expression of markers of adipogenesis such as peroxisome proliferator-activated-receptor-g (PPAR-g) and lipoprotein lipase (LPL)[65]. Similarly, in pellet culture a periodic heat shock enhanced the chondrogenic differentiation of human MSCs as depicted by increased sulfated glycosaminoglycan and increased expression of collagen type II and aggrecan in heat-shocked pellets than non-heat-shocked cell pellets[57]. Besides the above-mentioned effects, the novel effects of heat shock have been explored on *in vitro* wound healing[72], angiogenesis[73], neuroprotection and neurodegeneration[74]. Furthermore, heat shock treatment seems to be an effective way to protect the cells even after transplantation. Recently it has been shown that mild heat stress significantly enhanced the viability concomitant with reduced apoptosis and senescence of transplanted cells[65,70]. Chen *et al*[75], (2018) demonstrated that heat stressed bone marrow derived MSCs inhibited apoptosis of ovarian granulosa cells and enhanced their repair effect when transplanted in a chemotherapy induced rat model. In this study, the chemotherapy-induced rat model was established by intraperitoneal injection of cyclophosphamide by giving an initial dose of 50 mg/kg followed by a dose of 8 mg/kg for 14 d[75].

Heat shock response is an evolutionary conserved genetic response to various physiological, pathological, chemical and environmental stresses[76]. This response of heat shock (and other stressors) leads to the induction of special type of proteins in cells called heat shock proteins (HSPs). HSPs may function as molecular chaperones and can help in stabilization of intracellular proteins, repairing damaged proteins, and assisting in protein translocation[68,77-80]. Studies indicate that HSPs can interact with various transcription factors and thus are involved in various cell signaling pathways. Therefore, alterations in the expression of HSPs directly affect stem cell characteristics such as their proliferation capacity as well as differentiation and aging.

In conclusion, it is clear that hormetic effects of mild heat shock can affect the regenerative potential of adult stem cells *in vitro* and these effects help in better performance of these cells after transplantation (Figure 1). However, applying the correct hormetic conditions for stem cells from different sources is challenging. The temperature as well as the duration of heat shock treatment is important for optimal results. In addition, it is also important to select a method of application of heat shock in cell cultures. Instead of incubators, water baths may be more useful for this purpose for quick heat transfer.

GROWTH FACTORS AND CYTOKINES

The use of growth factors and cytokine preconditioning (Figure 1) can also influence the therapeutic potential of stem cells by improving self-renewal, cell survival, paracrine activity and differentiation potential concomitant with reduced senescence and apoptosis[81,82]. The growth factors interact with the receptors present on the cells and activate various downstream signaling pathways to influence numerous cell characteristics. Stem cells particularly MSCs release a number of growth factors and cytokines that influence the cells and tissues in an autocrine or paracrine manner. The half-life of these growth factors, however; is very short and therefore their stable therapeutic effects are limited.

BM-MSCs when preconditioned with stromal derived factor 1 showed enhanced survival, proliferation, migration, secretion of pro-survival genes (AKT-1, BCL-2, Erk) and pro-angiogenic factors (bFGF, VEGF) concomitant with reduced apoptosis and senescence[83]. In another study, BM-MSCs were treated with 0.05 μg/mL of SDF-1 that enhanced cell survival, engraftment and vascular density and suppressed apoptosis. Further, injection of the SDF-1 preconditioned MSCs in a rat model of left anterior descending artery ligation also improved myocardial function by increasing cell proliferation and reducing infarct size and fibrosis *via* SDF/CXCR4 signaling[84]. Preconditioning of BM-MSCs with 10 ng/mL to 100 ng/mL of SDF-1 also reduced hypoxia induced apoptosis[85]. TGF-Beta inhibits differentiation of BM-MSCs into adipocytes and osteocytes. Interestingly, however, the same growth factor promotes osteogenesis in the presence of IBMX (usually present in adipogenic differentiation medium). TGF-β1 is a potent stimulator of tissue regeneration[86] and it can switch adipogenic differentiation into osteogenic differentiation. Pretreatment of MSCs with TGF-β1 improves wound healing in a murine wound model by adhesion and migration to the wound site[87]. Further, TGF-β1 enhanced fibronectin production as well as survival of human umbilical cord-derived MSCs in a rat model of lipopolysaccharide-induced acute lung injury[88]. However, a previous study demonstrated that TGF-β1 induces senescence through production of ROS in periodontal ligament stem cells[89]. A 3 d preconditioning of AT-MSCs with tumor necrosis factor-alpha (TNF-α) significantly promoted proliferation, mobilization and differentiation into osteocytes *via* activation of ERK1/2 and MAPK signaling pathways. These results were confirmed by gene silencing with siRNA that partially inhibited ERK1/2 signaling and osteogenic differentiation of MSCs[90]. TNF-α preconditioning has been shown to improve *in vitro* bone regeneration by up-regulating BMP2. Further, it stimulated the cell proliferation and differentiation[91]. IFN-γ pretreatment improved the therapeutic efficacy of MSCs by enhancing the secretion of immunomodulatory molecules such as PGE2, HGF, TGF-β, and MCP-1[92]. MSCs pretreated with IFN-γ inhibited natural killer cell activation and NK mediated cytotoxicity by upregulating the synthesis of indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2[93]. In another study MSCs were pre-stimulated with IFN-γ to enhance their immunosuppressive and therapeutic properties *in vitro* and *in vivo*[94]. A combination of different growth factors may produce contrary results. For example, a combination of interleukin (IL)-1 and TNF-α in *in vitro* cultures of MSCs inhibited the osteogenesis and adipocyte *via* activating the canonical nuclear factor-kappa B (NF-kB) signaling[95]. Similarly, when cells were treated with a combination of bFGF and steroid hormones an enhanced neural differentiation was observed as indicated by upregulation of beta III-tubulin (β-III tubulin) and microtubule-associated proteins-2 (MAP-2) during 4 d of treatment[96].

Certain cytokines have also been shown to influence the regenerative potential of stem cells. IL-1β preconditioning of MSCs activated several biological processes such as cell survival, cell migration, cell adhesion, chemokine production, angiogenesis and modulation of the immune response[96]. More specifically MSC preconditioning with IL-1β significantly upregulated the expression of certain cytokines (TNF-α, IL-6, IL-8 and IL-23A), chemokines (CCL5, CCL20, CXCL1, CXCL3, CXCL5, CXCL6, CXCL10 and CXCL11) and adhesion molecules [vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1 and ICAM-4][96]. In another study, synovial MSCs when pretreated with IL-1β, showed significantly higher proliferation as well as chondrogenic potential[97]. To induce these results, TGF-β seemed to activate Akt, extracellular signal-regulated kinase 1/2 (ERK1/2), focal adhesion kinase (FAK), and p38, *via* TGF-β type I receptor in MSCs[97]. Xinaris *et al*[98], preconditioned MSCs with insulin-like growth factor-1 (IGF-1) before administration and found it effective in terms of migration and homing of cells which was required for the restoration of renal function following acute kidney injury[98]. Interestingly when the diabetic MSCs were preconditioned with a combination of IGF-1 (50 ng/mL) and fibroblast growth factor-2 (FGF-2) (50 ng/mL), upregulation of IGF-1, FGF-2, Akt, GATA-4, Nkx 2.5 and

downregulation of p16INK4a, p66shc, p53, Bax and Bak occurred[99].

In conclusion, preconditioning of cells with different growth factors and cytokines may enhance regenerative potential of stem cells (Figure 1). Although preconditioning of MSCs with different growth factors and cytokines can influence significantly the biological properties of MSCs, there are number of challenges to use this strategy successfully for optimum benefits. For example, will the same dose or concentration of cytokines and growth factors influence MSCs isolated from different sources? Some growth factors and cytokines may influence MSC function synergistically and antagonistically when used in combination[40]. Therefore, optimization of amalgamation of growth factors and cytokines as well as their concentrations is required for better results. Similarly, MSCs behave differently in culture conditions such as in 3D cultures and hypoxic conditions and therefore preconditioning in such conditions should be optimized (Figure 2).

CR

CR refers to consuming significantly reduced calories as compared to calories taken ad libitum. At the organismal level, it was first reported in 1935 that reduced caloric intake can extend the mean and maximum life span in rodents[100]. Since then beneficial effects of CR were observed in animals of other species such as rats, mice, dogs, fish, flies, worms, yeast and humans[101-103]. CR is now an established anti-aging strategy for prolonging lifespan and has also been applied on stem cells to rejuvenate them. CR as a non-genetic dietary intervention reduces the energy metabolism in cells and can positively affect regenerative potential of cells by extending their life span and making the cells healthy.

Glucose is an essential source of energy for all types of cells in the body although elevated levels of glucose have been shown to be associated with reduced mobilization, proliferation, homing and repair potential[104,105]. Similarly, stem cells isolated from diabetic patients and animals exhibited reduced yield, viability, proliferation, angiogenesis, differentiation and wound healing ability[106,107]. Cells are cultured in stem cell media that contain various components including glucose to ensure proper functioning and maintenance of cell characteristics. However, cells cultured *in vitro* in media with high glucose concentration show impaired regenerative potential of cells[108]. High glucose concentration in stem cell culture media was found to negatively impact a cell's viability, differentiation and self-renewal potential [109,110]. Based on the findings it was found that the conventional media used to expand cells was not appropriate for long term expansion of cells as it adversely impacted the biological properties of cells[110,111]. Thus induction of CR in cells by culturing in low glucose concentration is another area of interest for the enhancement of stem cell function before transplantation. Different protocols ranging from glucose depletion[109] to varying glucose levels[110] were adopted in this regard.

Al-Qarakhli *et al*[112] comprehensively studied the effect of glucose concentration on expansion as well as differentiation of mesenchymal stromal cells. They found that hyperglycemia negatively impact the proliferation, and osteogenic and adipogenic differentiation of cells with more senescence features in culture[112]. To investigate the effect of CR, Stolzing *et al*[110] (2006) used media with different glucose concentrations for MSC culturing. In this study MSCs cultured in medium with low glucose concentrations were functionally more active as evidenced by enhanced viability, proliferation and differentiation of cells when cultured in caloric restricted media[110]. When the biological characteristics of cells cultured in low glucose and` high glucose concentrations were compared, there was significantly more proliferation, colony-forming ability, homing and wound healing potential of cells in low glucose concentrations as compared to high glucose concentration. In addition, high glucose decreased expression of stemness genes (SOX-2, Nanog, Oct-4), survival genes (Sirt-1, Sirt-6, HIF-1α), glucose transporter 1 (Glut-1) concomitant with increases apoptosis and senescence in cells[113]. Choudhery *et al*[109], (2012) cultured the BM-MSCs in glucose free conditioned and optimized the time to perform further *in vitro* and in *in vivo* studies[109]. In this study aged MSCs were pre-conditioned with glucose depletion for 60 min to enhance the age depleted function of stem cells. Preconditioning of aged MSCs with glucose depletion resulted in upregulation of IGF-1, AKT and SIRT-1 concomitant with enhanced viability, proliferation and delayed senescence. Interestingly, the preconditioned aged MSCs after transplantation into heart showed increased expression of paracrine factors (IGF-1, FGF-2, VEGF and SDF-1a) that was associated with significantly improved cardiac performance in mouse

Table 2 Effect of glucose concentrations on cells

Glucose concentration	Major findings	Cell types	Ref.
Glucose free and 4.5 g/L	Glucose depletion enhances proliferation, delays senescence and restores ability of aged cells to repair senescent infarcted myocardium	Mouse bone marrow derived MSCs	[109]
0.25, 0.5, 1.0 and 4.5 g/L	High glucose decreases viability while low glucose concentration retains high proliferative and differentiation capability of cells	Rat bone marrow derived MSCs	[110]
5.56 mmol/L, 13.9 mmol/L, 27.8 mmol/L, and 55.6 mmol/L	Decrease in population doublings and CFUs. Increased senescence in high glucose	Human adipose tissue derived MSCs	[111]
5.5 mM and 25 mM	No negative impact on population doublings and expansion. Increased senescence, inhibit osteogenic and adipogenic differentiation potential	Endosteal niche lining compact bone cells (CB-MSCs)	[112]
1 g/L and 4.5 g/L	Decreased proliferation, increased apoptosis and senescence	Nucleus pulpous-derived MSCs	[113]
5.5 mM and 35 mM	Increased apoptosis	Human periodontal ligament fibroblasts	[114]
5 mM/L and 25 mM/L	Increased oxidative stress	Mesangial cells	[118]

MSCs: Mesenchymal stem cells.

model of myocardial infarction[109]. High glucose concentrations can impair cell function and induce apoptosis and represent a potential limitation for therapeutic strategies based on *ex vivo* expansion of stem cells[114]. In parallel to these findings some studies suggested a significantly increased apoptosis of β-cells in diabetic patients that resulted in β-cell dysfunction and reduced β-cell mass[115,116].

There are a number of cellular responses to high glucose (Table 2) that ultimately result in functional impairment and cell death[117]. High glucose results in generation of reactive oxygen and nitrogen species such as superoxide, nitric oxide and peroxynitrite and their derivatives[117,118]. This high glucose induced ROS species results in high glucose-mediated apoptosis and necrosis and ultimately cell death. ROS species produced by high glucose may increase the activity of NF-kB in various cell types and leads to cell apoptosis and death in a process that involves Bax and caspase activation[117]. In addition, high glucose concentration in the cell microenvironment activate those proteins that are related to apoptotic cell death including members of the caspase and Bcl-2 families[117].

In conclusion, the biological properties of cells are influenced by the glucose concentration in the culture medium (Figure 1). Previous studies indicate that low glucose concentration in the culture medium enhances cell proliferation, viability and differentiation potential of cells concurrent with reduced senescence and apoptosis. However, not only the glucose concentration but the duration of preconditioning of cells are important parameters to consider. For example, although 1 h preconditioning of MSCs with glucose depletion (0g/L) produced beneficial effects in Choudhery *et al*[109]'s study[109], culturing of cells without glucose for longer time will definitely produce deleterious effects in cell. Therefore evaluation of the effects of glucose concentrations with respect to time must the carefully considered for preconditioning of different types of cells (Figure 2).

CONCLUSION

Conclusion and future perspectives

Stem cell-based therapies are gaining interest of patients and doctors for their potential to treat diseases that cannot be cured with conventional medicines. Aged patients are the major candidates for stem cell-based therapies. However, studies clearly indicate that stem cell potential for autologous use deteriorates with donor age. The number of regenerative cells in aged and unhealthy individuals is very low, however, for the

success of stem cell based regenerative therapies large numbers of cells are required. Cells are usually expanded *in vitro* to obtain high numbers, however, this expansion further decreases stem cell function and does not give desired results after transplantation. Overall, with increasing donor age, disease condition of donors and *in vitro* expansion of cells, regenerative potential of stem cell decreases and it represents a major limitation for the success of cell therapies. To combat the problem of decline in regenerative potential of cells different strategies such as heat shock, hypoxia, caloric restriction and preconditioning with different factors can be applied *in vitro* before transplantation of cells. The correct application of these strategies have a profound effect on stem cell characteristics to enhance their therapeutic functions. These strategies may be used to enhance the self-renewal, repair and differentiation potential of cells and to keep the cells healthy. Use of these strategies also enhances cell survival and engraftment in hostile microenvironment of the target tissue. The inconsistent reports are due to the use of different levels of factors (hypoxia, glucose, temperature, growth factors & cytokine), variable durations and variety of cell types used in studies. The question is not if these strategies alters stem cell function but rather the use of the correct strategy and condition for an accurate period of time that is most important.

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REVIEW

Dental mesenchymal stromal/stem cells in different microenvironments— implications in regenerative therapy

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Abstract

Current research data reveal microenvironment as a significant modifier of physical functions, pathologic changes, as well as the therapeutic effects of stem cells. When comparing regeneration potential of various stem cell types used for cytotherapy and tissue engineering, mesenchymal stem cells (MSCs) are currently the most attractive cell source for bone and tooth regeneration due to their differentiation and immunomodulatory potential and lack of ethical issues associated with their use. The microenvironment of donors and recipients selected in cytotherapy plays a crucial role in regenerative potential of transplanted MSCs, indicating interactions of cells with their microenvironment indispensable in MSC-mediated bone and dental regeneration. Since a variety of MSC populations have been procured from different parts of the tooth and tooth-supporting tissues, MSCs of dental origin and their achievements in capacity to reconstitute various dental tissues have gained attention of many research groups over the years. This review discusses recent advances in comparative analyses of dental MSC regeneration potential with regards to their tissue origin and specific microenvironmental conditions, giving additional insight into the current clinical application of these cells.

Key Words: Microenvironment; Dental mesenchymal stem cells; Modulation of regenerative potential; Tissue origin; Hypoxia microenvironment; Inflammatory microenvironment; Clinical application

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Core Tip: This review discusses recent advances in comparative analyses of dental mesenchymal stem cell (MSC) regeneration potential. We have summarized the available research evidence concerning the effects of hypoxic and inflammatory microenvironmental factors on dental MSC differentiation capacity. Existing investigations indicate the very important aspect of the recipient microenvironment niche in terms of therapeutic efficacy of transplanted dental MSCs. However, some of the data for the same cell type (especially in hypoxic *in vitro* conditions) are conflicting, so it is important to point out that the biology of MSCs is not yet fully known, and further research in this area is needed.

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INTRODUCTION

Bone defects and dental loss connected with either disease or trauma seriously influence the quality of life of the whole population, including emotional, physical, and financial load on the society. For the medical treatment of oral diseases, periodontal treatment, dental implants, and dental protheses are the gold standards [1]. Since these oral therapies can only maintain the current state and stop further complications of the disease, failing to influence complete tissue regeneration, new technologies are needed to overcome these limitations at various tissue regeneration steps[2]. Currently, tissue engineering represents a promising future approach for recovering the function and integrity of tooth's hard tissue[3]. The microenvironment of dental tissues, containing dental immune cells, blood vessels, extracellular matrix (ECM), numerous secreted soluble mediators, and various stromal cells, essentially influences the healing process of diseased dental tissue[4,5]. In terms of regenerative features, mesenchymal stem cells (MSCs) are the most prominent among stromal cells. Their clinical application in the treatment of dental diseases is still at the beginning since the exact mechanism of their therapeutic properties is not yet clear.

MSCs are the population of multipotent stromal cells present in many adults, perinatal and fetal tissues, where they participate in homeostasis maintenance. They were initially isolated from bone marrow and characterized as fibroblast-like cells[6]. After a while, their presence has been demonstrated in various fetal and adult tissues, such as peripheral blood, umbilical cord, placenta, adipose tissue, and others[7,8]. In the past two decades, a variety of MSC populations have also been procured from different parts of the tooth and tooth-supporting tissues (Figure 1): Dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), dental follicle stem cells (DFSCs), gingival MSCs (GMSCs), and stem cells from the dental apical papilla (SCAPs)[9]. These cells are particularly suitable for research, given the easy availability of tissues through non-invasive dental procedures and simple methods for their isolation. According to the International Society for Cellular Therapy, minimal criteria to characterize MSC population consider the positive expression of surface markers CD73, CD90, and CD105 and negative expression of CD11b, CD19, CD79α, CD34, CD31, CD45, and human leukocyte antigen-DR isotype, along with their self-renewal and multilineage differentiation capacity into cells of osteogenic, chondrogenic, and adipogenic lineages [10] (Figure 2). Yet, the defining characteristics of MSCs are inconsistent among researchers. In addition to their regenerative role, MSCs exert immunomodulatory properties by affecting cells of the innate and adaptive immune system through direct intercellular contacts and/or secretion of soluble mediators (Figure 2). Moreover, MSCs express multiple paracrine functions, thus modulating surrounding microenvironment response to numerous autoimmune and inflammatory diseases[11]. Previous research has shown that MSCs suppress activation and proliferation of CD4+ helper and CD8+ cytotoxic T lymphocytes, B lymphocytes[12,13], dendritic cells, and natural killer cells[14], while increasing the production of regulatory T-lymphocytes[15]. In addition, it has been determined that MSCs modulate microenvironment in the injured

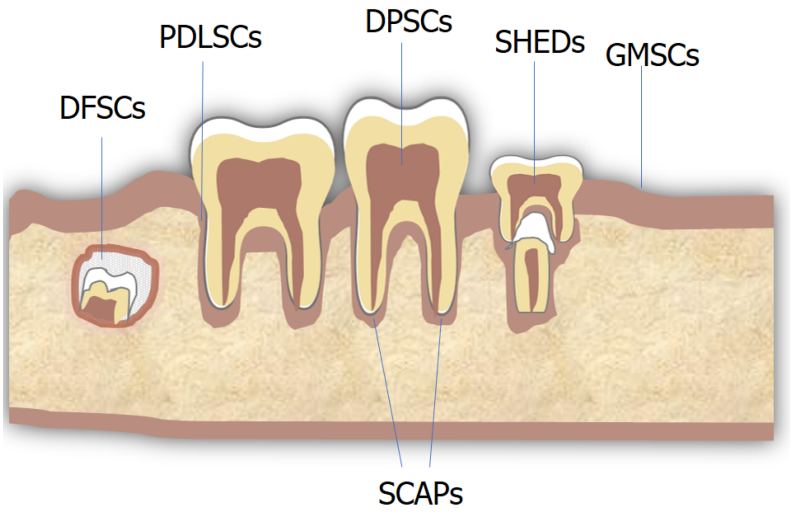

Figure 1 Schematic drawing illustrating sources of human dental tissue-derived mesenchymal stem cells. DFSCs: Dental follicle stem cells; DPSCs: Dental pulp stem cells; GMSCs: Gingival mesenchymal stem cells; PDLSCs: Periodontal ligament stem cells; SCAPs: Stem cells from the dental apical papilla; SHEDs: Stem cells from human exfoliated deciduous teeth.

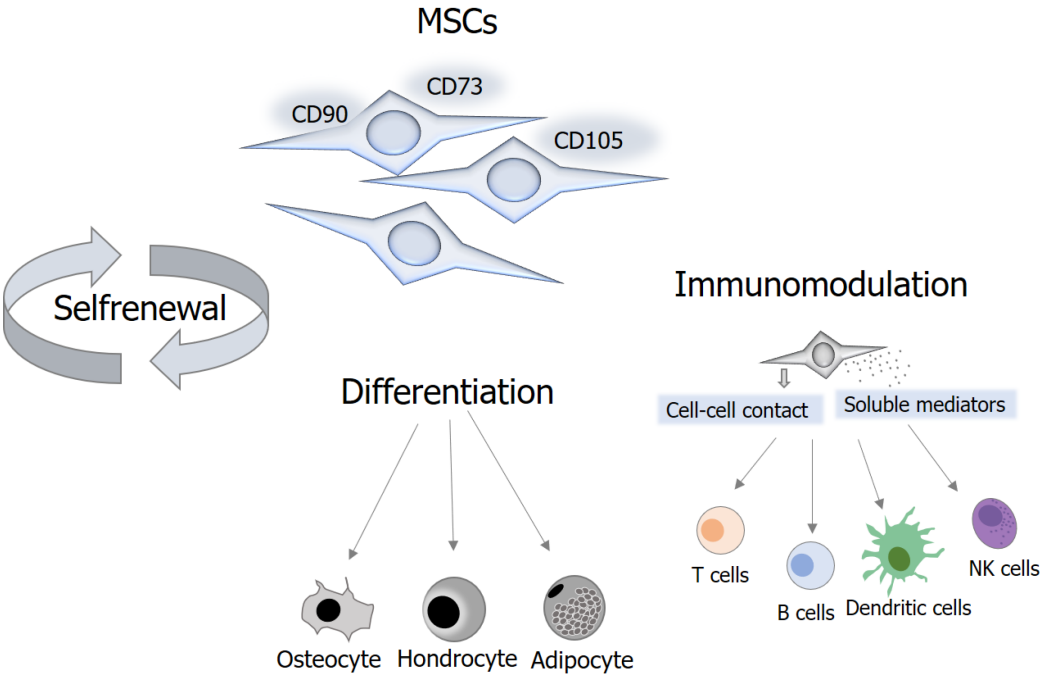

Figure 2 Properties of mesenchymal stem cells. mesenchymal stem cells are the population of multipotent stromal cells present in adult and perinatal tissues where they participate in maintaining of homeostasis. Due to their self-renewal capacity, differentiation potential into specialized cells of mesodermal origin and immunomodulatory features, these undifferentiated cells can be potentially applied in regenerative medicine and cell therapy.

tissue by releasing anti-inflammatory and anti-apoptotic molecules[16].

It is well known that MSCs' behavior depends on the context of the microenvironment in which they reside and function. These physiologically defined compartments, named stem cell niches, are the sites of external cues integration that determine the fate of MSCs and govern them towards specific functions[17]. Therefore, a better understanding of the effects of specific microenvironmental conditions on MSCs' fate is of great importance for getting a real insight into their biology and optimizing the conditions for their successful use.

Oxygen (O_2) represents an important factor in the stem cell niche necessary for cell activity and metabolism. Unlike laboratory standard conditions that correspond to the atmospheric (21%) O_2 levels, physiological oxygenation in tissues is hypoxic, ranging from 1%-14% with an average of about 5%[18]. Response to O_2 level changes in mammals is mainly regulated by hypoxia-inducible factor 1 (HIF-1)-α, an ubiquitously

expressed transcription factor subunit that translocates to the nucleus under hypoxic conditions where it binds to HIF-1β to regulate target genes[19]. Different O_2 levels affect various MSCs features[20,21]. Moreover, the hypoxia effects depend on cell type, oxygen concentration, and experimental design; thus, many studies gave contradictory results, especially considering short-term exposure to hypoxia[20]. In dental and dental-supporting tissue derived cells, low oxygen levels (1%) increase the formation of reactive oxygen species, leading to oxidative stress, specifically in periodontal ligament (PDL) cells[22]. The reactive oxygen species level multiplies when the bacterial inflammation occurs within a hypoxic environment[23]. A unique feature of the oral cavity is the presence of plenty of microorganisms such as bacteria, fungi, protozoa, or viruses, organized in the complex communities, termed as oral microbiome. These microorganisms coexist with the host in a symbiotic way. Depending on the mouth area, the microbiome's composition is diverse, with the dental tissues (including teeth and teeth supportive tissues) being the habitat of many microorganisms (dental plaque). As the composition of the oral microbiome changes dynamically[24,25], physiological balance can be disrupted, consequently encouraging the infection development in the host[26,27].

This review provides a detailed summary of currently available data concerning dental MSC regeneration potential in terms of the tissue origin and influence of hypoxic and proinflammatory microenvironments. Furthermore, it analyzes current evidence regarding clinical applicability of dental MSCs.

DENTAL MSCS REGENERATIVE PROPERTIES WITH REGARDS TO THEIR TISSUE ORIGIN

The term dental tissue refers to the tooth and tooth-supporting tissues (periodontium). There are two major constitutive parts of the tooth, a crown (mostly visible part of the tooth) and a root. The crown is formed of three hard tissues, enamel, dentin, and cementum, and one soft tissue, dental pulp[28]. Enamel, dentin, and cementum are tissues with limited or no possibility to regenerate[29], while the dental pulp is a loose connective tissue profusely vascularized and innervated. Structurally, dental pulp is divided into three main regions that form a continuum: The peripheral odontoblastic and the sub-odontoblastic layer and the central pulp[30,31]. In terms of cellularity, the most common dental pulp cell types are fibroblasts, peripheral odontoblasts, and DPSCs, whereas collagens I and III represent extracellular pulpal matrix key protein components[32]. Moreover, the proportion of cellularity to collagen with aging favors collagen[30].

Dental pulp MSCs, with their extensive proliferation and multipotential differentiation capability, have an intrinsic role in dental pulp regeneration potential (Figure 3). They were first discovered by Gronthos *et al*[33] in 2000 as MSCs derived from the pulp of the permanent, impacted third molars and supernumerary teeth, commonly considered as medical waste. Like other dental MSCs, DPSCs express osteoblastic markers such as alkaline phosphatase (ALP), collagen type I (COL1A1), and osteocalcin (OCN) and are able to differentiate into osteoblast-like cells. Furthermore, DPSCs are essential for postnatal tooth homeostasis through implementation of odontoblasts in the dentin restoration process. When dental injuries or odontoblast apoptosis occur, DPSCs rapidly proliferate, migrate, and differentiate into odontoblast cells. Moreover, being of neural crest origin, DPSCs can differentiate into functionally active neurons and glial cells[34]. Importantly, recent studies have revealed that DPSCs reside in neurovascular niche where they secrete an array of angiogenic regulatory factors and generate capillary-like structures demonstrating strong angiogenic ability[35]. Overall, neurovascular and MSC-like properties make DPSCs good candidates for bone and tooth regeneration.

Stem cells from the pulp tissue of human exfoliated deciduous teeth (Figure 3), firstly isolated by Miura *et al*[36] in 2003, are capable of forming dentin-like structures. Moreover, they show higher proliferative, odontogenic, and osteogenic differentiation potential than DPSCs[37]. Furthermore, these cells have a higher doubling time[38] than DPSCs and higher expression of collagens I and III as well as of pluripotency markers such as octamer-binding transcription factor-3/4, sex determining region Y-box-2, and Nanog homeobox[39]. SHEDs are also able to differentiate into neural and glial cells under appropriate conditions. Being able to demonstrate regenerative potential even 2 years after cryopreservation[40] and, because of their easy accessibility, SHEDs represent good candidates for bone and tooth regeneration.

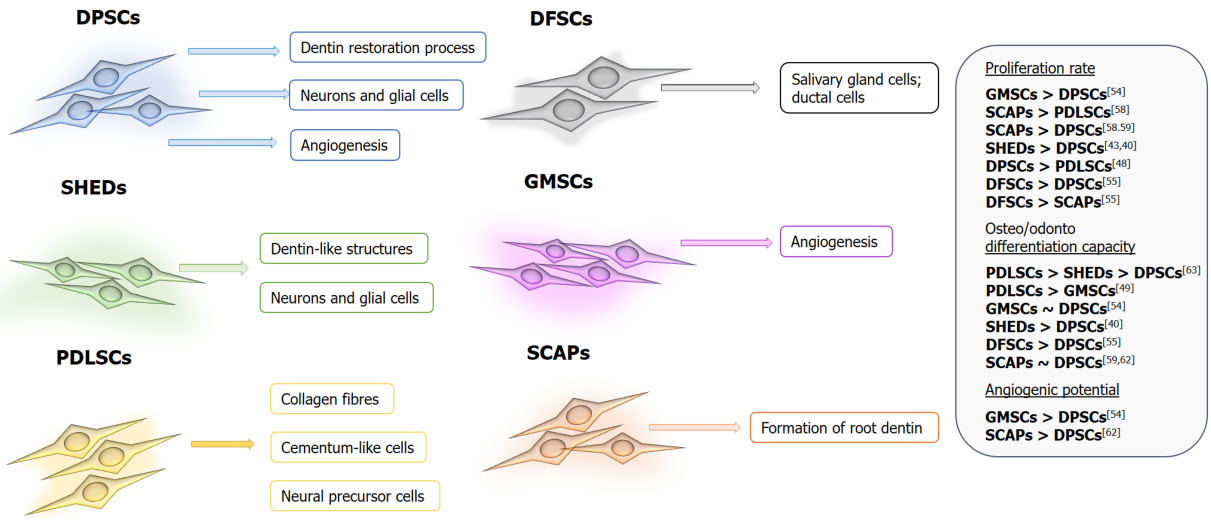

Figure 3 Functional properties and differences among dental mesenchymal stem cells. Dental mesenchymal stem cells (MSCs) are involved in dental tissues regeneration which is influenced by local microenvironment of the tissues they reside in. Overall, all dental MSCs represent good candidates for tissue regeneration, however their capacities differ (shown in table on the right). > , < and ~ represent higher, lower or similar capacity/rate respectively; The numbers in square brackets indicate the references. DFSCs: Dental follicle stem cells; DPSCs: Dental pulp stem cells; GMSCs: Gingival mesenchymal stem cells; PDLSCs: Periodontal ligament stem cells; SCAPs: Stem cells from the dental apical papilla; SHEDs: Stem cells from human exfoliated deciduous teeth.

However, the possibility of dental pulp tissue regeneration is restricted by several factors. Since dental pulp has collateral blood circulation due to the anatomical organization of the pulp chamber, the efficacy of the immune response to infection is limited[41]. Thus, the localization of DPSCs in perivascular and perineural pulp regions and consequent contacts with different microenvironments, along with their high immunomodulatory activity[42], makes them good candidates for modulating the immune response to infection. Furthermore, when comparing cellular density in specific tooth regions of dental pulp tissue from different teeth, higher density was observed in the coronal region of deciduous and premolar teeth compared with supernumerary and third molars. In contrast, a high cell density was observed in the apical region of supernumeraries and premolars compared with third molar teeth[43]. Overall, supernumerary dental pulp as source for DPSCs has the best morphometric parameters, and its cell density is comparable to that of deciduous tooth pulp.

Periodontium is a specialized connective tooth-supporting tissue that surrounds the root of the tooth. It has the role of attaching the tooth to the jawbone, amortizing the mechanical pressure that occurs during chewing and speech but also in the formation and resorption of bone tissue. Periodontium involves two soft tissues—PDL and gums, and two hard tissues—alveolar bone and cementum[44]. The PDL is a connective tissue with high cellularity and amount of ECM components. As for cellular constituents, PDL consists mainly of fibroblasts, osteoblasts, osteoclasts, cementoblasts, and cementocytes. Other cells present in PDL include epithelial cell rests of Malassez, macrophages, nerve cells, endothelial cells, as well as MSCs. PDLSCs represent a unique population of somatic stem cells of mesenchymal origin with the regenerative potential reflected by self-renewal and multipotent differentiation ability as well as potency for the formation of tissues that support the teeth, including the PDL and cement (Figure 3). On the other hand, the rich ECM of PDL is formed of collagen type I, II, and XII fibers, proteoglycans, and a vast vascular network[45]. Interestingly, collagen type I is the most widely used scaffold material for dental pulp regeneration [46], and PDLSCs have shown higher expression of *COL1A1* compared to DPSCs[47]. Taken together, these features qualify PDLSCs for use in the regeneration/reconstruction of tooth-supporting tissue in periodontal disease.

Like PDL, the gingival connective tissue also has a rich ECM. Collagen fibers and ground substances make up 60% and 35% of gingival ECM, respectively, and only about 5% of gingival connective tissue consists of various cells like fibroblasts, mast cells, macrophages, and inflammatory cells[48]. Structurally and functionally, the gingiva is different from PDL, displaying an even higher collagen turnover rate than PDL and having a distinct composition and organization of ECM. It was noticed that compared to PDL fibroblasts, gingival fibroblasts have a significantly lower level of ALP expression, an increased potential to stimulate epithelial growth, as well as a

distinctive property of regulating cytokeratin expression by epithelial cells[49]. Furthermore, the gingival tissue is highly vascularized, thanks to the high number of anastomoses[50]. Moreover, due to the activities of local microenvironment factors, including transforming growth factor-α, transforming growth factor-β, insulin-like growth factor, nerve growth factor, epidermal growth factor, and fibroblast growth factor, gingiva has a particularly high wound-healing capacity. Other local factors, such as mechanical signals from the ECM to the cells, may be involved as well[49]. All of these structural characteristics provide gingival tissue with a great therapeutic potential in regenerative therapy especially in terms of GMSCs. In contrast to MSCs from other sources, GMSCs isolated from the gingival lamina propria are profuse and easily procured cells through minimally invasive cell isolation techniques, which make them suitable cells for regenerative purposes[51]. Compared to PDLSCs, GMSCs have a higher rate of proliferation while also exerting a higher capacity to proliferate, migrate, and form angiogenic tubules in comparison to DPSCs (Figure 3)[52].

The alveolar bone is located on the jaw bones that hold the teeth, and it arises in the process of immature tooth root development from the dental follicle (DF), one of the multipotent tissues[53]. The DF is made up of MSCs and fibers surrounding the enamel organ and dental papilla of a developing tooth[54]. It is a vascular fibrous sac of ectomesenchymal origin. Histologically, DF is characterized by fibrous connective tissue with variable amounts of lining epithelium, including enamel, cuboidal, squamous, and, rarely, respiratory epithelium. The type of lining epithelium seems to be related to the patient's age[55]. DFSCs, originating from this developing tissue, possess higher plasticity than other dental stem cells[56]. Recently, isolated DFSCs were also found to have the ability to form salivary gland cells and ductal cells[56] (Figure 3).

Another immature dental tissue is the apical papilla, a tissue only present during root development before the tooth erupts into the oral cavity[57]. In comparison to the dental pulp, the apical papilla has less cellular and vascular components[57], and the tissue is more immature since it contains a higher number of MSCs than mature dental pulp tissue. Moreover, the apical papilla performs a key role in the differentiation of odontoblasts into cells capable of secreting the primary dentin matrix[58] . Furthermore, the localization of the apical papilla in the apical root of the tooth can benefit by its apical collateral circulation and thus survive during the pulp necrosis process, which additionally explains why immature teeth with necrotic pulps can undergo completion of root development[59]. SCAPs reside in the apical papilla of permanent immature teeth and appear to be the source of odontoblasts that are responsible for the formation of root dentin (Figure 3). Conservation of SCAPs when treating immature teeth may allow the continuous formation of the root to completion.

Overall, among adult tissues easily available through non-invasive dental procedures, SHEDs have a higher doubling time than DPSCs[39]. However, PDLSCs showed a significantly higher *in vitro* osteogenic differentiation potential than both SHEDs and DPSCs (Figure 3), as evidenced by functional studies and gene expression indicating the complex influence of stem cell origin on their regenerative potential[60].

MODULATION OF DENTAL MSCS REGENERATIVE PROPERTIES IN HYPOXIC MICROENVIRONMENT

In the oral cavity, O_2 levels are shown to range from 0.7% and 3.8% in human periodontal pockets[61] and approximately 3% O_2 in the pulp tissue of rats[62]; therefore, given the therapeutic potential of dental MSCs, a number of studies have focused on examining their functions in such microenvironments. According to the published data, dental MSCs have been intensively investigated with regard to different O_2 levels, and detailed outcomes are shown in Table 1.

Although most studies showed that hypoxia stimulated proliferation of DPSCs[63], PDLSCs[64-67], SHEDs[68,69], and SCAPs[70], some reported it had no[71,72] or a negative effect[73,74]. Also, results concerning the influence of hypoxia on differentiation capacity of MSCs derived from dental and dental supportive tissues are diverse. It was shown that under hypoxia, osteogenic differentiation of DPSCs was strongly suppressed compared to normoxia[75]; however, there are studies showing increased osteogenic[63,71], adipogenic and chondrogenic differentiation[71] at low O_2 levels. In order to mimic the native microenvironment of DPSCs, Labedz-Maslowska *et al*[73] grew hydrogel-encapsulated cells in the presence of 2% O_2. They showed up-regulation of osteogenic differentiation in hypoxic conditions, but calcium deposition was, in the case of two-dimensional culture, more prominent under normoxia.

Table 1 Effects of different oxygen levels on regenerative potential of human dental mesenchymal stem cells

Hypoxia level	Outcome	Ref.
DPSCs		
< 1% O_2	Weak ALP activity, weak calcium deposition	Janjić *et al*[75], 2019
2% O_2	Proliferation↑; odontogenic differentiation↑; angiogenesis↑; *in vivo*: Angiogenesis inside the pulp chamber↑, the formation of odontoblast-like cells lining along the dentin–pulp interface↑	Kuang *et al*[76], 2016
	No change in proliferation; calcium deposits↑; proteoglycan deposition↑; lipid droplets↑; PPARγ2 mRNA↑	Zhou *et al*[71], 2014
	Proliferation↓; Runx2 mRNA expression↑ (both 2D and 3D conditions); Runx2 and Col1A mRNA expression, osteopontin in 2D culture↑, calcium deposition in 3D culture↑, calcium deposition in 2D culture↓	Labedz-Maslowska *et al*[73], 2020
3% O_2	BMP2, OCN and RUNX2 protein expression↑; calcium deposits↑; RunX2 and Sp-7 mRNA expression↑; *in vivo*: In a mouse apical periodontitis bone destruction model, hDPSC recruitment and recovery of alveolar bone mass in infected periapical tissue↑, osteogenesis and bone mineralization↑	Wu *et al*[77], 2016
5% O_2	Proliferation↑; mineralization↑	Kwon *et al*[63], 2017
PDLSCs		
1.5%-2% O_2	ALP activity↓; SPARC protein expression↓; ALP, OCN, and BMP-2 mRNA expression↓; proliferation↓	Hou *et al*[79], 2009
2% O_2	Calcium deposition, proteoglycan deposition↑; lipid droplets↑; Runx2, Sox9 mRNA expression↑	Zhou *et al*[71], 2014
	ALP activity↑; Runx2 and Sp7 mRNA and protein expression↑; mineralization↑	Wu *et al*[80], 2013
	SPP1, RUNX2, SP7 mRNAs and protein expression↑	Li *et al*[65], 2014
	Proliferation↑; RUNX-2 and ALP protein expression↑; no effect on adipogenic differentiation; *in vivo*: Stronger bone regeneration region in male nude mice, more mineralized tissue in a periodontal defect model	Yu *et al*[64], 2016
	Proliferation↑; Runx2, osteopontin and osteocalcin mRNA expression↑; *in vivo*: After 12 wk of transplantation, hypoxia-treated cells differentiated into osteoblast-like cells that formed bone-like structures	Zhang *et al*[66], 2014
	ALP activity↑; Runx2 mRNA expression↑	Chen *et al*[81], 2017
3% O_2	Proliferation↑; osteogenic and adipogenic differentiation↓; chondrogenic differentiation↑; preconditioning: Osteogenic and adipogenic differentiation↑	Murabayashi *et al*[72], 2017
	Runx2, Alp, Col1, and Ocn mRNA expression↑; RUNX2 protein *ex vivo* and *in situ*↑	Xu *et al*[82], 2019
5% O_2	ALP activity↓	Matsuda *et al*[78], 1998
5% O_2; 1% O_2	Proliferation↓; ALP activity↑; Opn, Alp mRNA expression↑; Cemp1, Cap mRNA expression↑	Xiao *et al*[74], 2017
8% O_2	Proliferation↑; Cemp1, Ocn mRNA expression↑; CEMP protein expression ↑; mineral deposition↑; ALP activity↑; *in vivo*: CEMP1 protein expression in mouse PDL spaces↑	Choi *et al*[67], 2014
SHEDs		
1% O_2	Proliferation↑; mineralization↑; ALP activity↑; OPN and DMP1 protein expression↑; *in vivo*: After implantation in immunodeficient mice, the tissue-engineered constructs seeded with hypoxia primed SHED mediated faster intramembranous bone formation into critical size calvarial defects	Novais *et al*[69], 2019
2.3% O_2	Proliferation↑; no effect on adipogenic and osteogenic differentiation	Kanafi *et al*[68], 2013
SCAPs		
1% O_2	No effect on proliferation; RunX2, Alp, TGF-1↑; neuronal differentiation (CNP, NSE, and SNAIL mRNA expression↑; angiogenesis (VEGF A and B) ↑; adipocyte lipid binding protein (ALBP)↓	Vanacker *et al*[70], 2014
3% O_2	ALP activity↑; mineralization↑; Dspp, Dmp1 and Bsp mRNA expression↑	Yang *et al*[83], 2020
Chemical hypoxia		

DPSCs		
100 μM $CoCl_2$	No effect on proliferation; SOX9 and VCAN; no expression Col2a1, Acan↑, Col 10 mRNA expression↓; proteoglycans↓	Teti *et al*[85], 2018
100 mM $CoCl_2$	Short term: RUNX2, ALP, OCN, COL1A1 mRNA and protein expression↑; long term: RUNX2, ALP, OCN, COL1A1 mRNA and protein expression↓	Zheng *et al*[89], 2021
	ALP activity↓; Alp, Ocn, and Runx2 mRNA expression↓; mineralization↓	Osathanon *et al*[86], 2014
200 μM $CoCl_2$	ALP activity↓; Runx2, Alp, Ocn and Col-1 mRNA and protein expression↓; mineralization↓	Song *et al*[87], 2017
PDLCs		
200 μM; 400 μM $CoCl_2$	Proliferation↓; ALP, RUNX2, collagen I↓	Dong *et al*[88], 2014
1 mM DMOG	No effect on proliferation; COL1, RUNX2 and CEMP1 protein expression↑	Li *et al*[92], 2016
0, 5, 10, 20 μM deferoxamine	Proliferation↓; Runx2, Opn and Col1 mRNA expression↑; calcium deposition↑	Mu *et al*[91], 2017
SHED		
50 or 100 μM $CoCl_2$	ALP activity↓; calcium deposition↓; Alp, Runx2, and ColI mRNA expression↓	Chen *et al*[90], 2019

↑ and ↓ represent increasing or decreasing effect, respectively. ACAN: Aggrecan; ALBP: Adipocyte lipid binding protein; ALP: Alkaline phosphatase; BMP-2: Bone morphogenetic protein 2; BSP: Bone sialoprotein; CEMP 1: Cementum protein 1; COL-1: Collagen-1; DMOG: Dimethyloxalylglycine; DMP1: Dentin matrix protein 1; DPSCs: Dental pulp stem cells; DSPP: Dentin sialophosphoprotein; OCN: Osteocalcin; OPN: Osteopontin; PDLSCs: Periodontal ligament stem cells; PPARγ2: Peroxisome proliferator-activated receptor gamma; RunX2: Runt-related transcription factor 2; SCAPs: Stem cells from the dental apical papilla; SHEDs: Stem cells from human exfoliated deciduous teeth; Sox-9: SRY-box transcription factor 9; Sp-7: Osterix; SPARC: Secreted protein acidic and rich in cysteine; TGF-β: Transforming growth factor-beta; VEGF: Vascular endothelial growth factor.

Another study seeded DPSCs into nanofibrous spongy microspheres and showed that their priming at 2% O_2 prior to implantation significantly promoted the formation of odontoblast-like cells lining along the dentin–pulp interface of mice[76]. On the other hand, Wu *et al*[77] showed that preconditioning of cells at 3% O_2 enhanced DPSC osteogenic differentiation *in vitro* and more importantly upregulated their recruitment in mouse apical periodontitis bone destruction model and enhanced osteogenesis and bone mineralization.

While there is evidence that hypoxia decreased osteogenic differentiation of PDLSCs[65,78,79], it was demonstrated that it can also increase it[66,71,74,80,81] as well as adipogenic and chondrogenic differentiation[71]. Interesting observations in some of these studies were that HIF1α promoted osteogenic differentiation of PDLSCs, while HIF1A antisense long noncoding RNA 2 had a negative effect on it[81] and that the stimulative effect of 2% O_2 on osteogenic differentiation of PDLSCs was mediated by extracellular signal-regulated kinase and even more rapidly and vigorously by p38 mitogen-activated protein kinase[80]. The 24 h long pretreatment of PDLSCs under 2% O_2 increased osteogenesis, whereas cotreatment with tumor necrosis factor (TNF)-α and interleukin (IL)-β significantly reduced this effect, and no significant effects on adipogenic differentiation were observed[64]. Importantly, the transplants containing hypoxic pretreated-PDLSCs led to significantly stronger bone regeneration when subcutaneously placed into the dorsal region of male nude mice as well as more mineralized tissue in a periodontal defect model[64]. Similarly, PDLSCs grown at 2% O_2 differentiated into osteoblast-like cells that formed bone-like structures after transplantation into the backs of mice[66]. Cultivation of these cells at 3% O_2 inhibited their differentiation capacity to osteogenic and adipogenic lineages, whereas it enhanced chondrogenic differentiation[72]. However, the authors indicated that subsequent switch of 2 wk hypoxic preconditioned PDLSCs to normoxia allowed successful differentiation into osteogenic and adipogenic lineages. In contrast, Xu *et al*[82] found that 3% O_2 increased osteogenic markers expression *in vitro* and augmented runt-related transcription factor (RUNX) 2 protein expression *ex vivo* and *in situ via* HIF-1α-induced vascular endothelial growth factor, suggesting a positive role for HIF-1α in the early stage of osteogenesis of PDLSCs. Reduced O_2 tension besides osteogenic, increased cementogenic differentiation capability of PDLSCs[67,74], probably *via* the Wnt/b-catenin signaling pathway[74]. It was found that HIF-1 activity is required to stimulate the differentiation response *in vitro* and, more importantly, cementum protein 1 expression in mouse PDL spaces *in vivo*[67].

Although no difference in adipogenic and osteogenic differentiation potential of SHEDs was detected at 2.3% O_2 in comparison to normoxia[68], significantly higher osteogenesis was documented at 1% O_2 compared to control in SHEDs incorporated into plastically compressed collagen hydrogels[69]. Moreover, after implantation in immunodeficient mice, these hypoxia-primed SHED constructs mediated faster intramembranous bone formation into critical size calvaria defects. Hypoxia significantly increased the osteogenic[70,83], neural, and angiogenic marker expression in SCAPs and suppressed their adipogenic differentiation[70].

When establishing hypoxic conditions in the laboratory, researchers sometimes encounter lots of technical difficulties (*e.g.*, media changes), especially in long-term cultures, and therefore the use of chemical mimetic to induce hypoxic response has been an attractive alternative. Cobalt chloride ($CoCl_2$) stabilizes HIF-1α and HIF-2α under normoxic conditions, and it is the most investigated hypoxia-mimetic agent[84]. Supplementation of DPSCs with $CoCl_2$ had no effect on cell proliferation and reduced their chondrogenic[85] and osteogenic differentiation[86-88]. Interestingly, it was observed that enamel matrix proteins[87] or apigenin, an HIF-1a inhibitor[86], could reverse the effect of $CoCl_2$ on osteogenic differentiation. Zheng *et al*[89] demonstrated that osteogenic differentiation of PDLSCs was activated by short-term exposure to $CoCl_2$ but was inhibited following prolonged exposure, which might be mediated by circular RNA circCDK8. $CoCl_2$ had the same osteo-reducing effect on SHEDs given the significantly decreased ALP activity, calcium deposition, and osteogenic marker messenger RNA expression[90]. It was confirmed that deferoxamine[91] and dimethyloxalylglycine[92] promoted HIF1α expression in PDLSCs, and it was demonstrated that while proliferation was inhibited by deferoxamine, osteogenic differentiation was significantly promoted by both agents. The Wnt signaling pathways might be involved in dimethyloxalylglycine-induced differentiation of cells[92].

These findings provide important insights into capacity of dental MSCs to adapt to physiological conditions of low oxygenation *in vitro* by changing their regenerative properties.

MODULATION OF MSC REGENERATIVE PROPERTIES IN THE INFLAMMATORY MICROENVIRONMENT

In the human population, dental chronic inflammatory diseases are very common. For example, caries that cause progressive destruction of dental hard tissue[93] are one of the most frequent conditions in the oral cavity[94], particularly in childhood[95], while periodontal diseases—oral infections of tooth supportive tissue (gingivitis and periodontitis)—affect 20%-50% of the world population[96]. If left untreated, these conditions can cause significant damage to the oral cavity and consequently cause major problems in the processes of chewing, swallowing, digestion, and speech and create aesthetic problems[93]. Therefore, to consider the possibilities for regeneration and recovery of damaged oral tissue, it is necessary to perceive the regenerative potential of dental MSCs in the context of the inflammatory microenvironment.

Viewed from the perspective of physiological or pathological conditions, endogenous, resident MSCs respond to factors present in the immediate vicinity[57]. Thus, inflammation caused by caries or periodontal disease may significantly affect regenerative capacity of dental MSCs, including their proliferation, migration, colony forming capacity, and differentiation. However, data related to the properties of MSCs isolated form inflamed dental tissues are not consistent (Table 2). Namely, recent results of Inostroza *et al*[97] showed no significant differences between immunophenotype, trilineage differentiation, colony-formation, and proliferation of the DPSCs derived from healthy and inflamed pulp, although immunomodulatory functions of DPSCs from inflamed pulp were altered—showing decreased capacity to suppress CD3 T cell proliferation. Absence of CD34 and CD45 markers, along with high expression of MSC-associated markers between DPSCs extracted from normal and diseased pulp, was also observed in Park *et al*[98]. Besides, in comparison to control cells, DPSCs from inflamed tissue manifested higher osteogenesis (stronger mineralization and expression of osteogenic markers *OCN* and *RUNX2*) but lower neurogenesis (decreased expression of neurogenic markers microtubule-associated protein 2, neuronal nuclear protein, and glutamate decarboxylase 6), along with higher level of IL-6 expression. This study also shows the significance of IL-6, as a strong inflammatory factor, to modulate DPSCs function. Namely, the IL-6 treatment of DPSCs derived from healthy tissue stimulated their osteogenic differentiation and reduced neurogenic differentiation, while IL-6 blocking in DPSCs of inflamed tissue

Table 2 Effects of inflammatory microenvironment on regenerative potential of human dental mesenchymal stem cells

Inflammation level	Outcome	Ref.
DPSCs		
Irreversible pulpitis	No change in proliferation; no change in differentiation; not being able to supress CD3 proliferation; IDO activity↓	Inostroza *et al*[97], 2020
Decayed and pain affected tissue	No change in immunophenotype; proliferation↓; Ca deposition↑, OCN and RunX2 protein expression↑; MAP2 and NeuN protein expression↓; IL-6 expression and IL-6-induced osteogenesis↑	Park *et al*[98], 2019
Irreversible pulpitis	Population doubling time↓; STRO□1, CD90, CD105 and CD146 levels↑; Ca deposition↓; OCN and RUNX2 mRNA expression↓; LPL and PPARγ2 mRNA expression↓; *in vivo*: Retaining their stem cell potency	Alongi *et al*[99], 2010
Severe periodontal disease	Proliferation↑; Stro-1+, CD146, SSEA-4 levels↑; Ca deposition↑, OCN, RUNX2 and mRNA expression↑ (also upon stimulation with proinflammatory cytokines IL-1β and TNF-α)	Tomasello *et al*[101], 2017
GMSCs		
Severe periodontal disease	Proliferation↑; Stro-1+, CD146, SSEA-4 levels↑; Ca deposition↑, OCN, RUNX2 and mRNA expression↑ (also upon stimulation with proinflammatory cytokines IL-1β and TNF-α)	Tomasello *et al*[101], 2017
Dental plaque-induced gingival hyperplasia	Proliferation↑; Runx2 and OCN mRNA expression↓; ALP activity↓; PPARγ mRNA expression↓	Li *et al*[102], 2013
PDLSCs		
Chronic peridontitis	No change in Runx2, OCN and ALP mRNA expression; no change in PPARγ and aP2 mRNA expression; no change in proliferation; *in vivo*: Smaller newly formed cementum	Park *et al*[105], 2011
Chronic periodontitis	Proliferation↑; migration↑; Ca deposition↓, Runx2, ALP and OCN mRNA expression↓; no change in lipid droplets level and PPARγ mRNA expression	Tang *et al*[106], 2016
Periodontitis with alveolar bone loss	Proliferation↑; RUNX2 mRNA expression↓; PPARγ mRNA expression↓	Liu *et al*[107], 2011
Chronic periodontitis	viability and proliferation↓	Soheilifar *et al*[108], 2016

↑ and ↓ represent increasing or decreasing effect, respectively. ALP: Alkaline phosphatase; aP2: Adipocyte protein 2; DPSCs: Dental pulp stem cells; GMSCs: Gingival mesenchymal stem cells; IDO: Indoleamine 2,3-dioxygenase; NeuN: Neuronal nuclei; OCN: Osteocalcin; PDLSCs: Periodontal ligament stem cells; PPARγ2: Peroxisome proliferator-activated receptor gamma; RunX2: Runt-related transcription factor 2.

annulled their osteogenic/neurogenic capacity[98].

Contrary to these studies, compared to the control cells, DPSCs isolated from inflamed pulp expressed higher levels of MSC markers, while their capacity to proliferate was increased with reduced osteo/dentinogenic differentiation potential *in vitro*. Still, as well as DPSCs from healthy pulp, DPSCs of inflamed tissue retained their capacity to form pulp/dentin complexes after transplantation into immunocompromised mice, *i.e.* their regenerative capacity was preserved[99]. In the context of optimal dental MSC isolation, Tsai *et al*[100] demonstrated that dental diseases represent significant factors that affect MSC isolation and quality, given that successful MSCs yield was less pronounced from carious deciduous tooth or tooth with pulpitis. As for dental MSCs isolated from inflamed tooth supportive tissue, Tomasello *et al*[101] showed that DPSCs and GMSCs derived from the tissue affected by periodontitis proliferate faster and possess more pronounced mineralization. Also, no changes in negative MSC marker expression or in high expression of a positive MSC marker (CD29) were detected, while expression of other positive MSCs markers, such as Stro-1, CD146, and stage-specific embryonic antigen-4, was higher in DPSCs and GMSCs of inflamed tissues. In another study, increased proliferation, but with reduced osteogenic and adipogenic differentiation, of GMSCs under inflammatory conditions was observed[102]. Also, higher levels of matrix metalloproteinases (MMP)-1, MMP-2, IL-1, IL-6, TNF-α and type COL1A1 were detected in GMSCs collected from inflamed gingival tissues, indicating the potential of inflammatory environment to shift differentiation capacity toward pro-fibrotic phenotype, thus representing possible mechanism of gingival hyperplasia development during inflammation[102].

A recently published article provided a comprehensive review of papers related to the characteristics of GMSCs from healthy and inflamed tissue, altogether suggesting that GMSCs of inflamed tissues could be a reliable source of MSCs when compared to

the healthy gingiva[103]. Inflammatory environment can also alter properties of PDLSCs[104], however findings are not clear since other publications suggest that there are no significant differences between PDLSCs of healthy and diseased periodontal tissue[105]. Enhanced proliferation but reduced differentiation of dental MSCs derived from inflamed tissue has been detected for PDLSCs isolated from diseased tooth supportive tissue[106,107], while the study of Tang *et al*[106] also demonstrated increased migration of PDLSCs originating from inflamed periodontal tissue. Higher migratory potential with preserved osteogenic/cementogenic and adipogenic differentiation was reported in Park *et al*[105], and in an *in vivo* transplantation model, they showed that PDLSCs from inflamed periodontal tissue possess a preserved ability to form new cementum-like tissue and related periodontal fibers. However, similar to the findings of Tsai *et al*[100], related to the yield of MSCs derived from diseased tooth, Soheilifar *et al*[108] reported that viability and proliferation rate of PDLSCs isolated from the periodontitis-affected teeth were significantly lower in comparison to the control PDLSCs.

Having in mind that the regenerative ability of transplanted dental MSCs strongly depend on the donor/recipient microenvironment niche, gaining successful therapeutic effect in diseased microenvironment is the biggest challenge[56]. Further understanding the influence of the diseased tissue microenvironment on MSC regenerative potential would help the establishment of healing procedures.

CLINICAL RELEVANCE OF DENTAL MSCS

Numerous *in vivo* studies on animals and human clinical trials with various types of dental stem cells show a way to encourage novel tissue engineering strategies for therapies of dental diseases[109]. However, a long road is still ahead, as clinical trials are in their early phases. Despite abundance of preclinical studies, only a few clinical trials have been completed and published. One of the main reasons that stalls successful clinical application of dental stem cells resides in the fact that the exact mechanism of their therapeutic properties is not yet clear. The other challenges for clinical use of dental stem cells are strict regulations, high costs of cell processing, and lack of uniformity in approaches for isolation, expansion, and application of these cells. The therapeutic efficacy of transplanted dental MSCs is also compromised by the diseased microenvironment of the recipients[56].

DPSCs, as the first discovered dental tissue-derived stem cells, are also the most studied stem cells for dental tissue reparation[110]. Even though there are many studies in large animal models showing promising results in dental tissue regeneration, especially in dentin and dental pulp regeneration[110], results of clinical studies are less conclusive. The first study to achieve successful reparation of alveolar bone defect in humans was done by d'Aquino *et al*[111]. They used autologous DPSCs from third maxillar molars seeded onto a collagen sponge scaffold to fill the space left after the extraction of an impacted mandibular molar from the same patient (7 patients in total). A contralateral extraction site filled with sponge without cells served as a control (a split-mouth study design). Clinical and radiographic assessment after 3 mo and 1 year revealed optimal vertical repair and complete restoration of periodontal tissue[111]. In a pilot clinical study, Nakashima *et al*[112] demonstrated a safe and efficacious method for complete pulp regeneration in 5 patients with irreversible pulpitis using autologous DPSCs transplanted with granulocyte colony-stimulating factor in atelocollagen into pulpectomized teeth. However, a split-mouth randomized clinical trial on 32 patients, aiming to assess the efficacy of autologous DPSCs delivered in a collagen matrix for post-extraction socket healing, failed to show significant reduction in the socket bone resorption in the treated group compared to control[113]. It is noteworthy that, unlike in the other cited studies, in this trial a proprietary medical device and protocol (Rigenera®) for direct isolation of DPSCs from dental pulp was used, without prior expansion in culture.

DPSCs from deciduous teeth are also frequently used in dental tissue regeneration studies. In a randomized control clinical trial, Xuan *et al*[114] isolated autologous SHEDs from deciduous canine tooth pulp and implanted them in the form of aggregates, into an injured incisor of the patient with pulp necrosis secondary to trauma. Control patients were treated with standard apexification (a procedure that induces tooth root development and closure of the root apex through hard tissue deposition). After 12 mo follow-up, SHED implantation treatment led to regeneration of functional dental pulp with blood vessels and sensory nerves. In another study, Tanikawa *et al*[115] used SHEDs associated with HA-collagen sponge for closing

alveolar defects in patients with unilateral alveolar cleft defects and demonstrated that this therapy leads to bone regeneration with dental eruption and reduced morbidity compared to traditional iliac crest bone grafting and rhBMP-2.

Among other dental stem cells, alveolar bone derived MSCs also represent great promise in regenerative therapy. A pilot clinical trial evaluated treatment of maxillary radicular cysts in 9 patients using autologous alveolar bone-derived MSCs seeded onto a glutaraldehyde-cross-linked autologous serum scaffold and subjected to osteogenic differentiation[116]. It demonstrated a significant promotion of bone growth in all MSC-treated cysts.

In addition to osteal defects and pulp necrosis, periodontal diseases make up a large proportion of dental ailments targeted for regenerative therapy. A retrospective pilot study examined feasibility and safety of reconstructing the periodontal intrabony defects in 16 teeth of 3 patients with implantation of autologous PDLSCs mixed with a HA-based bone-grafting material[117]. After 32-72 mo follow-up period, clinical examination indicated improvement of probing depth, clinical attachment level, and gingival recession. In a randomized controlled study, Ferrarotti *et al*[118] used a biocomplex of autologous dental pulp micrografts (Rigenera®) with collagen sponge to fill intrabony defects of 29 chronic periodontal patients randomly allocated to test (n = 15) and control (n = 14) groups[118]. This treatment significantly improved clinical parameters of periodontal regeneration 1 year after the procedure. A novel approach using cell sheets of cultured autologous PDLSCs to treat periodontitis was assessed in a case series study involving 10 patients with chronic periodontitis[119]. Triple layered PDL-derived cell sheets with PGA mesh were transplanted on the root surface, and β-tricalcium phosphate (β-TCP) granules were used to fill in bony defects. In all 10 cases, clinical as well as radiographic endpoint parameters improved 6 mo after the treatment. On the other hand, a randomized clinical trial on 30 patients, with 41 teeth in total, using autologous PDLSCs in combination with grafting materials to treat periodontal intraosseus defects, revealed radiologically and clinically greater, but not statistically significant, regeneration of alveolar bone in cell-treated group compared to control group[120].

A recent pilot trial evaluated the safety and efficacy of autologous PDLSC transplanted with a commercial xenogeneic (porcine) bone substitute as a matrix for the regeneration of intrabony defects of 19 patients with chronic periodontitis[121]. The study confirmed the safety of the treatment, but the results have not demonstrated a significant additional clinical benefit compared to control, after 12 mo follow-up. In a randomized clinical trial designed to investigate the use of gingival fibroblasts and GMSCs in the treatment of intrabony periodontal defects, a total of 20 patients with periodontitis were evenly assigned into two groups[122]. Experimental group received cultured autologous gingival fibroblasts/GMSCs on β-TCP scaffold, covered by a collagen membrane, and the control group received β-TCP without the cells. After 6 mo, the study showed significant improvement of clinical and radiological parameters in comparison to the control group.

CONCLUSION

Dental tissues represent valuable sources of MSCs for possible use in regenerative therapy, especially of diseases associated with bone defects and dental loss. Given the different structures and compositions of the tissues they reside in, various dental MSCs exhibit diverse biological and functional features. Therefore, in this review, we have summarized the available research evidence concerning the effects of hypoxic and inflammatory microenvironmental factors on dental MSCs differentiation capacity. We can conclude that the existing investigations indicate very important aspects of the recipient microenvironment niche in terms of therapeutic efficacy of transplanted dental MSCs. Moreover, the therapeutic potential of dental MSCs in *in vitro* conditions mimicking native hypoxic and inflammatory microenvironment can lead to significant development of cell-based therapies.

However, some of the data for the same cell type (especially in hypoxic *in vitro* conditions) are conflicting, which is a trend noticed for other MSCs as well[20]. Therefore, it is important to point out that the biology of MSCs is not yet fully known. As MSC populations exhibit functional heterogeneity and a hierarchy in the terms of proliferative and differentiation potential and metabolic properties of the cells composing the tissues[123], they can behave differently even within the same tissue of healthy individuals. Moreover, the cultivation conditions affect their characteristics, and not all studies covered by this review used the same cell isolation methods and/or

the same cell passages. It should be also taken into account that inflammation doesn't solely contribute to these contradictory findings, given the lack of data on specificity of the inflamed tissues in the terms of proinflammatory factors involved. In addition, the reason for inconsistencies in results related to the same O_2 levels can be found in different experimental settings such as the duration of the hypoxic treatment. Taken together, we suggest further research in this area, but with synchronized cell isolation methods, cultivation conditions, and experimental designs among research groups.

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REVIEW

Regulating the fate of stem cells for regenerating the intervertebral disc degeneration

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Abstract

Lower back pain is a leading cause of disability and is one of the reasons for the substantial socioeconomic burden. The etiology of intervertebral disc (IVD) degeneration is complicated, and its mechanism is still not completely understood. Factors such as aging, systemic inflammation, biochemical mediators, toxic environmental factors, physical injuries, and genetic factors are involved in the progression of its pathophysiology. Currently, no therapy for restoring degenerated IVD is available except pain management, reduced physical activities, and surgical intervention. Therefore, it is imperative to establish regenerative medicine-based approaches to heal and repair the injured disc, repopulate the cell types to retain water content, synthesize extracellular matrix, and strengthen the disc to restore normal spine flexion. Cellular therapy has gained attention for IVD management as an alternative therapeutic option. In this review, we present an overview of the anatomical and molecular structure and the surrounding pathophysiology of the IVD. Modern therapeutic approaches, including proteins and growth factors, cellular and gene therapy, and cell fate regulators are reviewed. Similarly, small molecules that modulate the fate of stem cells for their differentiation into chondrocytes and notochordal cell types are highlighted.

Key Words: Stem cell; Intervertebral disc; Degeneration; Inflammation; Cell therapy; Gene modification

Core Tip: In this review, we presented a precise overview of the anatomical and molecular structure and surrounding pathophysiology of the intervertebral disc (IVD).

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Modern therapeutic approaches including proteins and growth factors, cellular and gene therapy, and cell fate regulators are highlighted. In addition, different types of stem cells used for the implantation in IVD are reviewed. Furthermore, small molecules that modulate the fate of stem cells for their differentiation into chondrocytes and notochordal cell types are presented. In conclusion, this review highlights regenerative medicine-based approaches for the regeneration of intervertebral disc degeneration.

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INTRODUCTION

Intervertebral disc (IVD) degeneration is a progressive, inflammation-driven cascade that leads to structural and mechanical failure, strongly associated with lower back pain (LBP), representing a global health burden. The worst aspect(s) of degenerative disc disease (DDD) is/are pain, discomfort, emotional distress, and functional disability, affecting the quality of life and causing socioeconomic burden[1]. Altered cellular microenvironment within the disc, reduced cell viability due to structural failure, and functional inadequacy are the leading causes of the adverse condition in LBP[2,3]. IVD degeneration (IVDD) treatments can only mitigate painful symptoms and improve flexibility and body movements[4].

Around 84% of the population experience an event of LBP sooner or later in their life span; 50% of them are younger age group (18 to 44 years), otherwise adulthood (45 to 64-years), and generate almost 80% of health care expenditure[5]. Even though the correct etiology of LBP remains obscure[6], IVDD results due to the loss of nucleus pulposus (NP) and/or annulus fibrosus (AF), which leads to the reduction in water content, diminished glycosaminoglycans (GAGs), and extracellular matrix (ECM), and collagen II deterioration in the NP region[7]. This remodeling results in reduced IVD height, osteophyte development, facet joint arthritis, and bending of vertebral bodies, which are reflected through magnetic resonance imaging (MRI)[8]. Spine fusion is the only available option, but it greatly reduces the flexion of the body. With the disease advancement, pharmaceutical or otherwise postoperative intervention is needed to reduce symptomatic pain and reserve the flexion of the spine[9]. Despite the innovations in IVD surgery, patients with the progressive disorder cannot receive the benefits of surgical intervention because of the associated morbidities.

Perinatal stem cells and their derivatives can offer an improved therapeutic approach for the treatment of disc degenerated diseases. Mesenchymal stem cells (MSCs) are being utilized to rectify the pathogenesis of DDD[10]. This review presents an overview of IVD biology and how cellular signaling plays a role in IVD homeostasis. We also review the opportunities and challenges for the utilization of cell-based therapy for IVD regeneration.

CELLULAR SIGNAL IN IVD

The development of IVD in embryogenesis relies on the coordinated network of molecular signals arising in the notochord and neural tube plate[11]. Following signaling pathways are involved in the IVD.

Sonic hedgehog

Sonic hedgehog (Shh) signaling plays a vital role in tissue morphogenesis, regulation, presenting information about embryonic patterning, and degree of cell fate differentiation and proliferation[12,13]. Somite stalks evolve in response to Shh and Wnt (wingless-related integration site) dependent regulatory pathways, while a sclerotome tissue generates only under the activating impact of the Shh pathway[14]. A specific attribute of the Shh intracellular signaling cascade works through synergistic

interaction with Noggin-cascade, a direct antagonist of the bone morphogenetic proteins (BMPs) pathway in the induction of sclerotome growth[14,15]. Noggin molecules are primitively expressed by the notochord cells blocking BMP signaling from developing vertebral bodies till the formation of the AF[16,17].

Paired box genes

Paired box (*Pax*) genes encode transcription regulators for proliferation, differentiation, apoptosis, and migration of pluripotent cells during embryogenesis. Expression of Pax genes plays an essential role in subsequent cell differentiation of distinct populations of IVD[18-20]. It is proved that *Pax1* and *Pax9* genes are entirely involved in the IVD formation. When these genes are obliterated, IVD and vertebral bodies do not develop, forming an irregular cartilaginous core[21]. *Pax1* gene expression in all sclerotome tissues is intervened by the activity of Shh and Noggin regulatory pathways in the notochord cells[22,23]. After IVD development, expression of the *Pax1* gene arises exclusively in the tissue of IVD primordium (precursor of the AF) enclosing the notochord. Hence, the *Pax1* gene impacts the notochord advancement by activating cell expansion which turns into the NP.

SRY-box genes

The SRY-box (Sox) family is involved in developing the vertebral column[24,25]. *Sox5*, *Sox6*, and *Sox9* genes are of significant importance for IVD development and growth. Sox5 and Sox6 are present in the cells of the notochord and the sclerotome[26]. In the mice deprived of *Sox5* and *Sox6* genes, the development of the notochordal membrane was weakened. This is associated with the evidence that these genes are key players in genesis IVD and intercellular proteins, including collagen II and aggrecan[26,27]. Lack of notochordal membrane prompts apoptosis of the notochordal cells (NCs) and disrupts the development of IVD segments. In the cells with knockout Sox9, notochord development starts, which is degraded due to the deprivation of the notochordal membrane matrix and inhibits the formation of sclerotome[28].

Transforming growth factor-β genes

Transforming growth factor-β (TGF-β) signaling pathways are effectively involved in advancing IVD and vertebral bodies. TGF-β intercellular signaling cascade stimulates cellular migration, proliferation, differentiation, and IVD matrix synthesis[29]. TGF-β3 is actively synthesized in the perichordal membrane during the condensation stage of embryogenesis and promotes the development of the AF and vertebral bodies. Blockage of the TGF-β2 receptors inhibits the synthesis of type II collagen leading to defective NP, the exterior part of the AF, and inadequate IVD mineralization. TGF-β2 receptors participate in the differentiation of IVD tissue and vertebral bodies, producing spine[30].

IVDD

DDD is a complex, multifactorial process, the etiology of which is not well known. Thus, there are no particular criteria to differentiate the IVDD from the physiological retardation of development, maturation, or adaptive tissue remodeling[31]. IVDD has perhaps been best defined as an "aberrant cell-mediated response to progressive structural failure"[32]. Heredities, ecological causes, mechanical factors, aging, systemic and toxic mediators are identified as risk factors[33]. This mechanism begins with alterations to the cellular IVD microenvironment leading to structural and functional failure[34]. Interestingly, evidence showed that the early disappearance of NC density in NP is crucial for IVD stability and induces impairment in the ECM anabolic/catabolic proportion, resulting in the change of the IVD mechanical properties[25,35]. IVDD is related to expanded ECM disruption[36], abnormal matrix formation[37], cellular apoptosis[38], inflammation[39], and regulation of sensory nerve and blood vessel in-growth into a normal avascular and neural tissue[40].

The onset of the IVDD is believed to be mainly in the NP[41]. The decline of the key essential proteoglycan, aggrecan[42], reduces additional ECM production in the NP, and causes decreased hydration[43], a deficit of IVD height, and general failure to resist compressive burden[44]. Compression pressures are hence dispensed through the NP to the adjacent AF, which leads to altered biomechanical function of AF and structural failure with radial and circumferential tears in the AF[45]. These fissures and tears facilitate the in-growth of nociceptive nerves and blood vessels, resulting in the secretion of inflammatory pain-related mediators, thus leads to radial disc bulges

or herniation of the NP into the contiguous spine, causing LBP[34].

Although the IVDs degenerate with aging and can be asymptomatic, a pathological process of IVDD is followed by pain. It has been revealed that a large number of people with no pain show degenerative disc changes that further complicate the differentiation of typical age-related degeneration from pathological conditions[46]. An increase in catabolic action of matrix-degrading proteases, pro-inflammatory cytokines, and contemporary immune cell infiltration is proposed to define disc degeneration factors[39]. Furthermore, lower disc pH, reduced nutrition, and calcified cartilaginous endplate (CEP) create an unfavorable environment for restoring the disc [47]. Presently, there are symptomatic cures for advanced phases of DDD but no effective disease-modifying therapies[48].

Inflammation in degenerated IVD

Degenerated IVD cells produce higher concentrations of pro-inflammatory mediators, which suggest their role in the pathogenesis of IVD. A variety of cytokines, chemokines, and enzymes have been associated with IVDD, including interleukins (IL), interferons, tumor necrosis factor-alpha (TNF-α), matrix metalloproteinases (MMPs), prostaglandin E2 (PGE2), nitric oxide (NO), and aggrecanase. Among these, TNF-α and cytokines of the IL-1 family have been most widely investigated. Both TNF-α and IL-1β are produced by IVD cells, and they acquire strong association in the pathogenesis of IVDD[49,50]. Degenerated and herniated discs exhibit upregulated expression of both pro-inflammatory chemokines, TNF-α and IL-1β[51]. Both have been found to activate ECM degrading enzymes and reduce ECM constituent synthesis *in vitro*[49,52]. Recent studies showed that both TNF-α and IL-1β molecules induce increased MMP expression, particularly MMP-1, -2, -3, -7, -8, and -13. These MMPs are well recognized for their proteolytic activity towards collagen and proteoglycans (PGs)[53]. Also, IL-1β, as a pro-inflammatory cytokine, upregulates the vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor, and nerve growth factor expressions to stimulate the neovascularization and neoinnervation of IVD that eventually lead to inflammation and discogenic pain[24]. Another study concluded that IL-1β is a master regulator in the disc cells that influence other cytokines and chemokines[54]. IL-1β and TNF-α in NP cells contribute to the secretion of chemoattractant molecules such as C-C motif ligand 5/regulated 5 (CCL5/CCR5), regulated upon activation, normal T cell expressed and presumably secreted (CCL5/RANTES) or chemokine C-X-C motif ligand 6 (CXCL6)[55], and are involved in the migration of MSCs.

Another pro-inflammatory cytokine that has been involved in the pathogenesis of IVDD is IL-6, which is also secreted by NP cells[56]. Indeed, degenerated IVD tissue samples contain a significantly higher expression of IL-6[57]. Notably, numerous genetic variations in cytokine genes have been correlated with IVD degeneration. Traditionally, inflammation has mainly been considered as a primary reaction to infection at the site of tissue injury; however, it is not sure if it is a cause or outcome of IVD degeneration and herniation[58]. During degeneration, increased aggrecan and collagen breakdown occur within the disc tissue with significant changes in IVD cell phenotype and increased levels of inflammatory cytokines[47]. With an advanced degeneration phase, clefts and tears are developed in the AF and NP, which leak into the external environment. This allows immune cell activation and the invading blood vessels to pervade the IVD through the clefts and tears of the AF[59].

THERAPEUTICS FOR DEGENERATIVE INTERVERTEBRAL DISCS

Modern treatments for IVDD remain a subject of debate. Despite the known consequences of the IVD pathological cascade, the treatment options for IVDD are limited. The traditional conservative therapy for chronic LBP involves a wide range of treatment modalities, including bed rest, physiotherapy, analgesic and anti-inflammatory medications, acupuncture, and chiropractic[60]. Approximately, 75%-90% of chronic LBP patients obtain satisfactory results with conservative treatment[34,61]. The pain symptoms can be overcome by administering anti-inflammatory mediators, for example, opioids, steroids, non-steroidal anti-inflammatory drugs, and muscle relaxants[39]. These anti-inflammatory drugs have effective short-term alleviation for back pain, but they cannot reverse the progression of IVDD[62]. If conservative management does not have the desired effect, the constant pain sensation progresses because of the nerve compression[63].

Interventional procedures for IVDD include spinal surgical interventions, such as discectomy, spine fusion, and total disc replacement to manage the degenerated disc. The main surgical treatment alternatives for IVDD are spinal fusion and the replacement of the whole disc. Spinal fusion surgery, fusing two vertebrae, provides stability to the spine, which can be attained by a range of surgical interventions, such as posterolateral fusion, anterior and posterior lumbar interbody fusion. The minimally invasive methods to the lumbar spine for interbody fusion include lateral lumbar interbody fusion[64]. Spinal fusion is considered as a gold standard treatment option for LBP[65]. The results of three randomized controlled trials, which compared spinal fusion with conservative treatment, showed substantial clinical improvement in only a limited number of patients[45].

Moreover, spinal fusion could accelerate the degenerative process in adjacent vertebrae[66], and it mitigates painful symptoms, irrespective of repairing disc structure and mechanics; therefore, its efficacy remains controversial. Disc arthroplasty has the advantage of removing the degenerated IVD and restoring it with a prosthesis that can permit flexibility between the discs[67]. Moreover, disc arthroplasty does not restore the mechanical movement of the native joint[61]. The additional motion-preserving surgical procedure includes posterior dynamic stabilization. These surgical procedures contain the installation of pedicle screws over a motion segment associated with a flexible graft. These devices intend to limit motion over the interspace to control discogenic pain[68]. The disadvantages of the surgical therapies can be extreme invasiveness, the increased possibility of recurrences, and failure of mechanical properties with contiguous segment degeneration. In most cases, some surgical intrusions and conservative treatments have low efficiency with lack of sustainable long-term effects. Instead of targeting the pathophysiology of the degenerative progression, they target the clinical symptoms[69].

Recent surgical treatment options for symptomatic degenerated IVD are still far from optimal outcomes. Hence, there is a substantial necessity for new therapies that focus on relieving painful symptoms and reestablishing IVD structure and mechanical loading capacity by explicitly addressing the underlying biological causes of DDD.

NOVEL THERAPEUTIC APPROACHES

The advancements in research and development have encouraged scientists to search for innovative pharmacological therapies in the regeneration of the IVD that mitigate painful symptoms by restoring and maintaining mechanical function. Depending on the stage of degeneration, different biological treatment options are used that alter the cascaded events at the molecular level. Figure 1 summarizes various therapeutic options for disc degeneration diseases. The three major groups of biological approaches for disc regeneration are divided as follows: (1) In the early stage of IVDD (grade II-III), growth factor injections may be effective; (2) In the intermediate stage of degeneration (grade IV), gene therapy or cell therapy may be required; and (3) In the advanced stage of IVDD (grade V), tissue engineering approaches are needed[70].

Growth factor therapy

The therapeutic use of growth factors enhances the matrix synthesis and delay degeneration by reducing inflammation[71,72]. Growth factors are the peptides or polypeptides that target specific receptors present on the surface of the cell, thereby influencing cell proliferation, differentiation and increasing their ability to synthesize the ECM[73,74]. Specific growth factors that include BMPs and TGF-β family members are used to stimulate osteogenic and chondrogenic differentiation[75,76].

The first successful exogenous administration of TGF-β1 in animal models showed the enhanced synthesis of PGs in the NP. Several *in vitro* and *in vivo* analyses on BMP-2 and -7, TGF-β, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), growth and differentiate factor 5 (GDF-5), and insulin-like growth factor 1 (IGF-1) revealed that they stimulate the synthesis of ECM[77-82].

In chronic conditions of IVDD, cocktails of growth factors may be needed because the growth factors have a short half-life and are unstable that limits their use as direct injection into the IVD. The administration of multiple injections of growth factors could enhance gradual release at target site or gene-based delivery system to obtain the desired effect. Currently, the primary focus is on platelet-rich plasma (PRP) that is used as a possible therapeutic option to promote IVD regeneration[83-86]. Some limitations like the absence of standardization of the dosage, the process of preparation, and identification of mode of action need to be settled[87].

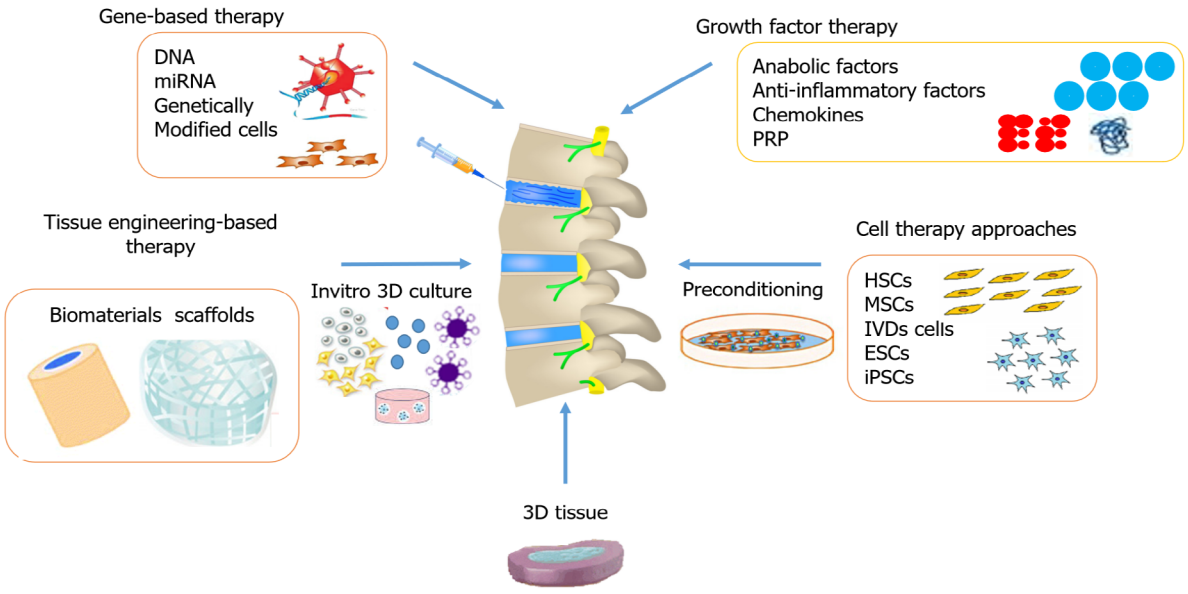

Figure 1 Different approaches used for restoring a degenerated disc. MSCs: Mesenchymal stem cells; ESCs: Embryonic stem cells; iPSCs: Induced pluripotent stem cells; IVD: Intervertebral disc; HSCs: Hematopoietic stem cells; PRP: Platelet-rich plasma.

Gene-based therapy

In the last few decades, gene-based therapy has achieved wide research applications to focus on the regeneration of the IVD structures. The introduction of genes encoding the chondrocyte-specific proteins is directly transferred into the effectual host tissues [88]. The gene-dose impact needs to be characterized for a safe and effective treatment. In contrast, certain findings have revealed inadequate outcomes of direct gene approach into the host cells[89]. Nonetheless, there are limited investigations that support the efficacy of this approach[90-93]. Recently, lentiviruses are believed to be competent vectors for gene transfer because they can deliver a substantial quantity of genetic material into the host cell's genome. The most frequently studied factors are TGF-β3, Sox-9, GDF-5, BMP family including 2, 7, and 12, connective tissue growth factor (CTGF), Wnt, IL-1, tissue inhibitor of metalloproteinases (TIMP-1), and LIM mineralization protein 1 (LMP-1), that are reported to enhance the synthesis of collagen type II and aggrecan in NP cells[94-106]. Genes involved in the development of IVD are summarized in Table 1.

Cell therapy approaches

Regardless of the development of various treatment alternatives, the conservative and surgical therapeutic approaches are not exceptionally valuable for treating degenerated disc disease. These are usually incapable of delivering any solution to reestablish the structural and mechanical function of degenerated IVD. This situation has prompted the advancement of a regenerative medicine-based approach that substitutes the apoptotic and necrotic cells and limits cell death in IVD by targeting different cellular and molecular events[107]. Out of several approved cellular and molecular approaches, the utilization of stem cell therapies has shown superior outcomes, and stem cell transplantation is being used to restore the degenerated IVDs [70]. Stem cells are undifferentiated cells that can differentiate into particular cell types and are broadly utilized as a cell therapy approach. Stem cells exist in a quiescent condition, and they self-renew in the propagation process. Stem cells are being researched *in vitro* and *in vivo* according to the need for the desired effect. Stem cell research has reformed the eventual fate of regenerative medicine because of its capability to recover impaired and damaged organs from treating various debilitating syndromes. The sources of stem cells and their properties are summarized in Table 2. Investigations are being made to comprehend the mechanism of regeneration at the molecular level to address the possible solutions for degenerative diseases and understand the basic pathogenesis and progression of different disorders.

Table 1 Modifying genes essential for the development of intervertebral disc

Ref.	Protein (Gene)	Key findings
Choi *et al*[14], 2012	Sonic Hedgehog (*SHH*)	Sclerotome tissue formation, annulus fibrosus formation, chondrogenesis of sclerotome cells
Wijgerde *et al*[15], 2005	Noggin (*NOG*)	Antagonist of the BMP pathway, promotes Shh intracellular signaling cascade and Pax1 gene activation
Murtaugh *et al*[16], 1999	Bone Morphogenetic Protein (*BMP*) family	In the presence of Shh, promotes chondrocyte differentiation of somite-derived IVD progenitors
Peters *et al*[21], 1999	Paired Box 1 (*PAX1*)	Chondrogenic commitment of sclerotome cells
Sugimoto *et al*[27], 2013	SRY-Box 9 (*SOX*)	Regulates IVD tissue growth and development
Sohn *et al*[30], 2010	Transforming growth factor-β (*TGF-β*)	Development of vertebral bodies
Pearson *et al*[31], 2005	Homeodomain Protein (*HOX*)	Somite Patterning

IVD: Intervertebral disc.

Table 2 Variation in properties of different sources of stem cell types

Properties	MSCs	ESCs	iPSCs
Sources	Perinatal and adult tissues	Embryo at blastocyst stage	Genetically reprogrammed specialized cells
Plasticity	Multipotent	Pluripotent	Pluripotent
Teratoma formation	No	Yes	Yes
Growth	Limited	High	High
Ethical concerns	No	Yes	No
Immune rejection	No	Yes	No
Cell transplantation	Autologous and allogenic	Allogenic	Autologous
Clinical trials in human patients	Ongoing	Limited	*In vitro/in vivo* only
Use in genetic disorder	Deficient (*Carry mutated gene*)	Superior	Deficient (*Carry mutated gene*)
Ease of isolation	Yes	No	No

MSCs: Mesenchymal stem cells; ESCs: Embryonic stem cells; iPSCs: Induced pluripotent stem cells.

STEM CELLS FOR IVD REGENERATION

Stem cells from different sources are involved in the regeneration of disc diseases. A comparison of MSCs and other cell types is presented in Table 3. Different cellular approaches used for the regeneration of IVDs are highlighted in Table 4.

Hematopoietic stem cells

Hematopoietic stem cells (HSCs) possess the capability to differentiate into blood cells. HSCs express CD34 molecules, while non-hematopoietic stem cells, including MSCs, do not show CD34 expression. These cells were injected into the rat IVDD model to investigate which population of cells might acquire disc-identical cells for treating IVDD. It is reported that HSCs can survive in the NP of host IVDs up to 42 d, while non-HSCs were detected up to 21 d only[108]. However, this was nullified by further confirmation that HSCs cannot cure DDD. Although HSCs can only induce blood cells and cannot differentiate into chondrocyte-like cells and repair disintegrated NP, this has begun a novel era of scientific investigation for tissue regeneration. It is demonstrated that HSC transplantation of autologous pelvic bone marrow (BM) cells for the degenerated disc in clinical trials yielded no efficient recovery[109].

MSCs

The therapeutic use of MSCs is based on their two basic characteristics, *i.e.*, they can be

Table 3 Human umbilical cord-derived mesenchymal stem cells compared with other stem cells sources

Properties	Perinatal	Adult	Embryonic
Ability to differentiate into various cell type	√	√	√
Plastic adherence	√	√	
High *in vitro* proliferation ability	√		√
Low risk of tumorigenicity	√	√	
Ethical issues			√
Lower risk of viral contamination	√		√
Capacity for autologous transplantation	√	√	
Established/proven treatment in human patients	√	√	
Ease of collection	√	√	
Less need for stringent antigen typing	√	√	

used to treat different diseases and can be isolated from the autologous source. MSCs are considered as a treatment choice for several diseases like DDD, stroke, myocardial ischemia, diabetes, and neurodegenerative diseases[110-113]. MSCs can be readily isolated due to their adherent property. MSCs possess the excellent capability to differentiate into three mature lineages, namely bone, adipose, and cartilage, as well as into endothelial, myogenic[114-116], epithelial[117], and neural cell types[118] under specific conditions when guided by appropriate growth factors or pharmacological inducers. They possess the remarkable proliferative capability in cell culture with excellent stability in their phenotype and differentiation potential[119].

Furthermore, they can be smoothly transformed with the ability to home at the transplantation site. MSCs are immunologically inactive, which makes them ideal candidates for transplantation[120]. MSCs have great capability to differentiate into chondrocyte-like cells that phenotypically resemble NP cells in chondrogenic induction conditions[121-123]. MSCs promote the regeneration of endogenous tissue by secreting cell survival factors[124].

Tissue-specific stem cells

CEP, AF, and NP-derived stem cells are isolated from the adult IVD, namely cartilage endplate stem cells, AF stem cells, and nucleus pulposus stem cells (NPSCs), respectively. These cells are effective candidates for IVD recovery. Trials with disc stem cells revealed remarkable advantages in homing and retention in the IVD niche, differentiation capability, and functional competency. However, limitations in harvesting, separation, and proliferation of disc stem cells and low potency hinder researchers from using them for therapy[125]. Studies to overcome IVD injury using disc derived stem cells showed their ability to replace affected tissue by producing disc-specific collagen type II and proteoglycan, and restoring disc hydration to physiological state[126,127].

Embryonic stem cells

Embryonic stem cells (ESCs) originate from the inner cell mass of blastula and possess an excellent tendency to differentiate into different cell types. They proved themselves as stable and relatively better source for disc regeneration involving *in vitro* production of NCs. These NCs are the first to form NP during the embryogenesis of the disc. Researchers have successfully differentiated ESCs into chondrocyte-like cells[128]. However, ESCs display tumorigenic properties, can cause teratoma formation, and also pose ethical concerns because of their embryonic origin, which limit their application for IVDD therapy[69].

Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) are derived from genetically reprogrammed somatic cells to an embryonic-like state. The introduction of pluripotency genes and factors in adult terminally differentiated cells is a major discovery of this era. In 2006, mouse iPSCs were first reported by Shinya Yamanaka together with his co-investigators who revealed that fibroblasts might be reprogrammed to an ESC-like cells by four pluripotent gene-induced expressions *i.e.* Sox2, octamer-binding transcription

Table 4 Summary of studies on cellular therapeutic approaches for regenerative potential of the degenerated disc

Type of stem cells	Gene	Preconditioning outcomes	Ref.
In vitro human cultured NP cells and MSCs	TGF-β1	TGF-β1 stimulates collagen-1 expression in cultured NP cells and in MSCs, increased collagen-1 and sox-9 expression. Co-cultured MSCs with NP cells showed high expression of collagen-1, aggrecan and sox-9 expression *via* TGF-β-dependent effect	[126]
Chick periosteum-derived MSCs Rabbit bone marrow-derived MSCs Rat MSCs	TGF-β1	Stimulate chondrogenesis and inhibits osteogenesis. Facilitates *in vitro* chondrogenic differentiation of rabbit BM-MSCs. Increased MAPK activity and upregulation of mRNA expression of sox-9, aggrecan, and collagen type II	[190,122,123]
Human adipose-derived MSCs and bone marrow-derived MSCs	TGF-β3, GDF-5, or GDF-6	In the presence of GDF-6, AD-MSCs leads to differentiation into an NP-like phenotype and results in a richer proteoglycan matrix with low rigidity	[158]
Human bone marrow-derived MSCs	TGF-β1, and GDF-5	Hypoxic TGF-β1 and GDF-5 both increased aggrecan and collagen II mRNA levels and GAGs accumulation	[159]
In vitro human bone marrow-derived MSCs	TGF-β3, dexamethasone, and ascorbate	Preconditioned BM-MSCs expressed higher level of chondrocytes differentiation markers than culture-expanded human IVD cells and articular chondrocytes	[193]
In vivo murine IVD cells	TGF-β3, GDF-5, FGF, or IGF-1	After four weeks of GDF-5 treatment, showed significantly increase in IVD height	[72]
Human adipose-derived MSCs	TGF-β1 and GDF-5	Both distinctly efficient in promoting an NP cell phenotype	[160]
Human cultured NP cells	TGF-β1, and IL-1β	TGF-β1 improved NP cell proliferation, downregulation of mRNA expression of ADAMTS-4 and -5, upregulation expression of TIMP-3. IL-1β inhibited NP cells proliferation, increase of ADAMTS-4 and -5	[161]
Canine cultured NP cells	TGF-β, and IL-10	Suppressed IL1-β and TNF-α expression inhibiting inflammatory reaction	[200]
In vitro human cultured NP cells. E19 rat cultured AF cell	TGF-β1, and IGF-1	Stimulation of human NP cells in a dose and time-dependent manner. TGF-β1 pushed AF cells to fibrocartilaginous phenotype. IGF-1 showed an upregulation of ECM	[79,162]
Murine ESCs	TGF-β, IGF, ascorbic acid, and cis-retinoic acid	All promotes differentiation toward chondrogenic lineage	[175]
Human bone marrow-derived stromal cells	TGF-β1, rhGDF-5, or bovine NPCs	Stimulates cytokeratin-19 and aggrecan/type II collagen ratio distinguish chondrogenic from IVD cell phenotype	[163]
Human bone marrow-derived MSCs	TGF-β3, and dexamethasone	Notochordal cell conditioned medium expressed higher level of NP-like phenotype markers and GAGs deposition than chondrogenic medium or TGF-β groups	[194]
Human cultured NP cells	TGF-β3, and dexamethasone	Enhanced NP proliferation, cell metabolism and reduce catabolism	[195]
Rabbit cultured NP cells	TGF-β1, and BMP-2	Robust restoration of ECM. Increased mRNA expression of aggrecan, type I and type II collagen	[133]
In vitro porcine cultured AF cells	BMP-2, and TGF-β1	Decrease in MMP-1 and increase in aggrecan synthesis	[73]
Mouse MSCs	BMP-2, 7, 13	Proliferate and differentiate into osteoblastic and chondrogenic lineages and no adverse effects on proliferation on undifferentiated MSCs	[164]
Human bone marrow-derived MSCs	BMP-7	Promotes both chondrogenic and osteogenic differentiation of MSCs	[165]
In vitro rat cultured AF cells	BMP-2	Increased mRNA expression of aggrecan and type II collagen. Also, up-regulates BMP-7 and TGFβ-3 mRNA expression	[166]

Mouse embryonic-derived MSCs	BMP-4, Insulin, triiodothyronine, or TGF-β3	All BMP-4, Insulin, and triiodothyronine suppressed adipogenesis and develop osteogenic phenotype. TGFβ-3 promotes chondrogenesis	[128]
In vitro human bone marrow-derived MSC cocultured with human cultured NP cells	BMP2, BMP4, BMP6, and BMP7	BMP4 showed a high potential for IVDs regeneration. Although, BMP2 and BMP7 showed no potent inducer for degenerated human NP cell's regeneration	[167]
Human bone marrow-derived MSCs	BMP-13	Inhibited osteogenic differentiation of human BM-MSCs and increased proteoglycan synthesis	[168]
Human adult MSCs	BMP-3, and TGFβ-1	Enhanced cell proliferation, GAGs content and differentiation into NP-like phenotype. Upregulated smad-3 signaling pathway	[126]
Human adipose tissue-derived MSCs	BMP-2, BMP-6, BMP-7, and TGF-β2	Both TGFβ-2 and BMP-7 induces chondrogenic potential	[76]
Human cultured NP and AF IVD cells	rhBMP-2, rhBMP-12, and adenoviralBMP-12	Both rhBMP-2 and rhBMP-12 increased NP collagen and proteoglycan but least effects on AF. Though, adenoviral BMP-12 increased ECM protein formation in equally NP and AF	[99]
Human and bovine cultured NP cells	BMP-7/OP-1 with BMP-2	Enhanced GAGs production and NP cells proliferation	[77]
Human cultured NP cells	rhBMP-7	Inhibited apoptotic effects, decreased caspase-3 activity and maintained ECM production	[169]
Bovine cultured NP cells	BMP-7, and IGF-1	Both BMP-7 and IGF-1 induces Smad signaling pathways and suppresses noggin expression *via* PI3-kinase/Akt pathways	[170]
Human cultured NP and AF IVD cells	BMP-2	Improved newly synthesized proteoglycan and increased mRNA expression of aggrecan, type I and type II collagen	[171]
In vitro cultured NP cells	IGF-1	Increase of matrix synthesis in well-nourished regions	[180]
In vitro canine cultured IVD cells	IGF-1, FGF, EGF, or TGF-β3	TGF-β3 and EGF both produced higher proliferative responses than FGF. Also, IGF-1 showed a slightly significant responses in NP but no contribution in AF and transition zone	[74]
Horse cultured articular cartilage cells. Bovine cultured NP cells	IGF-1	Maintained differentiated chondrocyte morphology and enhanced synthesis of ECM molecules. Increased proteoglycan synthesis	[178,191]
Bovine cultured AF and NP cells	IGF-1, bFGF, and PDGF	Strengthened cell proliferation	[81]
Human cultured AF cells	IGF-1, and PDGF	Significant reduced in apoptotic cell level	[182]
Chondroitinase ABC injection rabbit model	OP-1	Increase in disk height and matrix synthesis	[172]
Rabbit cultured NP and AF IVD cells	OP-1	Restored collagens and upregulated proteoglycan synthesis	[173]
Human cultured NP and AF cells	OP-1	Improved in the proteoglycan contents, total DNA, and collagen	[174]
Human cultured NP cells	OP-1	Partially repaired GAGs content, depends on a very high doses	[175]
Gene therapy, *in vitro* human IVD cells. Gene therapy, *in vivo* rabbit IVD	TIMP-1	Increased proteoglycan synthesis. Less MRI and histologic evidence of degeneration	[102,103]
In vitro cultured AF cells and chondrocytes	LMP-1	Increased proteoglycan synthesis, upregulation of mRNA expression of aggrecan, collagen types I and II, BMP-2 and -7	[105]
Human synovium derived stem cells	FGF-2, and FGF-10	FGF-2 stimulates chondrogenic gene expression, GAGs deposition and promotes both chondrogenic and osteogenic lineages	[176]
Ovine bone marrow-derived MSCs	FGF-2, and FGF-18	Promotes both chondrogenic and osteogenic lineages of MSCs	[177]
In vitro cultured human NP cells	FGF2	Increased proliferative potential, redifferentiation gene expression and GAGs deposition	[178]
Bone marrow-derived MSCs	bFGF, TGFβ-1 and TCH gel	Greater survival and repair effect on the	[179]

		degenerated IVDs	
In vitro rat cultured NP cells	rGDF-5	Dose-dependency high expression of aggrecan and collagen type II genes was induced by rGDF-5 disc cells from GDF-5-deficient mouse	[82]
In vitro bovine cultured. NP and AF cells, *in vivo* rabbit IVD model	rhGDF-5	Increased DNA and proteoglycan level *in vitro*. *In vivo*, rhGDF-5 injection improved IVD height, MRI and histological grade score	[183]
In vivo mice and rabbit model	GDF-5	Structural and functional maintenance of IVD	[184]
Canine BM peri-adipocyte cells (BM-PACs)	GDF-5, TGFβ-1, BMP-2, and IGF-1	GDF-5 promoted GAGs production and collagen type II without increasing collagen-10 mRNA expression	[199]
Adult bone marrow-derived MSCs	EGF	In the presence of EGF, promotes osteogenic differentiation and enhance paracrine secretion of BM-MSCs both *in vitro* and *in vivo*	[80]
In vivo rat bone marrow-derived MSCs	rhGCSF	Increase of end plates cell proliferation but no contribution in IVD regeneration or maintenance	[185]
Human synovium-derived MSCs	IL-1β, and TNF-α	Enhanced synovial MSCs proliferation and chondrogenic ability	[205,206]
Human bone marrow-derived MSCs. *In vitro* cultured porcine AF cells	IL-1β, and TNF-α	Both IL-1β and TNF-α suppressed chondrogenesis in a dose-selective manner. Increased expression of MMP-1	[73,207]
Gene therapy, *in vitro* cultured NP cells	IL-1 and IL-1Ra	IL-1Ra decreased extracellular matrix degradation	[101]
Mouse bone marrow-derived MSCs	SOX-9	Stimulate chondrogenesis	[95]
Gene therapy, *in vivo* in rabbit IVD	SOX-9	Chondrocyte phenotype of IVD, restored architecture of NP	[96]
Gene therapy, *in vitro* bovine AF cells	Sox-9, and BMP	Increased proteoglycan and/or collagen type II synthesis	[97]
Gene therapy, *in vitro* human NP cells	WNT-3A, WNT-5A, and WNT-11	Increased expression of redifferentiation NP genes and GAGs accumulation	[100]
Human bone marrow-derived MSCs	WNT-3A and FGF2	Synergistically both promoted MSC proliferation, chondrogenesis and cartilage formation	[186]
VEGFR-1 and VEGFR-2 lacZ/+ NP cells	VEGF	Raise NP survival	[208]
Rhesus monkey cultured NP cells	CTGF	Stimulation of collagen type II and proteoglycan synthesis	[187]
Human cultured NP cells	PRP	Enhanced NP proliferation and differentiation into chondrogenic lineage	[134]
Porcine cultured NP and AF cells; Porcine IVDD organ	PRP	Stimulation of IVDD cells proliferation. Increased mRNA expression levels of chondrogenesis and matrix formation	[83,84]
Bovine cultured AF cells	PRP	Upregulation of cell numbers and matrix synthesis	[85]
In vitro porcine cultured AF cells	PRP and other cytokines	Decreased enzymes expression causing degradation and increased matrix proteins synthesis	[86]

IVD: Intervertebral disc; BMP: Bone morphogenetic protein; EGF: Epidermal growth factor; FGF: Fibroblast growth factor; IGF-1: Insulin-like growth factor-1; OP-1: Osteogenic protein-1; PDGF: Platelet-derived growth factor; TGF-β1: Transforming growth factor-β1; ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs; TIMP: Tissue inhibitor of metalloproteinases; TNF-α: Tumor necrosis factor-α; MMP: Matrix metalloproteinase; IL-1β: Interleukin-1 beta; IL-1Ra: IL-1 receptor antagonist; SOX-9: SRY-box transcription factor-9; rhGDF-5: Recombinant human growth and differentiation factor-5; LMP-1: LIM mineralization protein-1; WNTs: Wingless-related integration site; VEGFR: Vascular endothelial growth factor receptor; LacZ: β-galactosidase; CTGF: Connective tissue growth factor; GCSF: Granulocyte colony-stimulating factor; PRP: Platelet-rich plasma; AF: Annulus fibrosus; GAGs: Glycosaminoglycans; NP: Nucleus pulposus; ECM: Extracellular matrix; IVDD: Intervertebral disc degeneration; MSCs: Mesenchymal stem cells; BM: Bone marrow; AD: Adipose tissue; ESCs: Embryonic stem cells; NPCs: Nucleus pulposus cells; MRI: Magnetic resonance imaging; DNA: Deoxyribonucleic acid; mRNA: Messenger ribonucleic acid; TCH: Temperature-responsive chitosan hydrogel; MAPK: Mitogen-activated protein kinase; PI3: Phosphatidylinositol 3; Akt: Protein kinase B.

factor 3/4 (Oct3/4), Kruppel-like factor 4 (Klf4) and Myelocytomatosis (c-myc). These iPSCs were identical to the mouse ESCs because they express pluripotent markers and can differentiate into any cell lineage[119,129]. In subsequent years, they performed

several experiments using human fibroblasts and successfully reprogrammed them to iPSCs by applying the same factors. A different team of researchers attained a similar achievement with minor alterations of Lin-28 and Nanog rather than c-myc and Klf4 [130]. iPSCs possess a great tendency to differentiate into each of the three germ layer cells containing NCs[131,132]. Despite their ability to induce chondrogenesis, iPSCs might be susceptible to tumorigenesis because of their extreme pluripotent nature.

Tissue engineering-based therapy

MSCs face challenges like survival following transplantation, inadequate paracrine secretion, and limitations in cell homing. These hindrances in the effectiveness of MSCs can be overcome by improving their potential of migration, homing, propagation, and differentiation into the preferred cell type. Thus, selecting an appropriate scaffold for stem cells can better serve for the re-development of the lost tissue. Injectable bio-materials or micro and nanoscale scaffolds are preferable for biocompatibility, cell infiltration, and remodeling of the transplanted cells. Upon preconditioning, the fully biocompatible material can also target cell attachment, proliferation, normal morphology, and elevated expression of desired factors. Thus, the strategy has the advantage of inducing differentiation *in vitro* and transplanting cells *in vivo*[133,134].

CURRENT ISSUES RELATED TO TREATING DEGENERATIVE INTERVERTEBRAL DISC

IVD is the largest avascular structure in the human body that has limited efficiency for regeneration. Due to a vascular nature of IVD, tendency to develop strategy for their treatment and regeneration is low[135]. Rehabilitation, surgical interventions, post-trial treatment, and standardized procedures for the subjects should be deemed mandatory. In the case of the local treatment, a small incision should be made[136]. Therefore, surgeries for injecting therapeutic cells should be minimally invasive. In addition, safety concerns such as high intensity of neuropathic pain and secondary infections and genuine diagnosis of complications are significant. One of the critical aspects of designing clinical trials with lower back injuries is the level of injury-induced cases[137]. In selecting subjects with an exclusively specific level of damage, the distance of the injured spinal segment, route of administration, and phenomenal interaction of cell or drug action should be considered[138]. Therefore, long term patient follow-up with standardized measurement scales, such as the American Spinal Injury Association Scale for neurological levels, Normal Rating Scale (pain and spinal cord independence level), Modified Ashworth Scale (for spasticity), and International Association of Neurorestoratology Spinal Cord Injury Functional Rating Scale (for the report of functionality) are essential[139]. Current IVDD animal models are of limited significance as most are different from human disc degeneration[140]. Factual information can be obtained from animal models; however, the limitations are that the studies were generally applied on young rodents with the recently damaged disc in which normal tissue repair mechanisms are still active to heal the degeneration. It is also difficult to quantify the amount of pain. Therefore, researchers use alternate methods to examine disc regeneration or repair success by performing biochemical, molecular, and histological assessments.

Few ethical concerns should be considered while performing pre-clinical studies to translate into clinical trials. Using scientific validity, fair subject selection, favorable distribution of risks-benefits ratio, and informed consent is necessary to make clinical research ethical, which is considered challenging in disc diseases[141]. Typical successful measurements comprise proportions of morphology (*e.g.*, IVDs height, AF delamination, and IVD degeneration grade through MRI and histology), cellularity, ECM quality and quantity, cytokine levels, and biomechanics (*e.g.* pressure/volume testing, compressive strength, and range of motion)[142]. Further, leakage of the delivering cells or drugs is a concern because small escape is possible while injecting. Cell therapy may upregulate the production of some growth factors, which may not be suitable for disc repair, as the cells intrinsically express a high level of growth factors, for example, TGF-β1 and bFGF, that can mediate blood vessel formation, trigger inflammatory mechanism and regulate abnormal disc cell differentiation. Therefore, extensive studies related to the toxicity of biochemical factors in the intervertebral disc are necessary before they are applied in clinical trials. Furthermore, safety with any type of gene therapy is a major consideration. These limitations make direct application of biological approaches difficult to treat disc injuries from animals to

humans[143,144].

ENHANCING THE IVD REGENERATION POTENTIAL BY HUMAN PERINATAL MSCs

The implantation of MSCs is considered a promising therapeutic approach for IVD regeneration. MSCs are primarily found in adipose tissue, dental pulp, BM, and peripheral blood. Recent advances with MSCs have shown that they can be isolated from a variety of postnatal organs such as skin, bone, cartilage, periodontium, pancreatic islets, skeletal muscle, periosteum, and synovial membrane/fluid as well as from perinatal tissues like umbilical cord tissue, umbilical cord blood (UCB), AF, and placenta[107,145,146]. The human perinatal umbilical cord is an optimistic source of MSCs. Like BM stem cells, human umbilical cord-derived MSCs (hUC-MSCs) are the noncontroversial source. The cells have rapid self-renewal properties and possess various advantages, making them promising therapeutic candidates[147]. Some of the advantages are as follows: (1) They are accessible in massive amounts, considering plenty of umbilical cord (UC) with around 135 million births globally every year; (2) They can be effectively collected and manipulated without any adverse effect on the infant or mother; (3) There are no predetermined ethical issues that need to be managed in contrast with ESCs; (4) They show more significant proliferative potential compared to BM-MSCs[148]; (5) They possess minimal immunogenicity[149]; (6) There is minimal possibility of viral contamination[150]; (7) They possess a relatively large harvest size as compared to MSCs from BM[151]; and (8) They need less stringent antigenic typing, and there may be less rejection[152].

Studies have shown that MSC isolation and characterization from Wharton's jelly (WJ) tissue can be easily performed[153,154]. In addition, several current clinical trials explain the utilization of UC matrix-derived MSCs. It is early to relate *in vivo* research of tissue regeneration utilizing MSCs derived from UCB compared to other sources to understand better the capability of hUC-MSCs to regenerate degenerative discs. Clinical trials showed that hUC-MSC transplantation could be a promising substitute for the treatment of prolonged discogenic LBP[155] due to better survival in the avascular niche of the IVD[156] with differently manipulating transplanting cells[157].

DIFFERENTIATION of MSCs TOWARDS CHONDROGENESIS

Stem cells have been treated with small molecules to improve their renewing capability. Numerous proteins and small molecules have been examined in this perspective such as TGF-β[158-163], BMPs[164-171], osteogenic protein (OP)[172-175], bFGF[176-179], IGF[180-182], GDF-5[183,184], granulocyte colony-stimulating factor (GCSF)[185], Wnt[186], CTGF[187], decalpenic acid, β-glycerophosphate, isobutyl methylxanthine, purmorphamine, ascorbic acid, and heparin-binding growth-associated molecule (HB-GAM)[188,189]. TGF-β has been found to lead periosteum-derived stem cells towards chondrogenic lineage and inhibit osteogenic differentiation in extreme density culture[190]. High concentrations of IGF-1 can impose the expression of chondrogenic proteins in BM-derived MSCs[191]. Ascorbic acid, non-organic phosphates, and dexamethasone increase the differentiation potential of BM-derived stem cells towards osteoblasts in CEPs[192-195]. Similarly, pleiotrophin (PTN) has also been reported to differentiate stem cells derived from human BM into chondrocytes[196]. Dexamethasone, insulin, and soluble factors have also been shown to stimulate chondrogenic differentiation of MSCs *in vitro*[197].

Chemical treatment to improve cell survival

Cell survival at the transplantation site is the most critical challenge. Numerous cells die soon after implantation at the site of injury[156]. Direct stimulation of stem cells into specific lineage by using growth factors and small molecules to increase their survival in host tissue is the most practical approach. Investigations showed that the expression of particular cell survival factors could enhance cell feasibility and survival in diseased tissue[198,199]. TGF-β is a growth factor associated with several cellular processes including cell proliferation and differentiation[200]. The rabbit model of IVDD induced through nucleus aspiration and infused with a combination of TGF-β1, fibrin glue, and rabbit MSCs, produced improved results[201]. Similarly, *in vitro* trans-differentiation phenomenon of MSCs into different cell types showed that tra-

nsplanted cells could combine with native cells to give better performance in the damaged tissue[202].

Chemical treatment to improve stem cell homing

For enhanced regeneration, proficient cell homing is essential because the curative impact primarily depends on the effective cell engraftment following transplantation. Various investigators have utilized chemokine/cytokines receptors associated with MSC homing to enhance cell attachment at degenerated tissues[203], including CCR1, 2, 4, 7, 9, and in addition, CXC chemokine receptor-5, -6[204]. CCL5/RANTES has been identified as a chemoattractant secreted by degenerative IVD in organ culture [55]. Moreover, the possibility of different cytokines associated with the pathogenesis of IVD degeneration, specifically TNF-α and IL-1β, play an important role in controlling MSC recruitment to the IVD[101,205-207]. *In vitro* and *in vivo* research studies showed that molecular pre-requisite of MSCs with growth factors like TNF-α and stromal-derived-factor-1 (SDF-1) represent primary signaling cues to elevate VEGF production[208]. MSC conditioned medium improved neuronal survival in several neurological disorders such as neurodegenerative diseases, stroke, and spinal injuries[209]. Moreover, the conditioned medium acquired from articular cartilage stimulated the chondrogenic potential of MSCs and ECM development. The paracrine influence of prominin-1 or CD133+ endothelial progenitor cells from cord blood releases biologically active molecules in the conditioned medium along with microvesicles, which stimulate cell growth and homing. CD133+ cell derivatives with microvesicles possess messenger RNAs for various pro-angiopoietins and anti-apoptotic factors, containing bFGF, receptor tyrosine kinase (c-kit) ligand, IGF-1, VEGF, and IL-8, contributing to withstand harsh microenvironment of the disc[210].

CONCLUSION

In conclusion, this review highlights regenerative medicine-based approaches for the regeneration of IVDD. Numerous potential therapeutic options were identified for the development of cellular therapies. The harsh microenvironment of the degenerative disc poses challenge to the survival of implanted cells. Therefore, possible strategies are needed to enhance the ability of the transplanted cells by preconditioning, chemical modification, genetic manipulation, and augmentation of growth and survival factors to help cells withstand the harsh disc microenvironment. The ultimate goal is to ensure that the transplanted cells survive, integrate and differentiate into desired cell types to regenerate and restore the normal physiological function of the IVD.

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ORIGINAL ARTICLE

Basic Study

Bone marrow mesenchymal stem cell therapy regulates gut microbiota to improve post-stroke neurological function recovery in rats

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Abstract

BACKGROUND

As a cellular mode of therapy, bone marrow mesenchymal stem cells (BMSCs) are used to treat stroke. However, their mechanisms in stroke treatment have not been established. Recent evidence suggests that regulation of dysregulated gut flora after stroke affects stroke outcomes.

AIM

To investigate the effects of BMSCs on gut microbiota after ischemic stroke.

METHODS

A total of 30 Sprague-Dawley rats were randomly divided into three groups, including sham operation control group, transient middle cerebral artery occlusion (MCAO) group, and MCAO with BMSC treatment group. The modified Neurological Severity Score (mNSS), beam walking test, and Morris water maze test were used to evaluate neurological function recovery after BMSC transplantation. Nissl staining was performed to elucidate on the pathology of nerve cells in the hippocampus. Feces from each group of rats were collected and analyzed by 16s rDNA sequencing.

RESULTS

BMSC transplantation significantly reduced mNSS ($P < 0.01$). Rats performed better in the beam walking test in the BMSC group than in the MCAO group ($P <$

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0.01). The Morris water maze test revealed that the BMSC treatment group exhibited a significant improvement in learning and memory. Nissl staining for neuronal damage assessment after stroke showed that in the BMSC group, cells were orderly arranged with significantly reduced necrosis. Moreover, BMSCs regulated microbial structure composition. In rats treated with BMSCs, the abundance of potential short-chain fatty acid producing bacteria and *Lactobacillus* was increased.

CONCLUSION
BMSC transplantation is a potential therapeutic option for ischemic stroke, and it promotes neurological functions by regulating gut microbiota dysbiosis.

Key Words: Ischemic stroke; Bone marrow mesenchymal stem cells; Neurological function; Gut microbiota

Core Tip: Bone marrow mesenchymal stem cell (BMSC) transplantation provides a novel approach for ischemic stroke therapy. Studies on the "gut-brain axis" indicate that gut microbiota dysbiosis affects stroke prognosis. We investigated the interactions between BMSCs and gut microbiota. Our findings indicate that the therapeutic mechanism of BMSCs on ischemic stroke treatment may involve the regulation of microbiome structure and function.

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INTRODUCTION

Globally, stroke is a lethal disability-causing disease that affects up to 13 million people annually[1]. The latest data from the American Heart Association shows that in the United States, one person suffers a stroke after every 40 s[2]. Stroke patients exhibit recurrent attacks, which exerts a huge socio-economic burden on the society and families. Ischemic stroke is the most prevalent stroke type, accounting for 70%-80% of all stroke types[3]. Intravenous thrombolysis and endovascular thrombectomy are the primary treatment options for stroke. However, they are associated with time and technical limitations[4,5]. Therefore, it is important to develop novel therapeutic approaches for ischemic stroke.

Stem cell transplantation is considered a potential therapeutic strategy for patients after ischemic stroke[6]. Bone marrow mesenchymal stem cells (BMSCs) are a group of stem cells with various characteristics, including autologous harvesting, rapid proliferation, easy *in vitro* culture, and low immunogenicity. Moreover, they are not limited by ethical restrictions. BMSCs have the effects of neuroprotection, modulation of inflammation, immune responses, endogenous neurogenesis, and astrogenesis[7]. Specifically, their inflammatory regulatory function has been investigated in various inflammatory diseases.

An estimated 100 trillion microorganisms reside in the human gut. They are closely associated with human health and diseases[8]. The understanding of gut microbiota is only at the rudimentary stage; however, studies have confirmed the existence of bidirectional communication in the microbiota-gut-brain axis, which influences stroke treatment and prognosis[9-11]. After a stroke, the central nervous system (CNS) is injured, then, as a stress response mechanism, the hypothalamic-pituitary-adrenal axis triggers the release of adrenocorticotropic hormone-releasing factor (CRF) and glucocorticoids[12]. Sympathetic and parasympathetic nerves directly affect gastrointestinal functions *via* communication with the enteric nervous system[10]. This induces suppressed gut motility, increased gut permeability, gut microbiota dysbiosis,

and immune cell activation. Studies have documented significant microbial diversity changes in feces of stroke patients[13,14]. Severe stroke destroys the intestinal barrier, therefore, commensal gut microbiota migrates to other organs; this is the primary cause of systemic infections after stroke[15]. A few bacterial species in gut microbiota or their metabolites regulate intestinal immunity, which regulates post-stroke immunity[16]. Animal model experiments have established that changing the gut microbiota improves the prognosis of stroke[17,18]. Despite the documented efficacy of stem cell therapy in altering the populations of gut microbiota in several inflammatory diseases, it has not determined whether it has a similar effect on ischemic stroke.

Therefore, we used a rat model of transient middle cerebral artery occlusion (MCAO) to investigate whether BMSCs can improve abnormal intestinal flora after ischemic stroke.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley (SD) rats, 5-6 week old, weighing 220–250 g, were purchased from Beijing Huafukang Biotechnology Company (Beijing, China). The rats were housed in pathogen-free conditions under a 12 h-light/12 h-dark cycle at 25 °C. The Ethics Committee of Tianjin University of Traditional Chinese Medicine approved this study (approval number: TCM-LAEC2019038).

BMSC isolation, culture, and identification

In this study, 4-wk-old SD rats were cervically dislocated. The femur and tibia were isolated and removed under sterile conditions. The Dulbecco's modified Eagle medium (DMEM) was used for flushing the bone marrow cavity, and the bone marrow flush was collected. The isolated cell suspension was sieved through a 200-mesh nylon sieve and then centrifuged (1000 r/min) for 10 min at 4 °C. The supernatant was discarded, and the cells were re-suspended with DMEM containing 10% fetal bovine serum (FBS; BI). The cell density was adjusted to 2×10^6 cells into 25 cm^2 culture flasks and incubated in a cell incubator (37 °C, 5% CO_2). The cells were passaged every 3-4 d, and the third-passage cells were used for further experiments. BMSCs were incubated with fluorescence antibodies, including CD90-PE, CD29-APC, CD45-PerCP, and CD31-FITC (1:100, Miltenyi, Germany), to identify the phenotype by flow cytometry (FACS Calibur, BD, San Jose, CA, United States).

Experimental design

Rats were randomly divided into three groups (n = 10 each): Sham operation control group (Sham), transient MCAO group, and MCAO with BMSC treatment group. The Sham and MCAO groups were injected with normal saline (PBS), and the BMSCs group was injected with 1×10^6 BMSCs through the tail vein 24 h after reperfusion. Rats were killed after 21 d of reperfusion to collect feces and brain tissue for analysis (Figure 1A).

MCAO

The intraluminal filament model was used to induce transient MCAO as described by Jackman *et al*[19]. Rats were anesthetized with 4% isoflurane and fixed in a supine position, and a longitudinal incision was made 0.3 cm to the right of the midline of their neck. Then, the muscles and tissues were separated to expose the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA). Subsequently, a filament nylon suture was inserted into the right ECA and pushed until the middle cerebral artery (MCA) was obstructed. After 90 min of ischemia, the filament was removed carefully and reperfusion performed. During surgery, the rats were placed on a thermostat system to maintain body temperature.

Neurobehavioral scores

The Longa 5-point scale was used to judge whether MCAO surgery is successful: 4, the animal died; 3, the animal could not walk in a straight line, and its body was tilted to one side; 2, the animal turned to one side during crawling; 1, the animal could not straighten its limbs and was stiff; 0, the animal was normal. If the score was 1-3, the model was considered successful, and the experiment can be carried out later; 0 and 4 were rejected. Animals with a score of 1 to 3 will be grouped for later experiments.

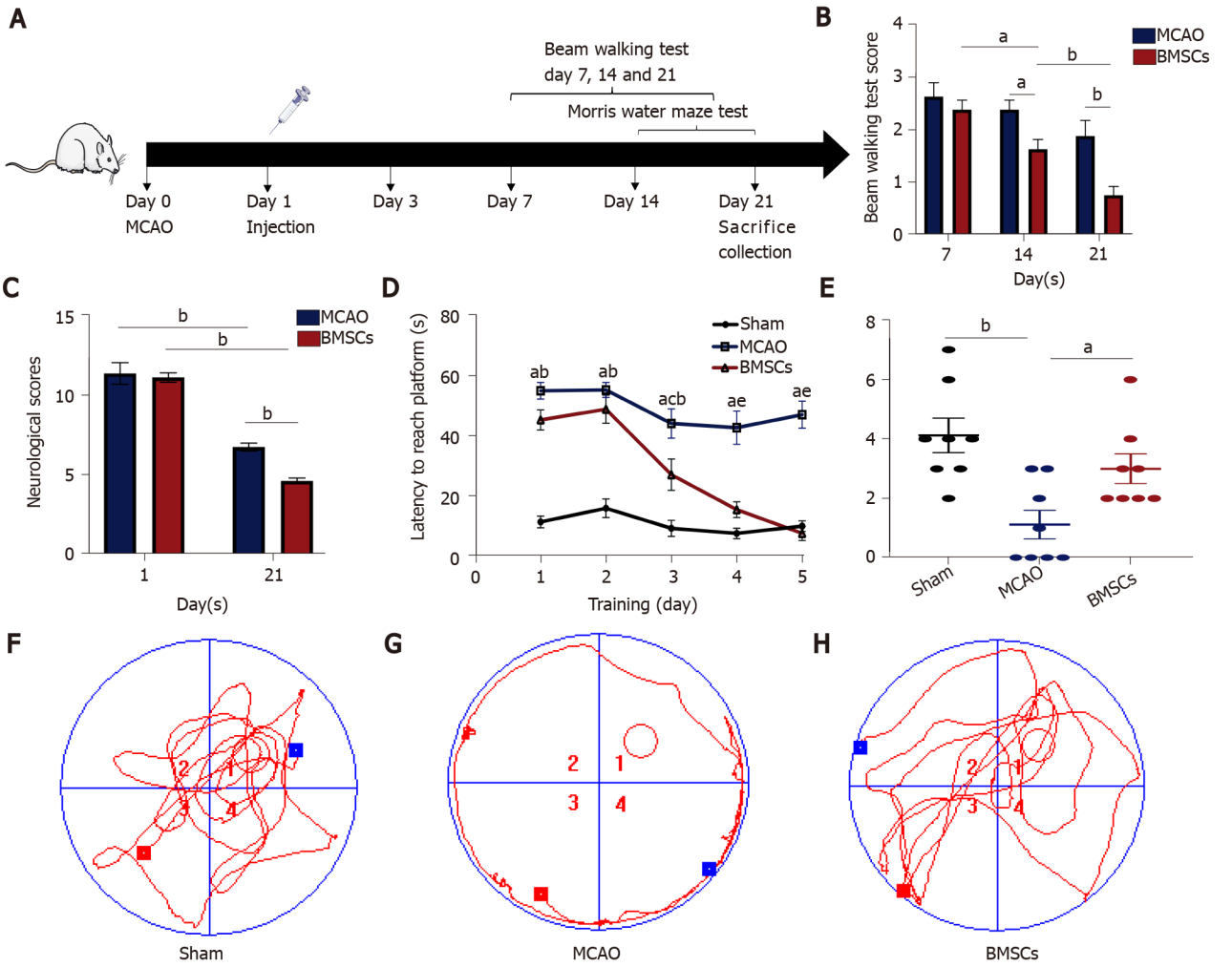

Figure 1 Bone marrow mesenchymal stem cells improve neurological function after stroke. A: Experimental design. Rats (3-5 wk) were randomly divided into three groups: Sham, middle cerebral artery occlusion (MCAO), and bone marrow mesenchymal stem cells (BMSCs). BMSCs or PBS were injected through the tail vein 1 d after MCAO. The modified Neurological Severity Score (mNSS), the beam walking test, and the Morris water maze test were evaluated before rats were killed after 21 d of reperfusion; B: mNSS was performed at days 1 and 21 after MCAO ($^aP < 0.05$; $^bP < 0.01$); C: Beam walking test were performed at days 7, 14, and 21 after MCAO ($^bP < 0.01$); D-H: Morris water maze test. The time that rats needed to escape latency to find the hidden platform (D). $^aP < 0.01$ when Sham *vs* MCAO; $^bP < 0.01$ when Sham *vs* BMSCs; $^cP < 0.01$ when Sham *vs* BMSCs; $^dP < 0.01$ when MCAO *vs* BMSCs; $^eP < 0.01$ when MCAO *vs* BMSCs. The number of rats crossing over the target platform on the sixth day ($^aP < 0.05$; $^bP < 0.01$) (E). The data are expressed as the mean ± SEM ($n = 10$). The tracks of each group on the sixth day (F-H). BMSCs: Bone marrow mesenchymal stem cells; MCAO: Middle cerebral artery occlusion.

The modified Neurological Severity Score (mNSS) was used to score the neurological function of the rats on days 1 and 21 after reperfusion, which included motor, sensory, reflex, and balance tests with a total score of 18[20]; the higher scores mean more severe injuries.

Behavioral analysis

Two blinded investigators observed all behavioral tests at regular times of the day. The apparatus was washed with 70% ethanol after each animal was tested to eliminate olfactory cues.

Beam walking test: For detecting motor coordination and balance, the beam walking test was evaluated at 7, 14, and 21 d after reperfusion. The rats were placed on a balance beam that was 1 m long, 2.5 cm wide, and 20 cm high from the ground. A soft cushion was placed under the balance beam to prevent the mouse from falling. Every mouse was scored according to the following rules: (1) If the rat crossed the balance beam smoothly without the hind limbs slipping; (2) If the rat gripped the edge of the balance beam, but the hind limbs did not dangle; (3) If the rat clutched the balance beam, and one limb dropped from the balance beam; (4) If the rat clutched the balance beam, and two limbs dropped from the balance beam or rotated on the balance beam (> 60 s); (5) If the rat tried to balance on the balance beam but fell (> 40 s); (6) If the rat tried to balance on the balance beam but failed (> 20 s); and (7) If the rats fell and did

not attempt to balance on the beam (< 20 s).

Morris water maze test: The Morris water maze test was performed 14 d after surgery for six consecutive days to test rats' spatial memory ability. The water maze was a circular black pool (Shanghai Xinsoft Information Technology Co., Ltd.), 150 cm in diameter, 50 cm high, and 25 cm deep, with the water temperature maintained at 20 ± 1 °C. The pool was divided into four quadrants (1, 2, 3, and 4), and the circular platform was located in quadrant 1, 2 cm below the water surface. The rats were tested twice daily for 60 s for the first 5 d and were allowed to remain on the platform for 10 s after each test. On day 6, a probe trial was performed by removing the platform and allowing the rat to swim freely in the pool for the 60 s. The time and route taken by the rats to complete the task were recorded. Finally, the data were exported and analyzed using Morris water maze analysis software.

Histological analysis of rat brain

The rats were fixed by perfusion in 4% paraformaldehyde (PFA). The brains were quickly removed and fixed in 4% PFA at 4 °C for 24 h. After dehydration, they were embedded in paraffin and serially sectioned into 4 μm tissue sections for histological analysis. Nissl staining was performed to evaluate neuron damage. The histopathology of the hippocampus of brain tissues was observed with a microscope (BX43; Olympus).

Microbiome 16S rDNA sequencing and analysis

The rat feces from each group were collected into 2 mL sterile freezing tubes on day 21 and stored at -80 °C until the bacterial DNA was extracted. Total bacterial DNA was extracted using DNA Extraction Kit (QIAGEN, Germany) following the manufacturer's instructions. To ensure the quality and quantity of DNA, extracted DNA was detected by agarose gel electrophoresis and stored at -20 °C until further processing. The diluted DNA was used as the template for PCR amplification of bacterial 16s rRNA genes with the barcoded primers (V3-V4 regions) and Takara Ex Taq (Takara). The PCR product was purified with AMPure XP beads (Beckman Coulter Genomics, United States) and quantified using a Qubit dsDNA assay kit (Life Technologies, United States). According to the standard protocols, equal amounts of purified amplicon were sequenced using the Illumina Miseq sequencer PE250 (Illumina, United States). The raw data were processed sequentially with the software Trimmomatic (version 0.35), Flash (version 1.2.11), QIIME (version 1.8.0), and UCHIME (version 2.4.2) to get the operational taxonomic units (OTUs). The valid tags were classified at a 97% similarity cutoff to analyze the gut microbiota diversity.

α-diversity is a measure of the abundance and diversity of microbial communities in a sample. In this paper, the Shannon index and Chao index were used to represent α-diversity[21,22]. The Shannon index is an alpha diversity statistic for estimating the index of microbial diversity in a sample. A higher value indicates that the community is more diverse. The Chao index assesses the number of OTUs in a sample. The larger the Chao index, the higher the number of OTUs, indicating that the number of species in the sample is more numerous. The functional pathways of microbial communities for each sample were inferred using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) software[23]. The PICRUSt software predicts the metabolic function of microorganisms by comparing the resulting 16S sequencing data with a genomic reference database of microorganisms with known metabolic functions.

Statistical analysis

The results are expressed as the mean ± SEM. The data were analyzed using one-way analysis of variance (ANOVA) and *t*-test. The difference was considered significant at $P < 0.05$.

RESULTS

BMSCs improve neurological function after ischemic stroke

The mNSS, beam walking test, and Morris water maze test were used to estimate the neurological function after ischemic stroke. The neurological deficit scores of each group of rats were evaluated at 1 and 21 d after ischemia-reperfusion (Figure 1B). Compared with the MCAO group, the BMSCs group had significantly improved

neurological function. The mNSS scores of both the MCAO and BMSCs groups were substantially lower at 21 d than on the first day ($P < 0.01$). However, the BMSCs group had a more significant decrease in mNSS scores at day 21 than the MCAO group ($P < 0.01$). Beam walking test showed that rats subjected to BMSCs transplantation presented a larger motor functional improvement (14 d, $P < 0.05$; 21 d, $P < 0.01$; Figure 1C)

To assess the spatial learning and memory capacity of BMSC-treated rats after stroke, the Morris water maze test was used to detect the escape latency of a random search for the hidden platform during the first 5 d. Compared to the MCAO group, the BMSCs group showed a significantly shorter duration of escaping latency ($P < 0.05$; Figure 1D). After removing the hidden platform at 6 d, rats of the BMSCs group were easier to find the previous location of the platform site compared to those of the MCAO group, which passed over the platform site more times ($P < 0.05$; Figure 1E). The typical swimming tracks of each group (Figure 1F-H) also indicated that rats treated with BMSCs had significantly improved spatial memory.

BMSCs alleviate neuronal loss in the hippocampus after ischemic stroke

Nissl staining demonstrated no significant changes in neurons in the hippocampal CA1 area of the brain in the Sham group on day 21. In the MCAO group, the boundaries of the hippocampal CA1 area were irregular, the number of Nissl bodies was reduced, and a large number of neurons underwent necrosis. Compared with the MCAO group, the rat hippocampal neurons in the group treated with BMSCs were arranged in an orderly manner, and necrotic cells were significantly reduced (Figure 2). These results suggest that stroke causes severe neuronal damage in rats and that BMSC treatment can effectively protect neurons and prevent neuronal loss.

Effect of BMSCs on microbial α-diversity and structure after ischemic stroke

To identify whether treatment with BMSCs influences the gut microbiota after ischemic stroke, we analyzed differences in species complexity and bacterial communities between populations based on OTUS and species annotation results. We obtained a total of 1494295 quality filtered 16s rRNA gene sequences from three groups of 30 samples, with an average of 49810 ± 1281 reads per sample. We compared microbial α-diversity between the Sham, MCAO, and BMSCs groups, and both Shannon and Chao index results showed no statistical difference between the three groups (Figure 3A and B).

We calculated inter-sample distances between the three groups to analyze the differences in community species composition among individual samples within each group. We demonstrate the nonmetric multi-dimensional scaling (nMDS) plot, and the principle co-ordinates analysis (PCoA) plots in Figure 3C and D. Different groups are presented in different colors in the figure, and samples from the same group are clustered together. The nMDS analysis and PCoA showed that MCAO and BMSCs could alter the microbiota composition significantly compared to the Sham group. However, there was no significant difference in microbiota structure between the two groups of MCAO and BMSCs. To further investigate the variability of microbial communities between the two groups, the ANOSIM test was used to test both Bray-Curtis and Unweighted Unifrac algorithms (Bray-Curtis, $r = 0.0769$, $P = 0.042$; Unweighted Unifrac, $r = 0.0679$, $P = 0.0415$, respectively). The results showed significant differences in the microbial communities between the two groups.

BMSCs modulate gut microbiota after ischemic stroke

We next sought to explore the effect of treatment with BMSCs on the composition of the microbial structure. Figure 4A shows the abundance of microorganisms in the three groups, in which Bacteroidetes, Firmicutes, Proteobacteria, and Epsilonbacteraeota were the most significant contributors at the microbial phylum level. Compared with the Sham group, MCAO and BMSC increased the relative abundance of Proteobacteria, suggesting significant differences in the gut microbiota structure after stroke. Furthermore, we analyzed the differences in the relative abundance of microorganisms between the three groups at the level of genus (Figure 4B). The data showed that the relative abundance of *Ruminococcaceae_UCG−005*, *Mycoplasma*, *Ruminiclostridium_5*, *Oceanimonas*, and *Marvinbryantia* was significantly decreased, and the relative abundance of *Escherichia−Shigella*, *Alloprevotella*, *Butyricimonas*, *ASF356*, and *Enterococcus* was increased in the MCAO group compared with the Sham group. BMSC treatment increased the relative abundance of *Ruminiclostridium_5* and decreased *Butyricimonas* and *ASF356* at the species level. The dominant bacteria of MCAO and BMSCs are shown separately at the species level in Figure 4C and D. We concluded that species enriched in the BH group included *Clostridium* spp and *Lachno-*

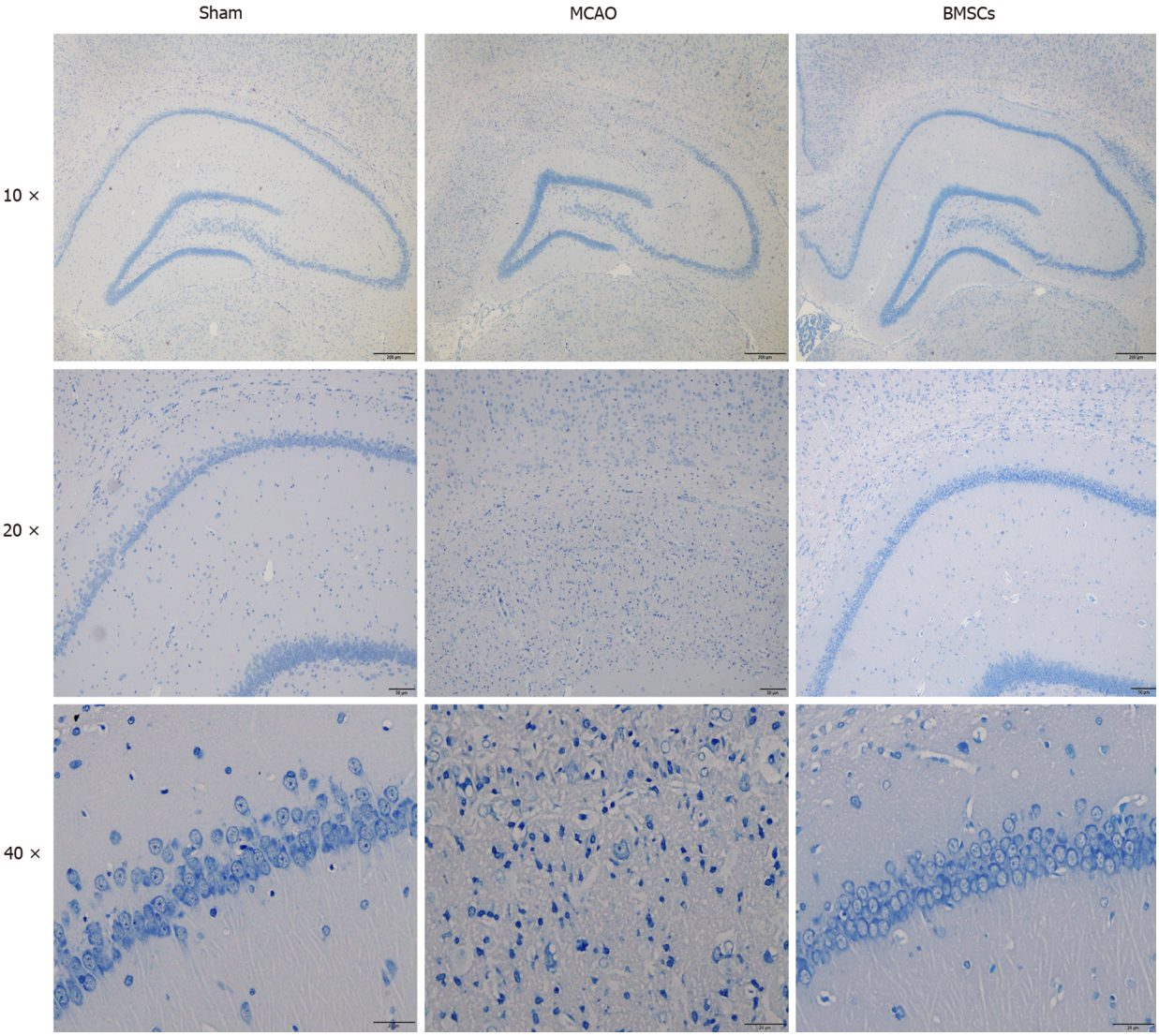

Figure 2 Histopathological changes in brain tissue of rats. Nissl staining in the hippocampal CA1 region for the Sham, middle cerebral artery occlusion, and bone marrow mesenchymal stem cells group exhibited brain injury after 21 d post-stroke (n = 3). Pathological observation of the hippocampus (magnification, × 40). CA1 region of the hippocampus (magnification, × 100). The morphologies of neurons in the hippocampal CA1 region (magnification, × 400). BMSCs: Bone marrow mesenchymal stem cells; MCAO: Middle cerebral artery occlusion.

spiraceae spp, which are the potential species to produce short-chain fatty acid (SCFA). A comparison of potential SCFA producing bacteria in the feces revealed that depletion occurred in the MCAO group (Figure 4E). Additionally, it was observed that the relative abundance of *Lactobacillus* was significantly increased at the genus level after BMSC treatment (Figure 4F).

Predictive analysis of gut microbiota function

PICRUSt functional prediction analysis was based on 16S sequencing data annotated in the Greengenes database. Using PICRUSt software can predict the composition of known microbial gene functions and thus statistically different functions between groups. In this study, the Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to assess microbial function, and 25 differentially KEGG functional pathways were identified between MCAO and BMSCs (Figure 5). The gut microbiota of BMSCs influenced the pathways of metabolism, including "Carbohydrate Metabolism", "Biosynthesis of Other Secondary Metabolites", "Glycan Biosynthesis and Metabolism", "Lipid Metabolism", "Metabolism of Cofactors and Vitamins", "Metabolism of Other Amino Acids", and "Xenobiotics Biodegradation and Metabolism". We also found that BMSCs-enriched function pathways were associated with "Membrane Transport", "Signaling Molecules and Interaction", "Transport and Catabolism", and "Transcription".

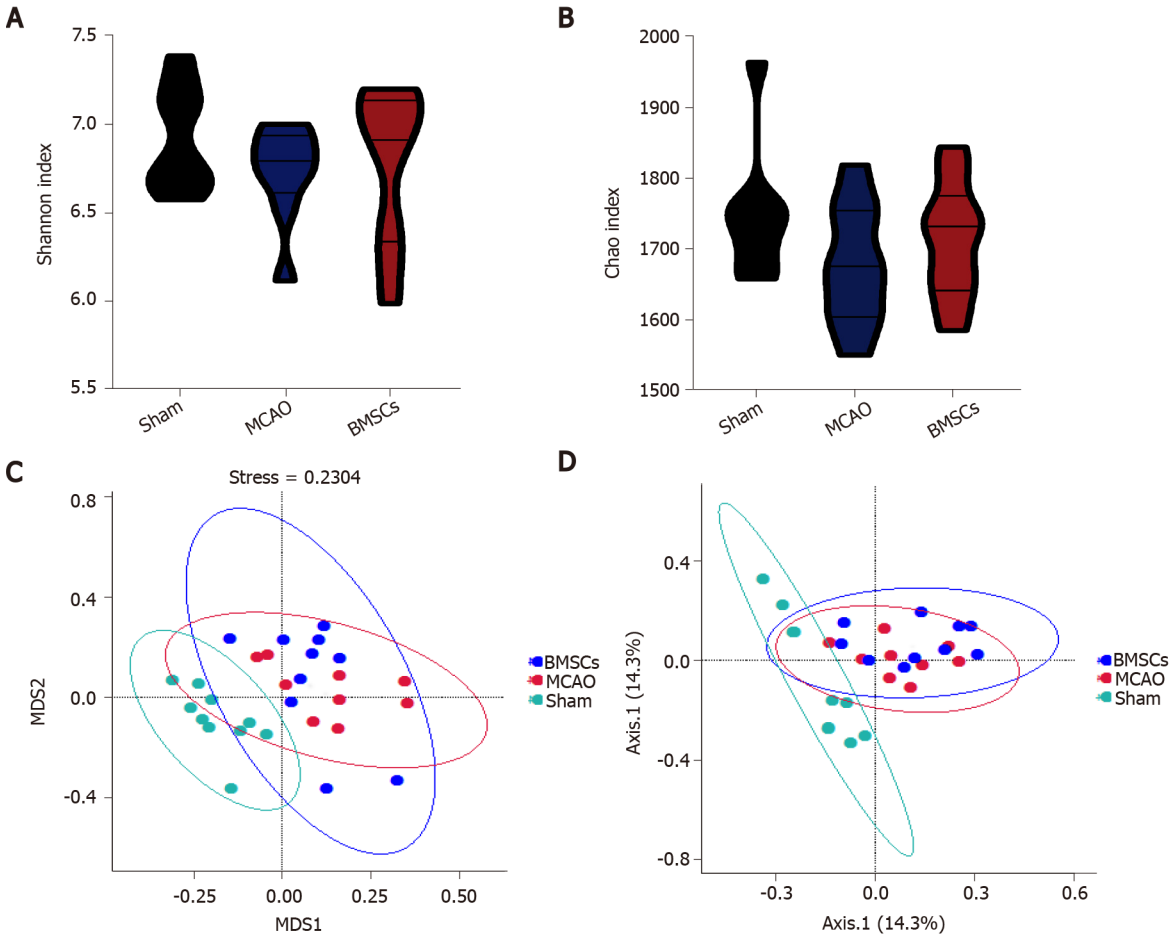

Figure 3 Abundance and structures analysis of intestinal microecology. A and B: Shannon and Chao index to present α-diversity of gut microbiota; C and D: mNDS and PCoA plot to illustrate the dissimilarities among microbiota structures. BMSCs: Bone marrow mesenchymal stem cells; MCAO: Middle cerebral artery occlusion.

DISCUSSION

For the first time, this study showed changes in gut microbiota after ischemic stroke treatment using BMSCs. BMSCs disrupted the composition and structure of gut microbiota, thereby affecting metabolic pathways in ischemic stroke.

Evidence from basic and clinical studies show that BMSCs can effectively treat patients with ischemic stroke[24]. Transplantation of BMSCs significantly enhances neurological functions after stroke[25], consistent with our results. We established that treatment with BMSCs significantly reduced mNSS scores and enhanced balance, coordination abilities, and learning memory in rats. Notably, cerebral ischemia caused neuronal damage in the hippocampus, striatum, thalamus, and cerebral and cerebellar cortices, with the CA1 region of the hippocampus being one of the most sensitive brain regions. Nissl staining revealed serious neuronal damage in rats after ischemic stroke, which explains memory impairment in the Morris water maze test. In contrast, BMSCs effectively protected the nerve cells.

Studies have confirmed complex interactions between gut microbiota and stroke. Xia *et al*[26] reported that *Parabacteroides*, *Oscillospira*, and *Enterobacteriaceae* among others were enriched in stroke patients, whereas *Prevotella*, *Roseburia*, and *Fecalibacterium* were enriched in healthy individuals[26]. In stroke patients, dysbiosis is closely associated with metabolism and inflammation. Besides, a specific genus of gut microbiota and associated metabolites are used as potential indicators for stroke prediction and prognosis[13,27]. In stroke animal models, similar alterations in gut microbiota have been detected. Singh *et al*[9] found that the most abundant phyla of *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* overgrew in MCAO mice[9]. Chen *et al*[28] reported that after stroke, rats exhibited an increase in the abundance of opportunistic pathogens, including *Alistipes*, *Bacteroides*, *Klebsiella*, *Shuttleworthia*, *Haemophilus*, *Fusobacterium*, *Faecalibacterium*, *Proteus*, and *Papillibacter*[28]. After transplantation of BMSCs, we analyzed the changes in gut microbiota to investigate the role of gut

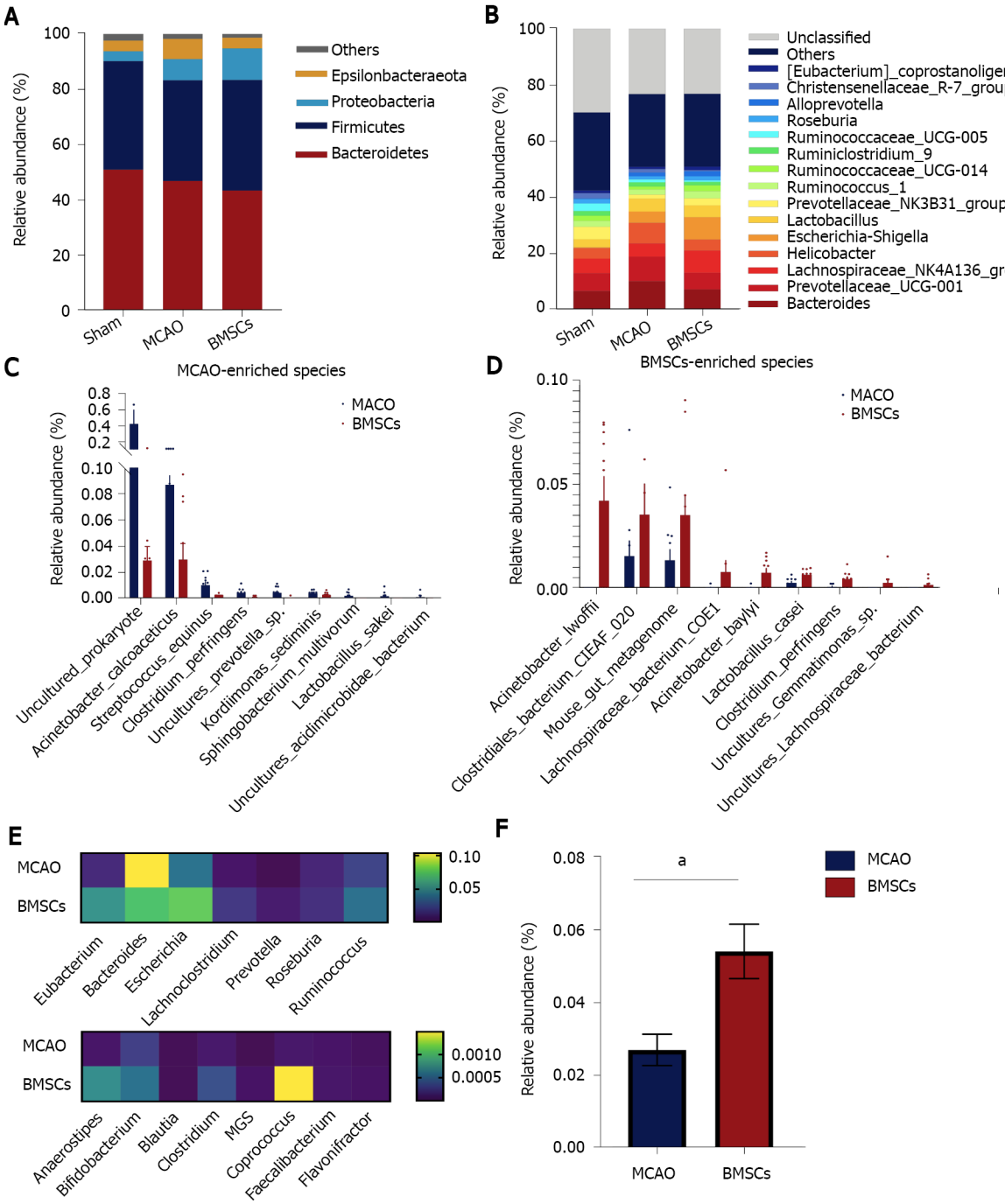

Figure 4 Bone marrow mesenchymal stem cells modulate the composition of gut microbiota. A: Taxonomic composition at the phylum level; B: Taxonomic composition at the genus level; C and D: Significantly different abundances at the species level between middle cerebral artery occlusion (MCAO) and bone marrow mesenchymal stem cells groups; E: Comparison of the abundance of potential short-chain fatty acid-producing species in the MCAO and BH groups; F: Relative abundances of *Lactobacillus* at the genus level between the MCAO and BH groups ($^aP < 0.05$). The data are expressed as the mean ± SEM ($n = 10$). BMSCs: Bone marrow mesenchymal stem cells; MCAO: Middle cerebral artery occlusion.

microbiota in post-stroke rats. We found that BMSCs did not alter the α-diversity and structure of gut microbiota after stroke. Further assessments of the composition of microbiota structure suggested that BMSCs significantly increased the abundance of potential SCFA-producing bacteria.

Lachnospiraceae and *Clostridium* are the main groups of SCFA-producing bacteria [29]. For mammals, SCFA is a critical gut microbial metabolite. It can be used as a substrate for the metabolism of cholesterol, glucose, and lipids, which provide nearly 10% of daily caloric requirements[30]. Besides, it achieves its anti-inflammatory effects by activating G protein-coupled receptors (GPCR) to regulate T cells[31]. Additionally, SCFA protects and repairs the intestinal mucosal barrier by secreting mucus and stimulating tight junction protein expression[32].

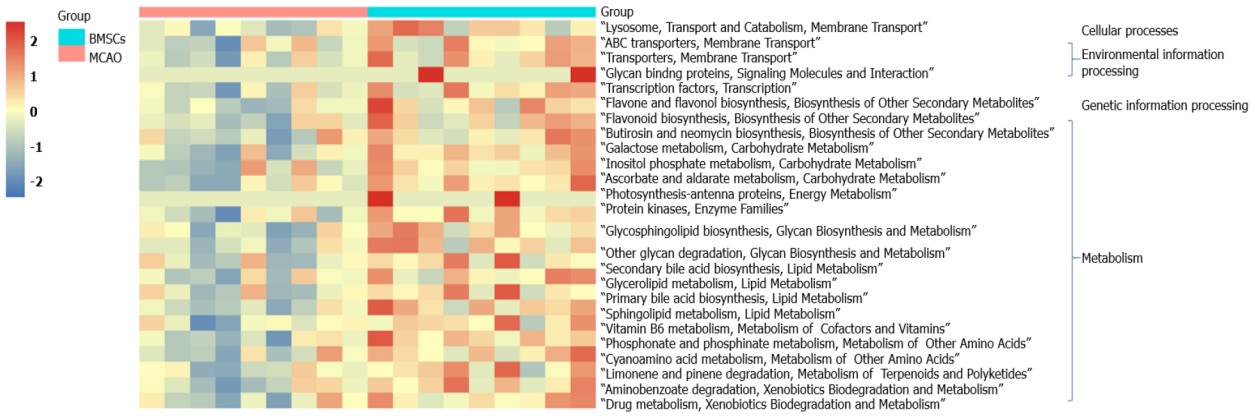

Figure 5 Alterations of microbial function. Heatmap illustrates the difference of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (KEGG level 3) between middle cerebral artery occlusion and bone marrow mesenchymal stem cells groups. Welch's t-test was used for statistical analysis, and the pathways is displayed when $P < 0.05$. BMSCs: Bone marrow mesenchymal stem cells; MCAO: Middle cerebral artery occlusion.

The abundance of *Lactobacillus* has been shown to be significantly increased in cerebral infarction patients[33]. Interestingly, we found a significantly high abundance of *Lactobacillus* in the fecal matter of the BMSCs group. Bourriaud *et al*[34] realized that butyrate-producing bacteria ferment lactic acid to produce butyrate, which reduces inflammatory responses, thereby protecting the injured brain[34]. Given that BMSCs increase the abundance of potential SCFA-producing bacteria, an increase in *Lactobacillus* leads to the production of more lactic acid to be fermented to butyrate, thereby improving neuroinflammation during stroke.

CONCLUSION

This is the first study to elucidate on alterations in gut microbiota after BMSC treatment in an ischemic stroke condition. We found that BMSCs potentially improve neurological damage after stroke by regulating gut microbiota. This provides a basis for future research into the role of BMSCs from the perspective of the "gut-brain axis".

ARTICLE HIGHLIGHTS

Research background

Ischemic stroke is a highly lethal and disabling disease that has a severe impact on the quality of life of patients. Gut microbiota is closely related to the treatment and prognosis of stroke. The improvement of neurological function by bone marrow mesenchymal stem cells (BMSCs) may be related to the regulation of gut microbiota.

Research motivation

Many studies have shown that gut microbiota plays an important role in immunity after stroke through the gut-brain axis.

Research objectives

To observe the regulation of gut microbiota after BMSC treatment.

Research methods

Rats were divided into three groups [Sham, middle cerebral artery occlusion (MCAO), and BMSCs]. Recovery of neurological function in rats after BMSC transplantation was observed by the modified Neurological Severity Scores (mNSS), beam walking test, and Morris water maze test. Pathological observation of hippocampal neuronal cells was conducted by Nissl staining. 16S rDNA sequencing was used to analyze the composition of gut microbiota.

Research results

Transplantation of BMSCs significantly reduced mNSS scores ($P < 0.01$), and improved balance and coordination ($P < 0.01$), learning, and memory in rats. The structure of the C1 region of the hippocampus was clear and necrotic cells were significantly reduced after the intervention of BMSCs. Compared with the MCAO group, BMSCs effectively increased the relative abundance of short-chain fatty acid-producing bacteria and *Lactobacillus* in feces.

Research conclusions

Transplantation of BMSCs can regulate gut microbiota, which provides a potential therapeutic mechanism for stroke treatment.

Research perspectives

We demonstrated the modulatory effect of BMSCs on the gut microbiota after stroke, which provided an experimental basis for elucidating the gut-brain axis.

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ORIGINAL ARTICLE

Basic Study

SmartFlare™ is a reliable method for assessing mRNA expression in single neural stem cells

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Abstract

BACKGROUND

One of the most challenging tasks of modern biology concerns the real-time tracking and quantification of mRNA expression in living cells. On this matter, a novel platform called SmartFlare™ has taken advantage of fluorophore-linked nanoconstructs for targeting RNA transcripts. Although fluorescence emission does not account for the spatial mRNA distribution, NanoFlare technology has grown a range of theranostic applications starting from detecting biomarkers related to diseases, such as cancer, neurodegenerative pathologies or embryonic developmental disorders.

AIM

To investigate the potential of SmartFlare™ in determining time-dependent mRNA expression of prominin 1 (*CD133*) and octamer-binding transcription factor 4 (*OCT4*) in single living cells through differentiation.

METHODS

Brain fragments from the striatum of aborted human fetuses aged 8 wk postconception were processed to obtain neurospheres. For the *in vitro* differentiation, neurospheres were gently dissociated with Accutase solution. Single cells were resuspended in a basic medium enriched with fetal bovine serum, plated on poly-L-lysine-coated glass coverslips, and grown in a lapse of time from 1 to 4 wk. Live cell mRNA detection was performed using SmartFlare™ probes (CD133, Oct4, Actin, and Scramble). All the samples were incubated at 37 °C for 24 h. For nuclear staining, Hoechst 33342 was added. SmartFlare™ CD133- and OCT4-

additional data are available.

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specific fluorescence signal was assessed using a semiquantitative visual approach, taking into account the fluorescence intensity and the number of labeled cells.

RESULTS
In agreement with previous PCR experiments, a unique expression trend was observed for *CD133* and *OCT4* genes until 7 d *in vitro* (DIV). Fluorescence resulted in a mixture of diffuse cytoplasmic and spotted-like pattern, also detectable in the contacting neural branches. From 15 to 30 DIV, only few cells showed a scattered fluorescent pattern, in line with the differentiation progression and coherent with mRNA downregulation of these stemness-related genes.

CONCLUSION
SmartFlare™ appears to be a reliable, easy-to-handle tool for investigating *CD133* and *OCT4* expression in a neural stem cell model, preserving cell biological properties in anticipation of downstream experiments.

Key Words: mRNA detection; SmartFlare™; NanoFlare; Live staining; Nanotechnology; Neural stem cell genes.

Core Tip: The detection of RNA transcripts in living cells is a challenge in embryonic development and cancer related studies. In the last decade, a straightforward and noninvasive approach has emerged, exploiting the combination of nanotechnology and the physiological behavior of stem cells. Although SmartFlare™ technology is far from providing an unambiguous localization of specific mRNAs, it might help in elucidating the time-dependent dynamics of RNA expression at single-cell level, where results are coherent with those coming from both qRT-PCR and fluorescence *in situ* hybridization (FISH), the gold standards for mRNA analysis.

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INTRODUCTION

During the last decades, the proper signature of neural stem cells and their derivatives has been accomplished by tracking both proteins and mRNAs. Thus, the experimental setting is a real challenge when the production of certain proteins is scarce and the sensitivity threshold of the laboratory methods is inadequate. Furthermore, a snapshot of this phenomenon does not account for the pathway dynamics, such as axonal transport, fast secretion, and developmental mechanisms orchestrated by molecular gradients.

Historically, simultaneous detection at single-cell level by means of immunochemical and FISH techniques can provide ultimate confirmation for the presence of a variety of signaling molecules. Nevertheless, the real-time monitoring of specific RNA transcripts and downstream proteins is limited by cell fixation and permeabilization dictated by the above techniques and the required lysis of tissues to extract RNA for qRT-PCR. This last molecular option provides information about gene expression levels, in heterogeneous populations, hiding the small but relevant differences and changes taking place in individual cells. Ultimately, the aforementioned methodologies make incompatible further analysis (*e.g.*, cell sorting and collection) particularly meaningful for addressing developmental issues. Within this context, an affordable and reproducible method aiming at encompassing both the kinetics and quantification of endogenous RNAs at cellular level has been brought by a group of researchers[1,2]. SmartFlare™ technology combines the high sensitivity of oligonucleotide-linked

nanoparticles with natural receptor-mediated endocytosis to uptake the same nanoconstructs. In particular, target RNA-specific complementary single stranded RNA (capture strand) is hybridized with a complementary "reporter" sequence bound to a fluorophore (Cy3 or Cy5) at its 5' -end that, for vicinity to the central gold particle, is permanently quenched. Only upon pairing with the target RNA sequence, the reporter strand can be released and consequently gain the feature to flare with fluorescent emission at the proper wavelength and intensity, consistent with the expression level of the target RNA. Since the introduction of the SmartFlare™ concept [2-4], this molecular procedure has been successfully exploited for the identification and assessment of both tumor and immune cell subsets[5-8]. Interestingly, the SmartFlare™ technique could provide a wide spectrum of research applications, as identifying RNAs into mammalian conceptuses at different developmental stages has already been used as a proper model[9]. Indeed, SmartFlare™ allows the detection of RNAs specific for hereditary diseases, sex determination, performance and conformation traits in early embryonic stages[1,10-13], and the expression of pluripotency genes in embryonic stem cells and induced pluripotent stem cells (iPSCs) of murine, porcine and human origin[14]. Nevertheless, the ultimate confirmation of these experiments still relies on detecting the same transcripts by qRT-PCR.

To answer to some developmental issues related to the expression of the transcription factor Octamer-Binding Transcription Factor 4 (*OCT4*), involved in the differentiation process of human neurospheres in a time-dependent fashion[15,16], the mRNA pattern of *OCT4* at single-cell level was analyzed from 3 to 30 d *in vitro* (DIV) using specific SmartFlare™ probes to assess a possible downregulation strictly linked to cellular maturation from stem/progenitor to neural phenotype. In parallel, a SmartFlare™ probe for Prominin 1 (*CD133*), encoding for a transmembrane glycoprotein widely recognized as a marker of neural progenitor cells, was tested[17,18].

Our findings suggest that SmartFlare™ technology is a straightforward tool for discriminating gene transcripts specifically related to some neural stem cell markers.

MATERIALS AND METHODS

Forebrain tissues were obtained from aborted human fetuses aged 8 wk postconception (Lund and Malmö University Hospitals) in accordance with guidelines approved by the Lund/Malmö Ethical Committee (ethical permit No. Dnr 6.1.8-2887/2017). Brain fragments from the striatum were subjected to microdissection under a stereomicroscope (Leica, Germany), incubated for 30 min in an expansion medium at 37 °C, and then mechanically dissociated in order to obtain a single-cell suspension. Expansion medium DMEM/F-12 (1:1; InVitrogen, Life Technologies, United States), 2.92 g/100 mL L-glutamine, 23.8 mg/100 mL HEPES, 7.5% $NaHCO_3$, 0.6% glucose, and 2% heparin (all from Sigma-Aldrich, United States) contained B27 supplement (1%; InVitrogen), human Leukemia Inhibitory Factor (LIF; 10 ng/mL; Sigma-Aldrich), Epidermal Growth Factor (EGF; R&D Systems, United States), and Fibroblast Growth Factor (20 ng/mL and 10 ng/mL, respectively; R&D Systems, United States). Live cells were thereafter counted by the Trypan Blue dye exclusion method before plating in culture flasks at the fixed density of 50.000 cells/mL, at 37 °C in a humidified atmosphere with 5% CO_2. After several weeks, neurospheres were developed and supplied by the Laboratory of Stem Cells and Restorative Neurology (Lund). To determine the capacity of cells to form secondary spheres, single neurospheres were first passaged and then plated for 1 wk. The newly shaped neurospheres were enzymatically dissociated with Accutase solution (Sigma-Aldrich) when at least 70% of them were below 100 μm in radial size or, if smaller, when before their inner core faded to dark, indicating an activated oxidative process and subsequent cell death.

For *in vitro* differentiation, pelleted neurospheres were incubated with Accutase solution for gentle dissociation for 10 min at room temperature (RT), followed by DMEM/F-12 addition for halting the enzymatic activity. After centrifugation, single cells were resuspended in 500 μL basic medium (without growth factors and heparin) containing 1% fetal bovine serum (FBS; differentiation medium) and plated on poly-L-lysine-coated glass coverslips (5000-10000 cells/cm^2)[16,19]. During the differentiation period (1-4 wk), the specific medium was refreshed every third day.

Live-cell mRNA detection was performed using SmartFlare™ probes, according to the manufacturer's protocol (Merck Millipore, Temecula, CA, United States). Briefly, all the used probes were rehydrated by 50 μL of sterile nuclease-free double-distilled water to each vial and kept in the dark until needed. Immediately before the use, the

stock solutions were diluted 1:20 in sterile phosphate-buffered saline. Four μL of the same solutions were added to 200 μL of the medium for each tested probe. For each experiment, performed in triplicate, two control samples were run in parallel: a negative one made of a scramble construct that, therefore, does not recognize any cellular sequence and used to quantify the unspecific background (Scramble SmartFlare™ Probe); a positive control (uptake SmartFlare™ Probe) that permanently emits fluorescence supplying the information that the SmartFlare™ particles are uptaken by the target cell type. The following reagents were used: CD133 Hu-Cy3 SmartFlare™ RNA Probe (SF-958), Oct4 Hu-Cy3 SmartFlare™ RNA Probe (SF-438), Actin-Cy3 SmartFlare™ RNA Probe (SF-145), Scramble-Cy3 SmartFlare™ RNA Probe (SF-103), and uptake-Cy3 SmartFlare™ RNA Probe (SF-114), all provided by Merck Millipore. All samples were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 24 h, since in previous experiments the suggested 16 h incubation was evaluated not sufficient for the complete probe internalization. For nuclear staining, 10 μg/mL Hoechst 33342 (InVitrogen) was added 5 min before evaluation. Observations were made using an inverted microscope (IX 71; Olympus, Tokyo, Japan) with a x40 planapochromatic objective (PlanApo series; Olympus), taking care to grab all images with the same exposure time and filter set. Images (12-bit) were taken with a cooled monochrome CCD camera (Moticam Pro285D, Motic, China) with a 1360 × 1024 pixel chip. Image processing and analysis were performed using the Image-Pro Plus software (Media Cybernetics, United States).

SmartFlare™ CD133- and OCT4-specific fluorescence signal was assessed using a semiquantitative visual approach by three observers in a blinded fashion. This evaluation took into account both the fluorescence intensity and the number of labeled cells.

RESULTS

CD133 and *OCT4* gene expression was analyzed by SmartFlare™ technology in dissociated human neurospheres upon differentiation commitment, accomplished by switching to growth factor withdrawn media along one-month time frame (from 3 to 30 DIV), with 3 DIV as the minimum time needed by cells both to adhere to the substrate and to grow cytoplasmic area and processes. At 3 DIV, after incubation with specific SmartFlare™ probes, the morphological expression pattern for *CD133* and *OCT4* mRNAs (Figure 1A and B) was consistent with the Actin-positive cells (Figure 1C). Remarkably, when Hoechst-stained cells were not massively clustered but discernible as single elements, it was possible to evaluate that all cells displayed a diffuse but strong fluorescent signal, sometimes visible as converging single dots filling the thin cytoplasmic processes too. Similarly, the fluorescence of the CD133 reporter probe was as intense as that of Oct4. The Actin housekeeping probe was clearly internalized as a fluorescent patch distributed from the perinuclear area to the peripheral branches, where it appeared as a granular content connecting distant cells (Figure 1C). Fluorescence detection in those living cells was considered a specific marker for mRNAs presence when compared to scramble experiments (Figure 1D), where any background was undetectable in most cells.

At 7 DIV, microscopic images exhibited a clear fluorescence both with CD133 and Oct4 probes (Figure 2A and B). Although the robust arborization network was still detected, in visible branches of very few cells it was observed the presence of fluorescent dots, representative of the molecular beacon-associated mRNAs. The reliability of the results was confirmed by the positive and negative controls (Figure 2C and D).

Cells grown for 15 DIV presented a marked decrease in the SmartFlare™ fluorescence signal, as it was limited to less than half of the analyzed cells, irrespective of the CD133 or Oct4 probe incubation (Figure 3A and B). In addition, the mRNA-like presence was confined to the cytoplasmic domain and always in the shape of tiny and few grains.

Finally, after 30 DIV, even fewer positive cells with specific signal were noticed and again the only morphological feature consisted of single dot-like elements, both in CD133 and Oct4 probe-treated cells (Figure 4A and B). Accordingly, in the last two experiments (15 and 30 DIV), Actin (Figure 3C and 4C, respectively) and Scramble (Figure 3D and 4D, respectively) signals were representative of the specificity of the resulting fluorescence.

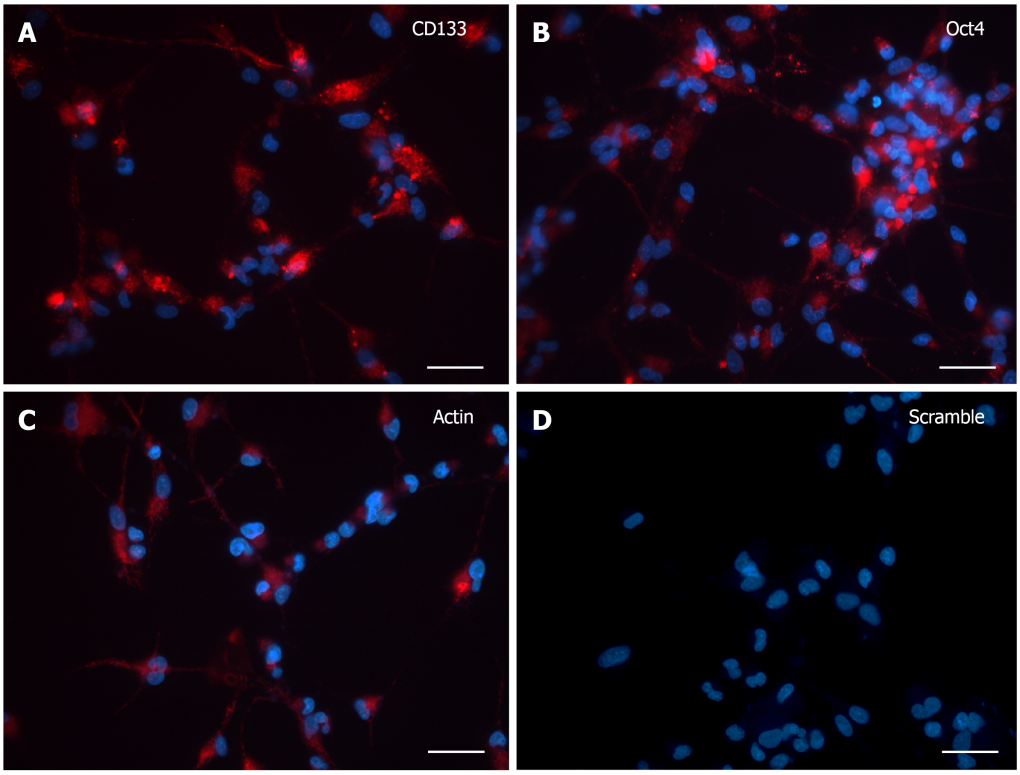

Figure 1 SmartFlare™ detection in 3 d *in vitro* neural stem cells. A and B: The expression pattern for both SmartFlare™ CD133 and Oct4 probes showed a diffuse and spotted-like fluorescence from the perinuclear area to the peripheral cytological processes; C: SmartFlare™ probe for Actin showed a robust and overall localization of red fluorescence as indicative of positive mRNA expression; D: Scramble probe-treated cells were almost completely lacking in unspecific red background. Nuclei were stained by Hoechst dye. Scale bar: 50 μm.

DISCUSSION

In this study, we carried out a simple and noninvasive RNA-based approach to monitor intracellular gene expression in living cells by fluorescent SmartFlare™ probes. In detail, this study focused on human neurospheres as neural stem cell reservoir, as this is a well-established model to study the progression of differentiation events giving rise to both neuronal and glial lineages. This is a very interesting topic to address, since it involves *OCT4*, one of the key genes implicated in encoding transcription factors prone to convert somatic cells into iPSCs and, therefore, necessary for the commitment of embryological events[20]. The rationale behind the present investigation dates back to a previous study, where the immunohistochemical presence of Oct4 protein was observed in neural stem cells during the first week of differentiation but disappeared after 4 wk. Coherently, in the same research, RT-PCR experiments supported *OCT4* mRNA downregulation, as illustrated by the blurred bands of the electrophoretic assay[16]. Therefore, the advantage of SmartFlare™ probe uptake has emerged for challenging the quantification of mRNA gradient in specific and individual cells. Moreover, the same technique could be useful for identifying neural stem/progenitor cells eventually sorted for further characterization, avoiding any minimal alteration of morpho-functional and biochemical properties. With regard to Oct4, there are some further but possibly misinterpreting studies describing cytoplasmic staining due to splicing variants that make it critical to distinguish transcriptional products[21-24]. For this reason, this study conceived the experimental design of choosing the cell surface antigen CD133 as an alternative positive marker of neural stem cells[25]. The localization of *OCT4* mRNA within cells has already been addressed by some researchers[26] using molecular beacon transfection in differentiated human mesencephalic-derived neurospheres. However, after dissociation, adherent differentiated monolayers resulted lacking *OCT4* expression. Interestingly, monolayered cells grown from neurospheres revealed the complete absence of mRNA expression just before the first week of differentiation, as further confirmed by immunocytochemistry. Indeed, the initial enthusiasm of the scientific community was damped by some studies reporting "A total lack of correlation between fluorescence intensities of SmartFlare probes and the level of corresponding RNAs assessed by RT-qPCR"[27]. Recent data might explain the resulting different amounts of mRNA

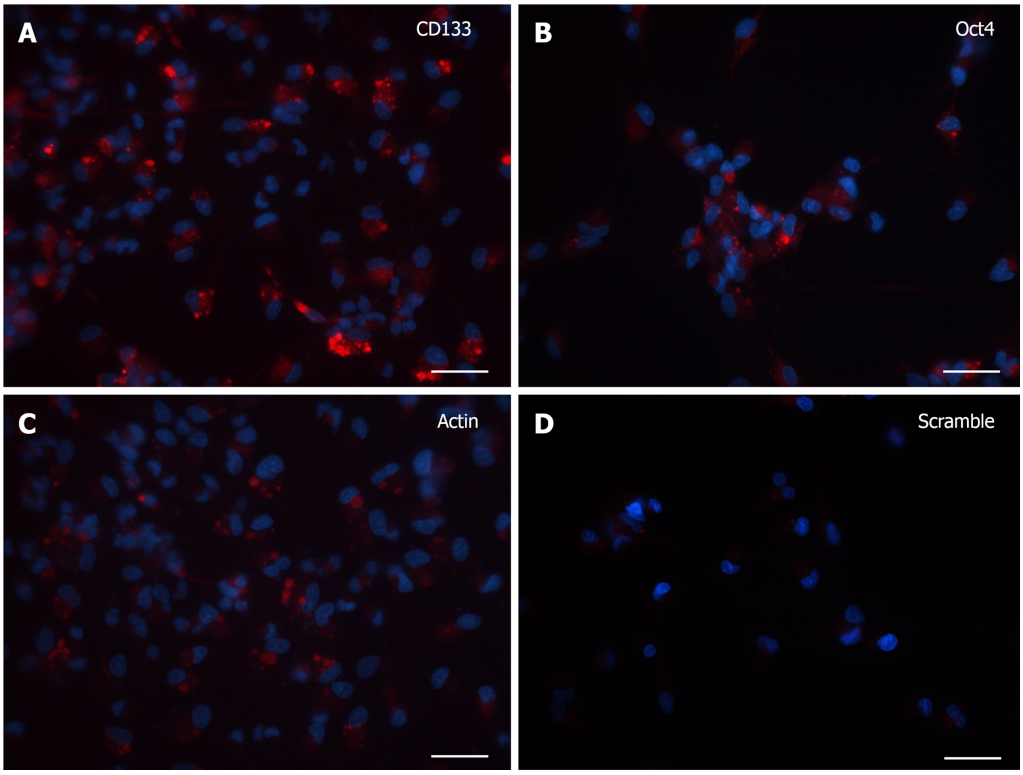

Figure 2 SmartFlare™ detection in 7 d *in vitro* neural stem cells. A and B: SmartFlare™ CD133 and Oct4 probes fluorescence was less intense than the signal detected in 3 d *in vitro* neural stem cells, but still present in almost all cells; C: SmartFlare™ probe for Actin showed a robust and overall localization of red fluorescence as indicative of positive mRNA expression; D: Scramble probe-treated cells showed a very faint unspecific red background. Nuclei were stained by Hoechst dye. Scale bar: 50 μm.

detected by SmartFlare™ and qRT-PCR, due to cytoplasmic stress granules where mRNA can be sequestered and made unavailable to be processed for translation[28].

Mason *et al*[29] argued about the SmartFlare™ probes sequestration by the lysosomal machinery. However, by specific matching Lysosome-associated Membrane Protein 1 (LAMP-1) SmartFlare™, these authors found a very low overlap (mean Manders' coefficient 0.26), concluding that the unspecific SmartFlare™ fluorescence localized in lysosomes could be negligible compared to cytoplasmic staining. Our findings agree with the heterogeneity of SmartFlare™ expression, either diffuse cytoplasmic or spotted from the perinuclear site to peripheral processes (dendrites and axons). Moreover, ultrastructural evidence of gold nanoparticles, encapsulated within endosomal/lysosomal compartments, does not explain the spotted fluorescent pattern, unless enzyme digestion would degrade and remove the nanostructure links, ultimately quenching the fluorescence signal. So far, there is still no experimental evidence for that degradative machinery, and, on the other hand, it cannot be ruled out whether there are some alternative routes either passively or actively driven by cells.

By means of a qualitative analysis, the strength of the SmartFlare™ technology would not be affected by the decrease of the fluorescence intensity as a reflection of a reduced lysosomal activity, which occurs during cell differentiation[30]. Actually, as shown by our results, it is unlikely to detect all the cells in the same stage of replication or differentiation within single timepoints.

Although FISH is a well-established and reliable qualitative molecular method, the advantages of SmartFlare™ technology could reside in the opportunity of analyzing unfixed single living cells, retaining their viability, morpho-functional and biochemical properties and allowing downstream experiments[31]. In particular, this approach could help to detect and count stem/progenitor living cells, expressing markers of stemness, in terms of differential expression of the relative mRNAs, as well as microRNAs, which could find application in the profiling of tumor cell heterogeneity [32,33]. Moreover, from an empirical perspective, the SmartFlare™ could be a quicker, easier and less expensive method than techniques involving RNA isolation. Thus, in agreement with the findings by Mason *et al*[29], our results might validate the SmartFlare™ technology as a reliable and easy-to-handle tool, at least in the qualitative analysis framework, although, in some cases, as usually happens, the possibility of an

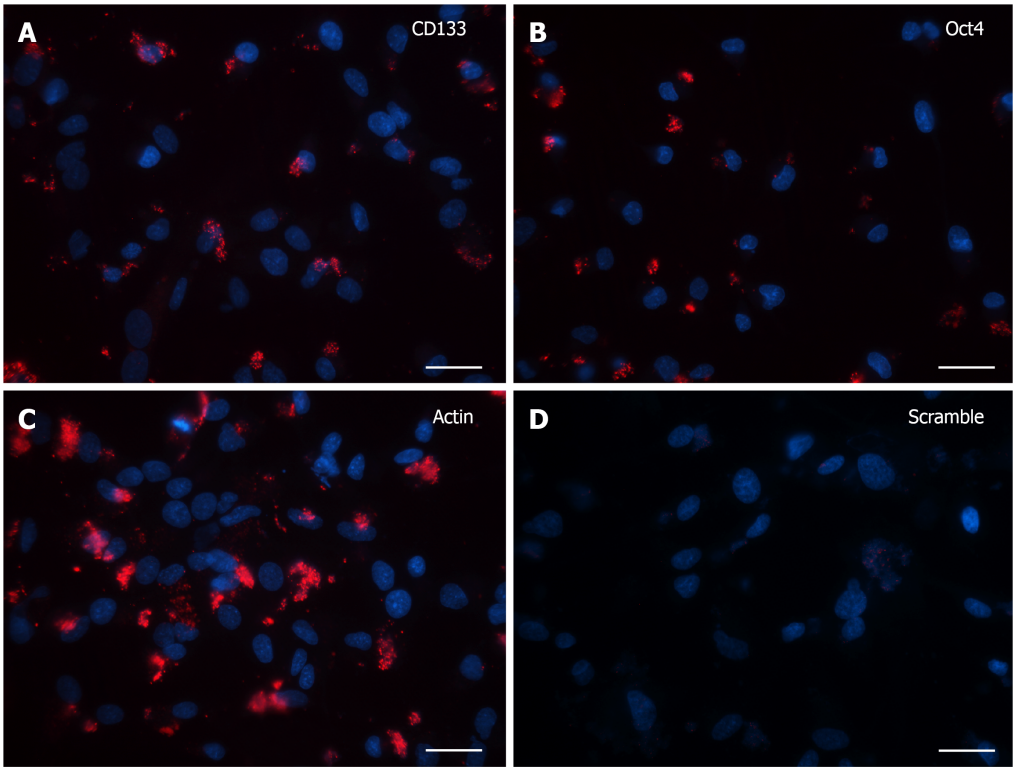

Figure 3 SmartFlare™ detection in 15 d *in vitro* neural stem cells. A and B: SmartFlare™ CD133 and Oct4 probes showed a dramatic fluorescence downregulation that was limited to small cytoplasmic granules in less than half of the observed cells; C: SmartFlare™ probe for Actin showed a robust and overall localization of red fluorescence as indicative of positive mRNA expression; D: Scramble probe-treated cells were almost completely lacking in unspecific red background. Nuclei were stained by Hoechst dye. Scale bar: 50 μm.

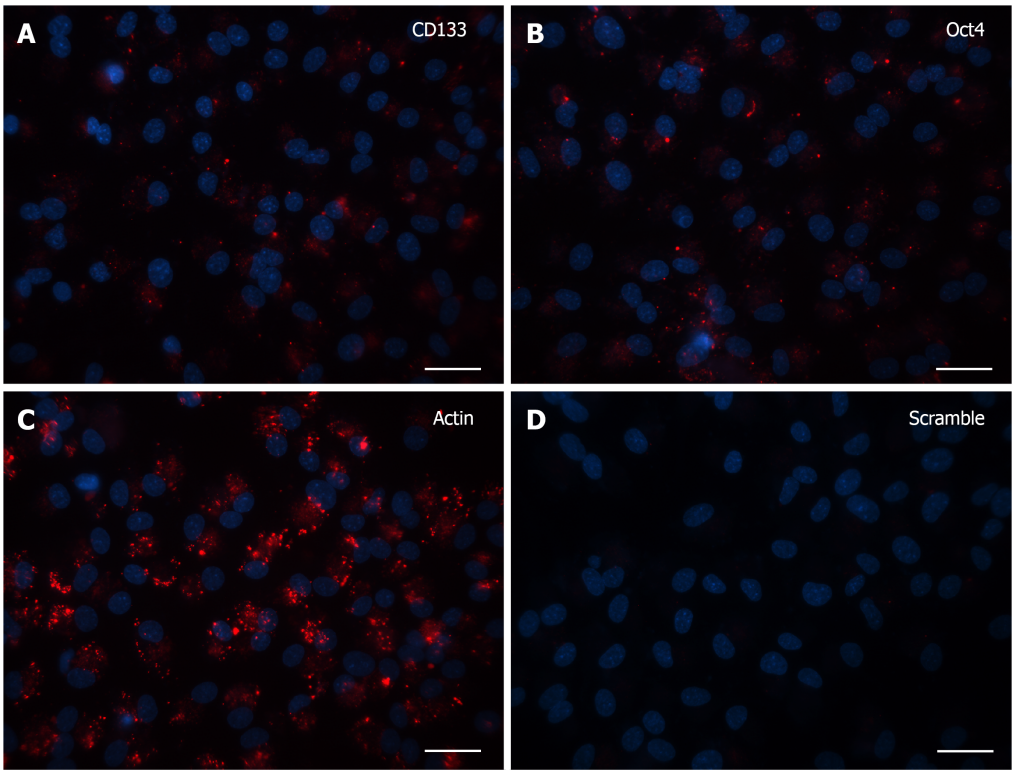

Figure 4 SmartFlare™ detection in 30 d *in vitro* neural stem cells. A and B: SmartFlare™ CD133 and Oct4 probes showed few cells expressing a tiny granular pattern in the cytoplasmic domain; C: SmartFlare™ probe for Actin showed an abundant red fluorescence in all observed cells; D: Scramble probe-treated cells were almost completely lacking in unspecific red background. Nuclei were stained by Hoechst dye. Scale bar: 50 μm.

artifact detection may arise.

In the prospect of controversial negative results, it should be considered that FBS supplementation in the culture medium could dramatically play a crucial role in the interpretation of target mRNA detection by SmartFlare™ technology, in terms of cytoplasmic distribution and localization. This methodological issue could partially explain the documentation failure by many research groups[19].

Despite the above-described unsolved criticism, some recent data on molecules and cells involved in immunological and inflammation response against cancer have renewed the interest in an innovative and effective platform to investigate some mRNA functions[34-36]. Besides, it cannot be denied that SmartFlare™ probe detection is not indicative of the real localization of single mRNA molecules. Nevertheless, those NanoFlare probes have paved the way to inspire a novel theranostic wave arising some new sticky-flares for *in situ* monitoring of human telomerase RNA[37], adopting photoactivation to detect mRNA in specific cells[38].

CONCLUSION

In conclusion, this new age of NanoFlare compounds has opened up or, at least, broadened biomedical applications, paying attention to preserving the physiological integrity of cellular systems with an excellent grade of selectivity and specificity[39].

ARTICLE HIGHLIGHTS

Research background

Although mRNA analysis is still conventionally achieved by fluorescence *in situ* hybridization and qRT-PCR, there is a strong need for real-time monitoring of specific RNA transcripts in living cells, both for a qualitative and quantitative assessment. Within this context, SmartFlare™ technology is a reliable tool for evaluating the presence and the upregulation/downregulation of mRNAs in individual living cells. In addition, this nanotechnology offers the advantages of retaining cell viability, morpho-functional and biochemical properties and allowing downstream experiments.

Research motivation

SmartFlare™ technology is a devoted and straightforward method for the spatio-temporal investigation of the *in situ* mRNA expression in living cells.

Research objectives

To study the dynamics of differentiation-related RNA transcripts in human neural stem cells.

Research methods

The presence of *CD133* and *OCT4* mRNA-linked nanoprobes in neurosphere-derived cells (from 3 to 30 DIV) was investigated by SmartFlare™ as a reliable insight into neural stem cell differentiation.

Research results

Until 7 DIV, all the cells displayed a strong SmartFlare™ fluorescent signal indicative of *CD133* and *OCT4* mRNA expression, as single dots encompassing both the cytoplasmic domain and the related processes. Upon 15 DIV, cells showed a marked decrease in the fluorescence, both for CD133 and Oct4 probes. In cells grown for 30 DIV, the CD133 and Oct4 probe uptake was very scant but still consisted of single dot-like elements, representative of a downregulation of the same genes.

Research conclusions

Our findings propose the SmartFlare™ technology as a reliable and straightforward tool in the context of a qualitative expression analysis applied to a broad panel of mRNAs in single living stem cells.

Research perspectives

The NanoFlare technology, such as SmartFlare™, could enhance the scenario of

biomedical applications in the field of marker identification mirroring both normal and pathological conditions, with the advantage of ensuring the physiological integrity of cellular systems.

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ORIGINAL ARTICLE

Basic Study

Urolithin a alleviates oxidative stress-induced senescence in nucleus pulposus-derived mesenchymal stem cells through SIRT1/PGC-1α pathway

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Abstract

BACKGROUND

In degenerative intervertebral disc (IVD), an unfavorable IVD environment leads to increased senescence of nucleus pulposus (NP)-derived mesenchymal stem cells (NPMSCs) and the inability to complete the differentiation from NPMSCs to NP cells, leading to further aggravation of IVD degeneration (IDD). Urolithin A (UA) has been proven to have obvious effects in delaying cell senescence and resisting oxidative stress.

AIM

To explore whether UA can alleviate NPMSCs senescence and to elucidate the underlying mechanism.

METHODS

In vitro, we harvested NPMSCs from rat tails, and divided NPMSCs into four groups: the control group, H_2O_2 group, H_2O_2 + UA group, and H_2O_2 + UA + SR-18292 group. Senescence-associated β-Galactosidase (SA-β-Gal) activity, cell cycle, cell proliferation ability, and the expression of senescence-related and silent information regulator of transcription 1/PPAR gamma coactivator-1α (SIRT1/PGC-1α) pathway-related proteins and mRNA were used to evaluate the protective effects of UA. *In vivo*, an animal model of IDD was constructed, and X-rays, magnetic resonance imaging, and histological analysis were used to assess whether UA could alleviate IDD *in vivo*.

RESULTS

We found that H_2O_2 can cause NPMSCs senescence changes, such as cell cycle arrest, reduced cell proliferation ability, increased SA-β-Gal activity, and increased expression of senescence-related proteins and mRNA. After UA pretreatment, the abovementioned senescence indicators were significantly alleviated. To further demonstrate the mechanism of UA, we evaluated the mitochondrial membrane potential and the SIRT1/PGC-1α pathway that regulates mitochondrial function. UA protected mitochondrial function and delayed NPMSCs senescence by activating the SIRT1/PGC-1α pathway. *In vivo*, we found that UA treatment alleviated an animal model of IDD by assessing the disc height index, Pfirrmann grade and the histological score.

CONCLUSION

In summary, UA could activate the SIRT1/PGC-1α signaling pathway to protect mitochondrial function and alleviate cell senescence and IDD *in vivo* and *vitro*.

Key Words: Urolithin A; Mitochondrial function; Oxidative stress; Senescence; Nucleus pulposus-derived Mesenchymal stem cells; The silent information regulator of transcription 1/PPAR gamma coactivator-1α pathway

Core Tip: In degenerative intervertebral disc (IVD), an unfavorable IVD environment leads to increased senescence of nucleus pulposus-derived mesenchymal stem cells (NPMSCs), which seriously affects endogenous repair of IVD. Urolithin A (UA) alleviated oxidative stress-induced NPMSCs senescence by activating the silent information regulator of transcription 1/PPAR gamma coactivator-1α signaling pathway and protecting mitochondrial function *in vitro*. UA could also delay extracellular matrix degradation and IVD degeneration (IDD) *in vivo*. The results provide the possibility to promote endogenous repair and retard IDD.

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INTRODUCTION

Low back pain seriously affects the quality of life and increases the economic burden on families and society[1,2]. Intervertebral disc degeneration (IDD) is the main pathogenic factor of low back pain, but its pathological mechanism has not yet been elucidated[3]. Therefore, exploring the pathological mechanism of IDD and seeking new methods for the prevention and treatment of degenerative disc diseases is of great significance to human health and social development.

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Nucleus pulposus (NP) cells play an important role by secreting a large amount of extracellular matrix (ECM) such as aggrecan and collagen type II to maintain the normal physiological function of the intervertebral disc (IVD) under physiological conditions. However, NP cells are terminal cells with low proliferation ability and no differentiation and self-renewal ability. In degenerative IVD, the number of NP cells is reduced, and their function is impaired, leading to a decrease in ECM secretion and aggravation of IDD. Endogenous repair is a specific repair mediated by tissue-specific stem cells and has been found to exist in a variety of tissues, such as the skin, liver and nervous system[4,5]. In 2007, Risbud *et al*[6] isolated and identified NP-derived mesenchymal stem cells (NPMSCs) in degenerative IVD, which provided a basis for the endogenous repair of IDD. However, the unfavorable microenvironment of degenerative IVD, such as inflammation, oxidative stress, and increased catabolism, leads to increased senescence and apoptosis of NPMSCs, which seriously affects endogenous repair[7]. Therefore, rescuing the activity of NPMSCs and delaying cell senescence is of great significance to alleviate IDD.

To date, natural metabolically active products have been widely found as important sources for drug discovery in antiaging and senescence-related diseases. Urolithin is a type of dibenzopyran-6-one derivative with different phenolic hydroxyl groups produced by intestinal microbial metabolism from foods rich in ellagitannins (pomegranate, strawberry, walnut, raspberry, *etc.*)[8]. Among them, urolithin A (UA) (Figure 1) was the first to be isolated and identified from the feces and urine of mice fed ellagic acid[9]. Previous studies found that UA can show biological effects, such as regulating estrogen secretion and antioxidant and anti-inflammatory activities[10-12]. Recently, the antiaging effect of UA has drawn considerable attention. Ryu *et al*[13] found that UA has a unique effect in inducing mitophagy, prolonging the lifespan of C. elegans and increasing muscle function in rodents. A previous study also found that UA could exert antiapoptotic and antiaging effects on NP cells[14,15].

However, there are few studies of the protective effect of UA on NPMSCs to date. In this study, we investigated whether UA could alleviate H_2O_2-induced NPMSCs senescence *in vitro* and in IDD animal models *in vivo* and elucidated the mechanisms involved in this progress.

MATERIALS AND METHODS

Isolation and culture of NPMSCs

This study was approved by the Ethical Committee of the Clinical Medical College of Yangzhou University (SBYY2020-023). Sprague-Dawley (SD) rats (weight, 200-300 g; age, 4-6 mo) were purchased from the Shanghai Institute of Family Planning Science [License No. SCXK (Hu) 2018-0006]. NPMSCs were harvested from the coccygeal IVD tissues of SD rats. Then, the NP tissues were isolated under a dissecting microscope and digested in 0.2% type II collagenase (Gibco, United States, catalog No. 17101015) for 12 h at 37 °C with 5% CO_2. Then, the obtained cells and partially digested tissues were washed with phosphate-buffered saline (PBS) twice, and centrifuged at 1000 r/min for 5 min, and then cultured in Mesenchymal Stem Cell Complete Medium (Cyagen, United States, catalog No. RASMX-90011) at 37 °C with 5% CO_2. The culture medium was changed every three days. The cells were passaged at a 1:3 ratio at 80%-90% confluence. NPMSCs used in the followed study was passage 3.

Surface marker identification of NPMSCs

The mesenchymal stem cell (MSC)-associated surface markers were examined by immunofluorescent staining. Cell slides with a diameter of 25 mm containing polylysine were placed in a 12-well plate, and NPMSCs were seeded and cultured in MSC complete medium. Then, the cells were fixed with 4% paraformaldehyde for 15 min and washed twice with PBS containing 0.5% Triton X-100 for 15 min. Then, the cells were blocked with 10% bovine serum albumin for 1 h at 37 °C and incubated with primary antibodies against CD 105 (Proteintech, China, catalog No. 10862-1-AP), CD90 (ABclonal, China, catalog No. A2126), CD73 (ABclonal, China, catalog No. A2029), CD34 (ABclonal, China, catalog No. A7429) and CD45 (ABclonal, China, catalog No. A2115) (1:100) at 4 °C overnight. The cell slides were washed twice with PBS and then incubated with secondary antibodies (Abcam, United Kingdom, catalog No. ab150077, ab150078) (1:500) for 1 h at room temperature. After treatment with the antifade mounting medium with 4′,6-diamidino-2′-phenylindole for 10 min, the cell slides were observed and recorded using a fluorescence microscope (Leica, Wetzlar, Germany).

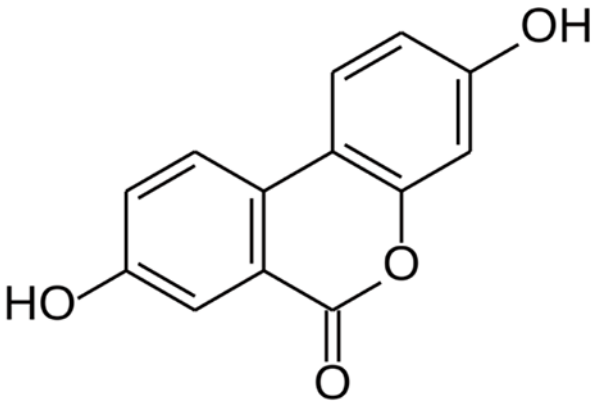

Figure 1 Chemical structure of Urolithin A.

Multilineage differentiation

To demonstrate the multilineage differentiation potential of NPMSCs, osteogenic, adipogenic and chondrogenic differentiation was induced. NPMSCs were seeded in 6-well plates and cultured until reaching approximately 80% confluency. Then, the culture medium was changed to osteogenic, cartilage and adipogenic differentiation medium (Cyagen, China, catalog No. RASMX-90021, RASMX-9004, RASMX-90031), and the medium was changed every 3 d according to the manufacturer's instructions. After reaching the deadline of induction, the cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min, and then the cells were processed with oil red O, alizarin red and alcian blue. Finally, the staining results were observed and imaged under a fluorescence microscope.

Cell viability assay

NPMSCs were seeded in 96-well plates at a density of 2 × 10^3 cells per well and incubated in complete medium overnight at 37 °C with 5% CO_2. Then, NPMSCs were treated with different concentrations ((0-80 μM, 0-48 h) of UA (MedChem Express, China, catalog No. 1143-70-0). After that, 10% cell counting kit-8 (CCK-8) (Dojindo, Japan, catalog No. CK04) was added to each well at different time points, and the optical density (OD) value was read after 1 h of incubation at 480 nm by a microplate reader (Bio-Rad, United States). Cell viability was calculated as follows: Cell viability (of control) = [(Ae-Ab)/(Ac-Ab)]. Ae, Ab, and Ac represent the OD value of the treatment, blank and control groups, respectively. Similarly, to further determine whether UA works through the silent information regulator of transcription 1/PPAR gamma coactivator-1α (SIRT1/PGC-1α) pathway, different concentrations (0-160 μM, 0-36 h) of the PGC-1α inhibitor SR-18292 (MedChem Express, China, catalog No. HY-101491) were cocultured with NPMSCs.

NPMSCs were divided into four groups in the following examinations: (1) Control group; (2) H_2O_2 group (80 μM H_2O_2); (3) H_2O_2 + UA group (80 μM H_2O_2 + 20 μM UA); and (4) H_2O_2 + UA + SR-18292 group (80 μM H_2O_2 + 20 μM UA + 20 μM SR-18292).

Cell proliferation assay

NPMSCs (5 × 10^4 cells/well) were seeded in a 12-well plate and cultured in MSC complete medium. An EdU Cell Proliferation Kit (Beyotime, China, catalog No. C0071S) was used to detect cell proliferation. Subsequently, NPMSCs were incubated with EdU for 2 h and fixed with 4% paraformaldehyde for 15 min, and then cells were incubated with 0.3% Triton X-100 for 10 min according to the manufacturer's instructions. Then, the cells were incubated with Click Reaction Mixture for 30 min and then incubated with Hoechst 33342 for 10 min in the dark. Finally, cells were observed and recorded using a fluorescence microscope and analyzed by ImageJ software (NIH, United States).

Cytotoxicity assay

Cytotoxicity was measured by the lactate dehydrogenase (LDH) activity in the supernatant using the LDH cytotoxicity assay kit (Beyotime, China, catalog No. C0016) according to the protocol. NPMSCs were seeded in 96-well plates (5 × 10^3 cells/well) and incubated in complete medium overnight at 37 °C with 5% CO_2. Then, NPMSCs were treated with 80 μM H_2O_2, 80 μM H_2O_2 + 20 μM UA and 80 μM H_2O_2 + 20 μM UA

+ 20 μM SR-18292 in the H_2O_2 group, H_2O_2 + UA group and H_2O_2 + UA + SR-18292 group, respectively. After that, 10% LDH release reagent was added to each well and incubated for 1 h. Then, the supernatant was transferred to a new 96-well plate and mixed with LDH detection working solution in the dark for 30 min. Finally, the OD value was detected at 490 nm by a microplate reader. The cytotoxicity was calculated as follows: Cytotoxicity (of control) = [(Ae-Ab)/(Ac-Ab)]. Ae, Ab, and Ac represent the OD values of the treatment, blank and control groups, respectively.

Senescence-associated β-Galactosidase staining

NPMSCs were seeded in a 6-well plate (1×10^4 cells/well), and senescence-associated β-Galactosidase (SA-β-Gal) staining was performed according to the manufacturer's instructions from the SA-β-Gal staining Kit (Beyotime, China, catalog No. C0602). NPMSCs were observed under a fluorescence microscope and analyzed by ImageJ software.

Cell cycle assay

NPMSCs were seeded in 6-well plates with serum-free medium overnight. After that, cells were collected and fixed with 75% ethanol overnight. Then, the cells were incubated with a mixed solution of propidium iodide (PI) dye and RNase A (Keygen, China, catalog No. KGA511) for 30 min, and the cell cycle phases were analyzed by flow cytometry (BD Company, United States).

JC-1 assay for mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was measured using the JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) Detection Kit (Keygen, China, catalog No. KGA603). NPMSCs from different groups were washed with PBS and incubated with 2 μM JC-1 dye for 20 min. Then, the cells were washed twice with incubation buffer and observed using a fluorescence microscope. The ratio of green (depolarization) to red (polarized) fluorescence intensity was calculated using ImageJ.

Reactive oxygen species

The level of intracellular reactive oxygen species (ROS) in NPMSCs was measured by a ROS detection fluorescent probe-DHE kit (Keygen, China, catalog No. KGAF019). After different interventions in a 12-well plate, NPMSCs were washed twice with PBS and incubated with 20 μM DHE for 1 h at 37 °C according to the manufacturer's instructions. Then, the cells were observed using a fluorescence microscope and analyzed by ImageJ.

Quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Invitrogen, United States, catalog No. 15596-026). Reverse transcription from whole RNA to complementary DNA (cDNA) and amplification of the cDNA were performed using a Prime Script-RT reagent kit (Vazyme Biotech, China, catalog No. R123-01) and SYBR Premix Ex Taq (Vazyme Biotech, China, catalog No. Q111-02) according to the manufacturer's instructions. The expression of target genes of NPMSCs in different groups was calculated by the comparative Ct method. The primers were designed according to the sequences in GenBank using Prime 5.0 software and are listed in Table 1.

Western blot assay

Total protein was extracted from NPMSCs by Whole Cell Lysis Assay (Keygen, China, catalog No. KGP250), and the protein concentration was measured using the BCA protein assay kit (Beyotime, China, catalog No. P0010). Then, an equal protein sample of each group was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. After that, the membranes were blocked with 5% nonfat milk for 2 h at room temperature and then incubated with primary antibodies against β-actin (Proteintech, China, catalog No. 20536-1-AP) (1:3000), p16 (Proteintech, China, catalog No. 10883-1-AP) (1:1000) and p21 (Proteintech, China, catalog 10355-1-AP) (1:1000) overnight at 4 °C. After washing three times with Tris-buffered saline and 0.1% Tween 20 (TBST), the membranes were incubated with secondary antibodies (Proteintech, China, catalog No. SA00001-2) (1:5000) for 2 h on a shaker at room temperature. Then, the membranes were visualized using an enhanced chemiluminescence system, and the relative amount of protein was analyzed using ImageJ software.

Table 1 Sequences of primers used for real-time PCR

Gene	Primer sequence
GAPDH	Forward 5'-CTGGAGAAACCTGCCAAGTATG-3'Reverse 5'-GGTGGAAGAATGGGAGTTGCT-3'
P16	Forward 5'-CCGATACAGGTGATGATGATGG-3'Reverse 5'-CGGAGGAGAGTAGATACCGCAAA-3'
P21	Forward 5'-AGTTGGAGCTGGTGGCGTAG-3'Reverse 5'-AATACACAAAGAAAGCCCTCCC-3'
SIRT1	Forward 5'-AGATTTCAAGGCTGTTGGTTCC-3'Reverse 5'-CAGCATCATCTTCCAAGCCATT-3'
PGC-1α	Forward 5'-GAGAAGCGGGAGTCTGAAAGG-3'Reverse 5'-GTCACAGGTGTAACGGTAGGTAATG-3'

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; SIRT1: Silent information regulator of transcription 1; PGC-1α: PPAR gamma coactivator-1α.

IDD animal model induction

Fifteen SD rats (weight, 200-300 g; age, 4-6 mo) were randomly divided into three groups (n = 5 per group): the control group (no operation), IDD group (punctured and DMSO treatment), and UA group (punctured and UA treatment). The SD rat IDD model was established according to a previous method[16]. Briefly, the rats were anesthetized by an overdose of pentobarbital. After sterilization with povidone iodine, the coccygeal IVD (C_O6-7) was percutaneously punctured by a 21 G needle at a depth of 5 mm, followed by rotation at 360° and holding for 30 s. The UA group was given water containing UA (25 mg/kg/d, dissolved in DMSO and diluted in water) for 4 wk from the first day after surgery[13]. The control group and IDD group were given an equivalent volume of DMSO for 4 wk.

Radiographic and magnetic resonance imaging evaluation

Radiographic and MRI scans were taken prepuncture and 4 wk after puncture, respectively. The rats were placed in a prone position after anesthesia by inhalation of 2% isoflurane. X-ray scans were performed, and the disc height index (DHI) was measured by ImageJ software[17].

The signal and structural change of the IVD were obtained by a 3.0-T clinical MR scanning system (Philips Intera Achieva 3.0 MR, Netherlands). Briefly, SD rats were maintained after inhalation of 2% isoflurane and then placed in a prone position. Sagittal T2-weighted images were evaluated according to the Pfirrmann grade[18].

Histologic analysis

All SD rats were euthanized by an overdose of pentobarbital after 4 wk of puncture. The IVD specimens were harvested and fixed with 4% paraformaldehyde, decalcified with 10% ethylenediaminetetraacetic acid solution, and embedded in paraffin. The specimens were cut into 5-μm sections, and the slices were stained with hematoxylin-eosin (HE), toluidine blue and safranin-O stains. Histologic images of HE were evaluated following histologic grading scale criteria reported by Norcross *et al*[19].

Immunofluorescent staining

After the rats were killed, IVD specimens were harvested and cut into 5 μm sections with a freezing microtome (Leica, Wetzlar, Germany). The sections were then fixed in 4% paraformaldehyde for 15 min and washed twice with PBS. Then, the sections were blocked with 10% bovine serum albumin for 1 h at 37 °C and incubated with primary antibodies at 4 °C overnight: rabbit polyclonal anti-collagen type II (ABclonal, China, Catalog No. A1560) (1:1000) and anti-aggrecan (ABclonal, China, Catalog No. A8536) (1:1000). After that, the sections were washed twice with PBS and incubated with secondary antibodies (Abcam, United Kingdom, catalog No. ab150077, ab150078) (1:500) for 1 h at room temperature in the dark. The sections were photographed by a fluorescence microscope and analyzed by ImageJ software.

Statistical analysis

All data were analyzed by Statistical Package for the Social Sciences (SPSS) software (version 26; IBM, Chicago, Illinois). The quantitative data are expressed as the mean ± SD. The data of multiple independent groups were analyzed by one-way ANOVA. Student's t-test was used to analyze the differences between the two groups. P value < 0.05 was significant.

RESULTS

Identification of NPMSCs

The cells isolated from the rat coccygeal IVD presented with a long spindle shape and grew in flower formation (Figure 2A). As shown in Figure 2B, MSC-associated surface markers were identified by cellular immunofluorescence. CD105, CD90, and CD73 showed high fluorescent expression, but CD34 and CD45 showed low fluorescent expression. The multilineage differentiation ability was confirmed by multilineage differentiation *in vitro* (Figure 2C). The results indicated that the cells isolated from the rat NP meet the appraisal standards of stem cells proposed by the International Society for Cellular Therapy (ISCT).

Measurement of cell viability

An appropriate concentration of H_2O_2 to induce oxidative stress damage in NPMSCs was confirmed in our previous study[20]. The viability effects of UA and the PGC-1α inhibitor SR-18292 on NPMSCs were analyzed using a CCK-8 assay. NPMSCs were cocultured with different concentrations of UA and SR-18292 supplemented culture media at different time points. As shown in Figure 3A, UA (0-20 μM) showed an appropriate inhibitory effect on cell viability for 24 h, but UA (> 40 μM) exerted a significant inhibitory effect ($P < 0.05$). Therefore, 20 μM UA was used as the final drug intervention concentration. Similarly, after incubation with different concentrations of SR-18292 at different time points, 20 μM SR-18292 for 24 h exhibited appropriate inhibition of cell viability and was used as the final drug concentration and intervention time (Figure 3B) ($P < 0.05$).

Measurement of cell proliferation and cytotoxicity

To elucidate the effect of H_2O_2, UA and SR-18292 on the proliferation ability of NPMSCs, we performed EdU staining of NPMSCs. As shown in Figure 3C-D, the EdU-positive rate of the H_2O_2 group was significantly lower than that of the control group (32.1% ± 5.4% *vs* 47.9% ± 5.8%, $P < 0.05$), and EdU-positive rate was increased by 12% after UA pretreatment. However, the protective effects of UA were reversed by SR-18292 (44.9% ± 5.7% *vs* 34.4% ± 5.9%, $P < 0.05$).

LDH release is regarded as an important indicator of cell membrane integrity and is widely used to assess cytotoxicity. As a common oxidative stress-inducing agent, 80 μM H_2O_2 still had cytotoxicity compared to the control group ($P < 0.01$). However, the cytotoxicity induced by H_2O_2 was alleviated after pretreatment with 20 μM UA, which indicates that LDH release was alleviated after UA pretreatment ($P < 0.05$). After treatment with SR-18292, the protective effect of UA was partly blocked (Figure 3E) ($P < 0.01$).

Measurement of SA-β-Gal staining

SA-β-Gal is a parameter evaluating cellular senescence, and senescence cells with high SA-β-Gal activity are stained blue. NPMSCs of the H_2O_2 group demonstrated a higher percentage of SA-β-Gal staining positive than the control group ($P < 0.01$). The percentage of positive cells was decreased after UA pretreatment ($P < 0.01$). The percentage of SA-β-Gal-positive cells was increased when NPMSCs were cocultured with UA and SR-18292 before H_2O_2 treatment (Figure 4A-B) ($P < 0.05$).

Measurement of cell cycle

Cell cycle arrest is one of the common features of senescent cells. As shown in Figure 4C-D, a higher percentage of NPMSCs showed cell cycle arrest in G2/M phase in the H_2O_2 group than in the control group. The percentage of NPMSCs arrested in G2/M phase decreased after pretreatment with UA, indicating that UA could attenuate H_2O_2-induced cell cycle arrest. However, the percentage of cells arrested in G2/M phase increased after treatment with SR-18292 compared with UA pretreatment alone.

Measurement of MMP and ROS levels

The polarized MMP was stained orange-red fluorescence in the control group, whereas the red fluorescence intensity was weakened, and the green fluorescence was enhanced after H_2O_2 treatment ($P < 0.01$). Compared with the H_2O_2 group, the MMP of NPMSCs pretreated with UA was still in the orange-red polarization state (Figure 5A-B) ($P < 0.01$).

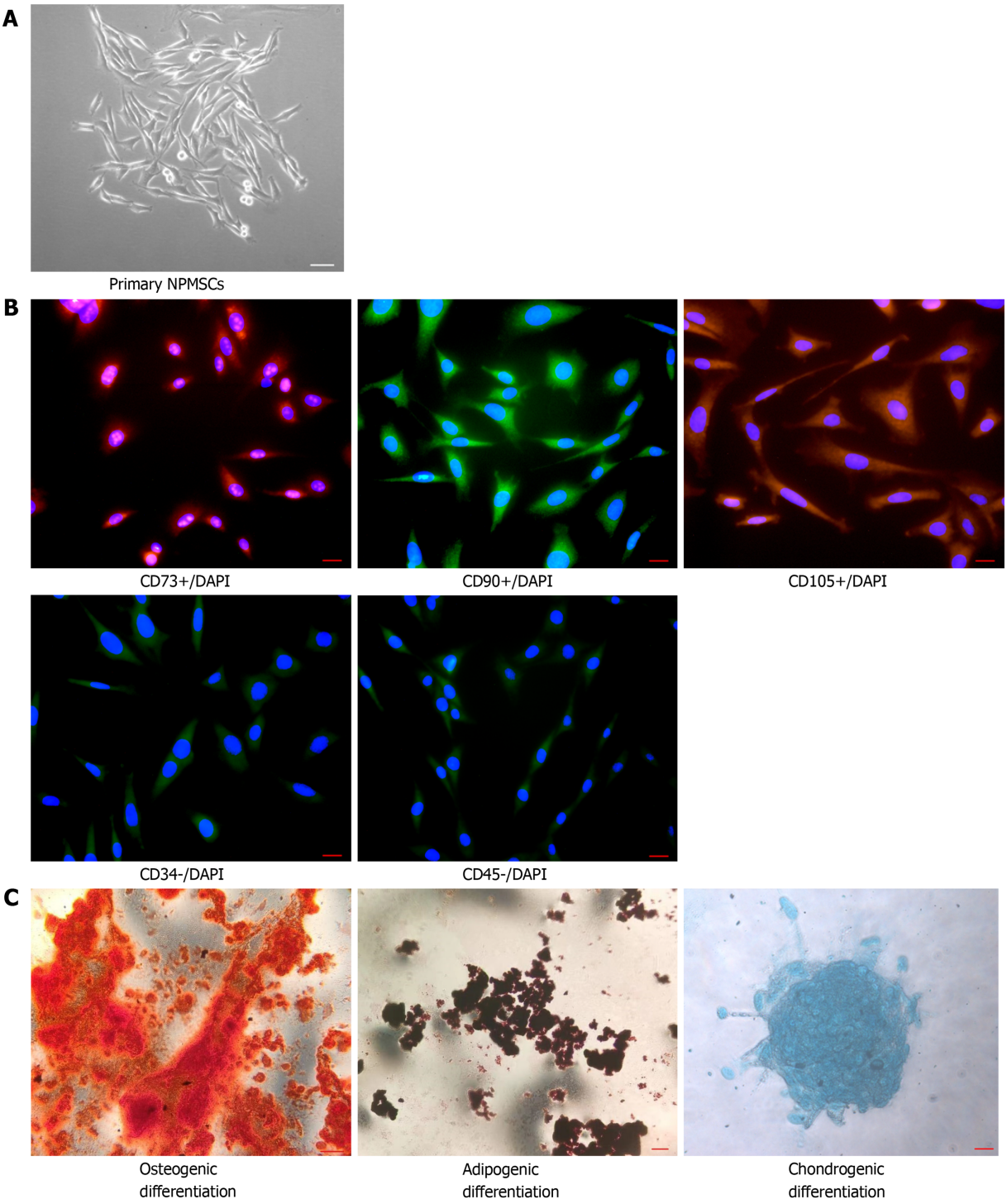

Figure 2 Identification of nucleus pulposus-derived mesenchymal stem cells. A: Isolated primary cells presented with a long spindle shape and grew in flower formation; B: Nucleus pulposus-derived mesenchymal stem cells (NPMSCs) exhibited high fluorescent expression of CD73, CD90, and CD105, but low fluorescent expression of CD34 and CD45; C: NPMSCs was positive for Alizarin red, Oil Red O, and Alcian blue staining after induced differentiation. Scale bar = 50 μm. NPMSCs: Nucleus pulposus-derived mesenchymal stem cells.

Excessive generation of ROS damages mitochondrial dynamics. As shown in Figure 5C-D, the ROS level of NPMSCs in the H_2O_2 group was significantly higher than that of the control group, and the ROS level of the H_2O_2 + UA group was significantly lower than that of the H_2O_2 group ($P < 0.01$). However, the protective effect of UA decreased after treatment with SR-18292 ($P < 0.01$). This result indicated that UA protected the dynamics of mitochondria through the PGC-1α signaling pathway and avoided the accumulation of ROS.

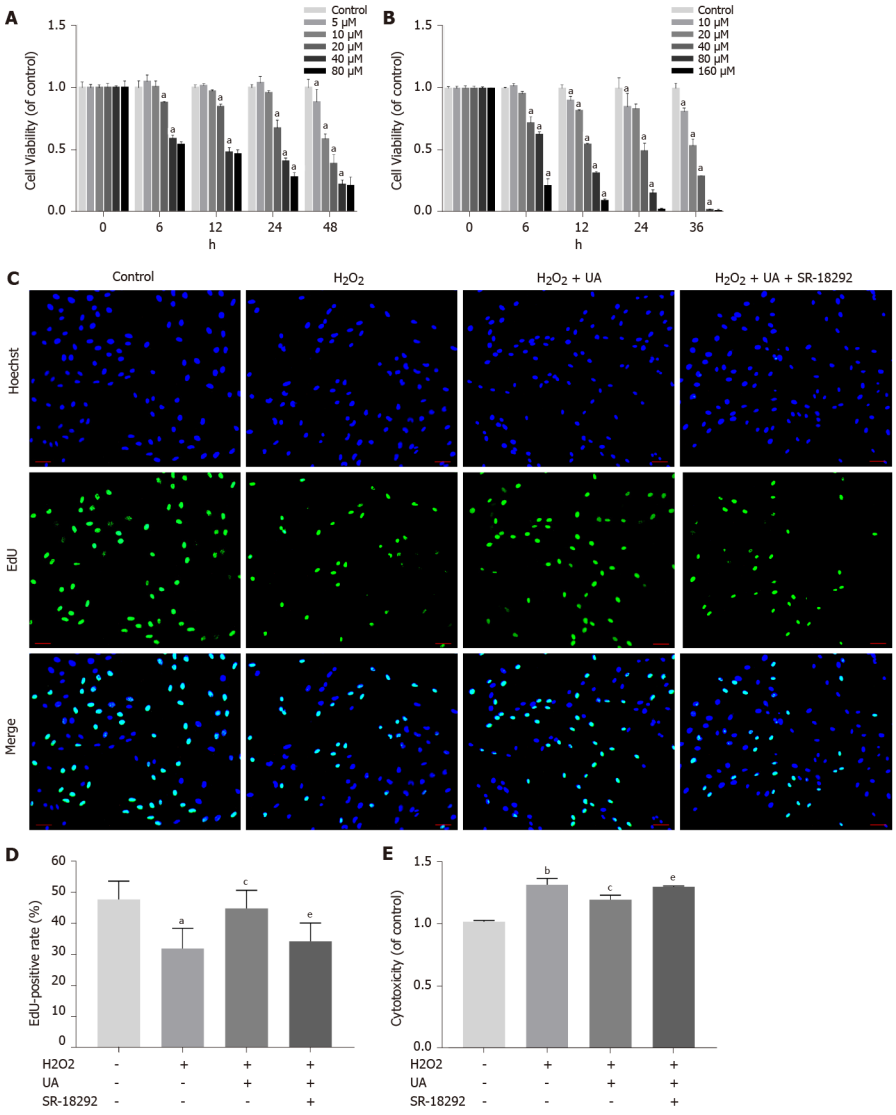

Figure 3 Cell viability assay, cell proliferation assay and cytotoxicity assay. A: Cell counting kit-8 (CCK-8) results of nucleus pulposus-derived mesenchymal stem cells (NPMSCs) treated with different concentrations and times of urolithin A (UA); B: CCK-8 results of NPMSCs treated with different concentrations and times of SR-18292; C: EdU assay results of NPMSCs in different groups. Green fluorescence represents cells in a proliferating state, and blue fluorescence represents cell nucleus (scale bar = 25 μm); D: Quantitative analysis of EdU results; E: Cytotoxicity results of NPMSCs treated with H_2O_2, H_2O_2 + UA and H_2O_2 + UA + SR-18292. All data are expressed as the mean ± SD. $^aP < 0.05$, $^bP < 0.01$ compared with control group; $^cP < 0.05$, $^dP < 0.01$ compared with H_2O_2 group; $^eP < 0.05$, $^fP < 0.01$ compared with H_2O_2 + UA group. CCK-8: Cell counting kit-8; NPMSCs: Nucleus pulposus-derived mesenchymal stem cells; UA: Urolithin A.

Measurement of senescence-related and SIRT1/PGC-1α pathway-related mRNA and proteins

We further evaluated the expression of senescence-related mRNA and proteins (P16 and P21) by western blotting and quantitative real-time polymerase chain reaction. As shown in Figure 6A-E, the expression of P16 and P21 in the H_2O_2 group was significantly increased compared with that in the control group ($P < 0.05$). However, the increased expression of P16 and P21 was alleviated by pretreatment with UA ($P < 0.05$). Moreover, pretreatment with SR-18292 weakened this protective effect of UA and decreased the expression of P16 and P21 ($P < 0.05$).

To investigate whether UA plays a role by activating the SIRT1/PGC-1α signaling pathway, related mRNA expression was evaluated. The results showed that the mRNA expression of SIRT1 and PGC-1α decreased after H_2O_2 treatment, but their expression was upregulated after UA treatment ($P < 0.05$). Then, pathway-related mRNA expression was reversed by treatment with the inhibitor SR-18292, which indicated that UA might exert a protective effect by activating the SIRT1/PGC-1α pathway (Figure 6F-G) ($P < 0.05$).

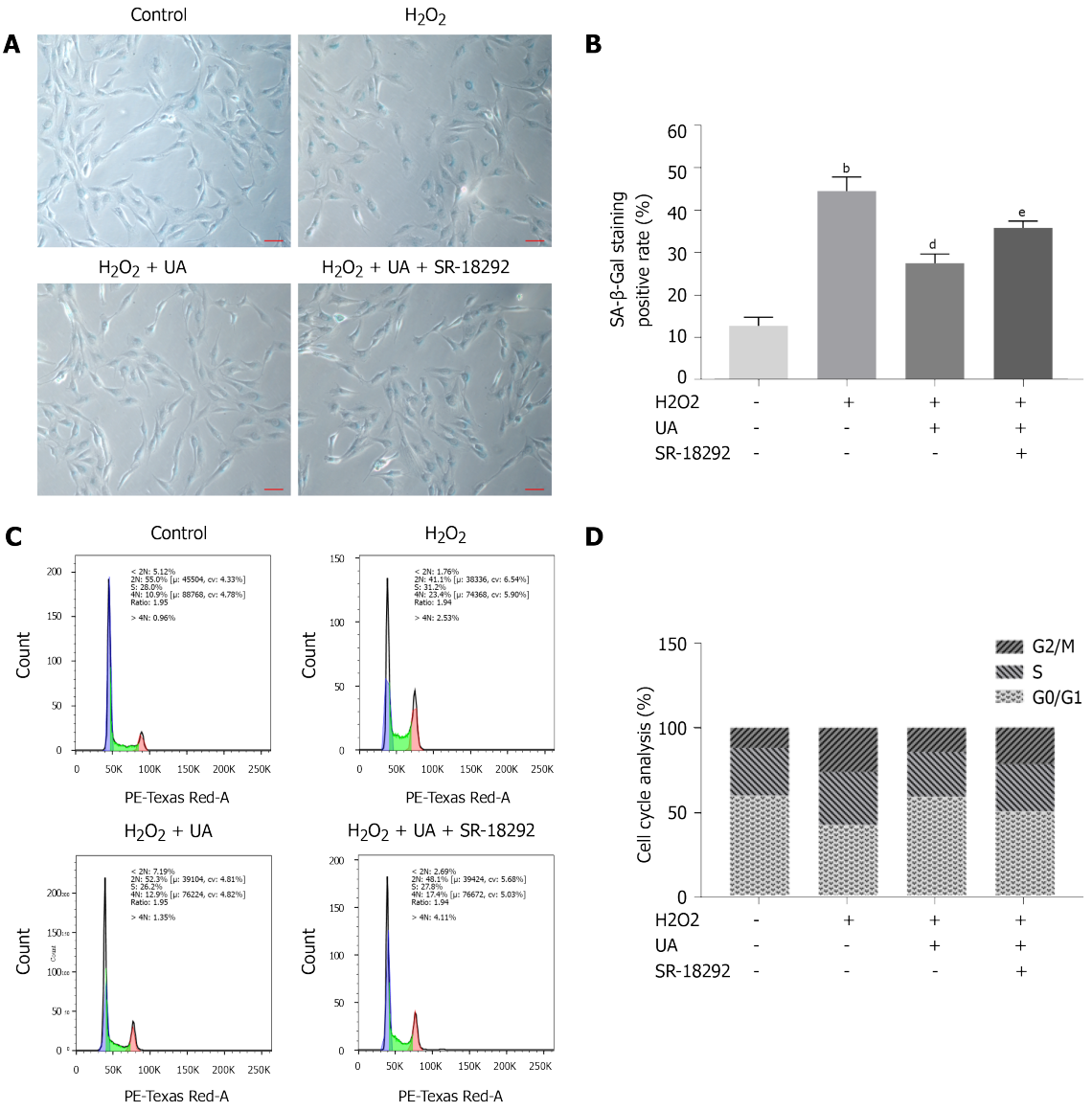

Figure 4 Senescence-associated β-Galactosidase staining assay and cell cycle assay. A: Senescence-associated β-Galactosidase (SA-β-Gal) staining results of nucleus pulposus-derived mesenchymal stem cells (NPMSCs) in different groups. Senescent cells exhibit blue-stained high expression of SA-β-Gal. (scale bar = 25 μm); B: Quantitative analysis of SA-β-Gal staining results; C: Cell cycle results of NPMSCs in different groups; D: Quantitative analysis of cell cycle results. All data are expressed as the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ compared with control group; $^{c}P < 0.05$, $^{d}P < 0.01$ compared with H_2O_2 group; $^{e}P < 0.05$, $^{f}P < 0.01$ compared with H_2O_2 + UA group. SA-β-Gal: Senescence-associated β-Galactosidase; NPMSCs: Nucleus pulposus-derived mesenchymal stem cells; UA: Urolithin A.

Radiographic and MRI evaluation

X-ray images were performed to evaluate disc height at 0 wk and 4 wk after needle puncture, and DHI was used to assess the disc height. As shown in Figure 7A-B, there was no significant difference in DHI among the three groups at 0 wk ($P > 0.05$). However, the DHI of the IDD group (0.040 ± 0.001) was significantly decreased compared with that of the control group (0.104 ± 0.005) at 4 wk ($P < 0.01$). Furthermore, the DHI of the UA group (0.068 ± 0.003) was significantly higher than that of the IDD group ($P < 0.01$).

The degree of IVD degeneration was measured by MRI according to the Pfirrmann grade at 0 and 4 wk after puncture. As shown in Figure 7C-D, the Pfirrmann grade scores at 0 wk among the three groups did not show any significant difference ($P > 0.05$). However, the Pfirrmann grade scores of the IDD group were significantly higher than those of the control group, and the Pfirrmann grade scores of the UA group were lower than those of the IDD group at 4 wk ($P < 0.01$). The results indicated that UA intervention could alleviate IDD *in vivo*.

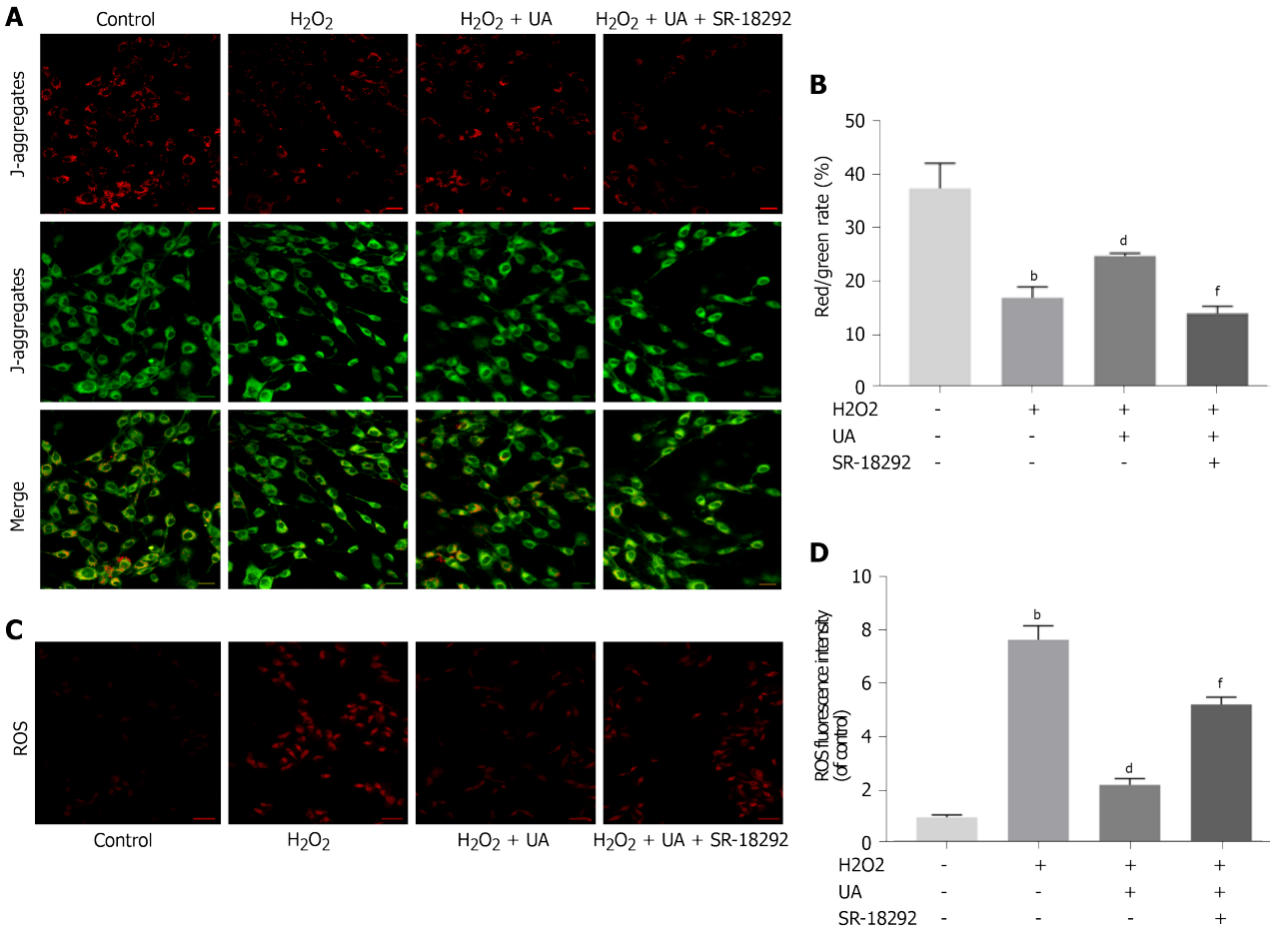

Figure 5 Mitochondrial membrane potential assay and reactive oxygen species assay. A: Results of mitochondrial membrane potential (MMP) in different groups detected by fluorescence. Red fluorescence represents the mitochondrial aggregate JC-1 and green fluorescence indicates the monomeric JC-1 (scale bar = 50 μm); B: Quantitative analysis of MMP results; C: Results of reactive oxygen species (ROS) in different groups detected by fluorescence. Red fluorescence represents high level of ROS (scale bar = 50 μm); D: Quantitative analysis of ROS results. All data are expressed as the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ compared with control group; $^{c}P < 0.05$, $^{d}P < 0.01$ compared with H_2O_2 group; $^{e}P < 0.05$, $^{f}P < 0.01$ compared with H_2O_2 + UA group. MMP: Mitochondrial membrane potential; ROS: Reactive oxygen species; UA: Urolithin A.

Histological analysis

As shown in Figure 8A-B, HE staining clearly showed inner well-structured gel-like NP and outer concentric ring-like annulus fibrosis, inner NP and cartilage endplates in the control group. In contrast, the well-structured IVD tissue was destroyed, and the NP tissue almost disappeared in the IDD group. However, a small number of NP cells and ECM still existed in the UA group. The histological score of the IDD group was also significantly lower than that of the control group, but UA treatment increased the histological score ($P < 0.01$).

Safranin-O staining showed that the proteoglycan matrix (red positive tissue) level was significantly lower in the IDD group than in the control group. However, UA treatment protected the proteoglycan matrix from decreasing, and the proteoglycan matrix level in the UA group was higher than that of the IDD group. Similarly, toluidine blue staining revealed more NP chondrocytes in the UA group than in the IDD group.

Measurement of collagen type II and aggrecan

The expression of collagen type II and aggrecan in the disc tissue was assessed by immunofluorescence. As shown in Figure 8C-E, only a small portion of NP tissues showed positive expression of collagen type II and aggrecan in the IDD group, which were significantly lower than those of the control group ($P < 0.01$). However, the expression of collagen type II and aggrecan was higher than that of the IDD group after treatment with UA for 4 wk, which indicates that UA treatment can delay the downward trend of collagen type II and aggrecan ($P < 0.05$).

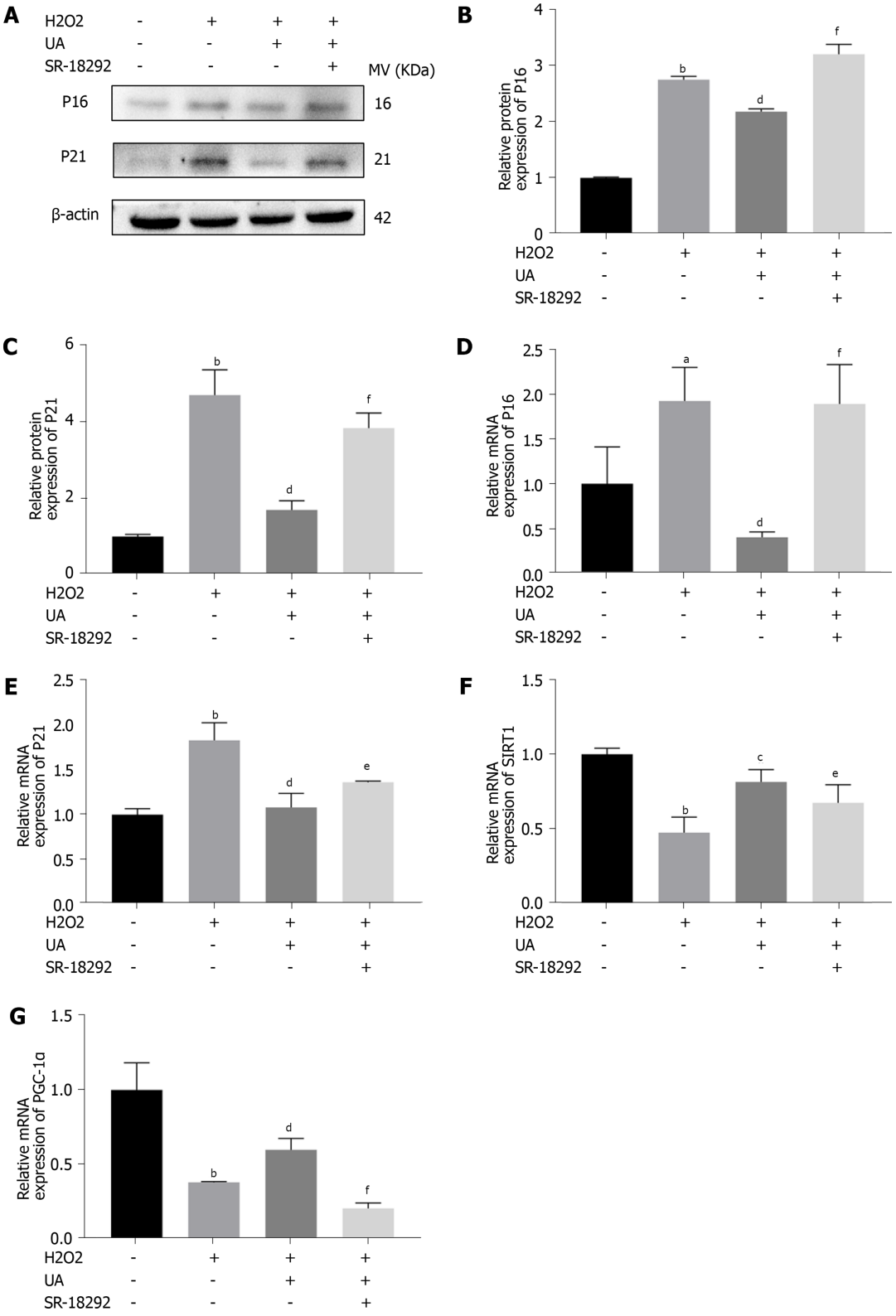

Figure 6 Senescence-related and SIRT1/PGC-1α pathway-related mRNA and proteins assay. A: The expression of senescence-related proteins (P16 and P21) in different groups; B-C: Quantitative analysis of P16 and P21 protein expression results; D-E: Quantitative analysis of P16 and P21 mRNA expression results; F-G: Quantitative analysis SIRT1/PGC-1α pathway-related mRNA. All data are expressed as the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ compared with control group; $^{c}P < 0.05$, $^{d}P < 0.01$ compared with H_2O_2 group; $^{e}P < 0.05$, $^{f}P < 0.01$ compared with H_2O_2 + UA group. SIRT1/PGC-1α: Silent information regulator of transcription 1/PPAR gamma coactivator-1α; UA: Urolithin A.

DISCUSSION

Our data demonstrated that UA could alleviate oxidative stress-induced NPMSCs senescence by activating the SIRT1/PGC-1α signaling pathway. NPMSCs harvested

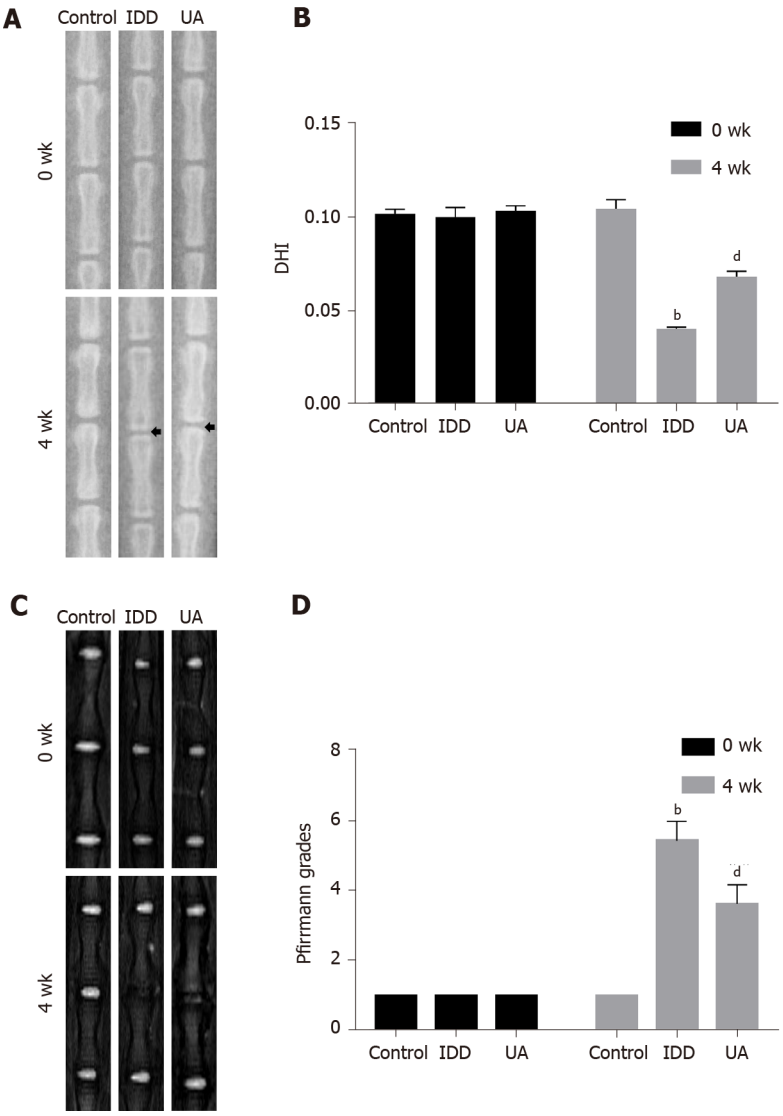

Figure 7 X-ray and magnetic resonance imaging evaluation in intervertebral disc degeneration animal models. A: The X-ray in different groups at 0 wk and 4 wk after puncturing; B: Quantitative analysis of the disc height index in different groups; C: The magnetic resonance imaging results of different groups at 0 wk and 4 wk after puncture; D: Quantitative analysis of Pfirrmann grades in different groups. All data are expressed as the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ compared with control group; $^{c}P < 0.05$, $^{d}P < 0.01$ compared with H_2O_2 group; $^{e}P < 0.05$, $^{f}P < 0.01$ compared with UA group. DHI: Disc height index; MRI: Magnetic resonance imaging; IDD: Intervertebral disc degeneration; UA: Urolithin A.

from the rat tails presented long spindle shapes and grew in flower formation. The ISCT for MCS has proposed the minimal criteria to define MSCs: (1) Plastic adherence characteristics; (2) Expression of CD105, CD73 and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules; and (3) Multilineage differentiation potential to osteoblasts, adipocytes and chondroblasts *in vitro*[21]. Acquired NPMSCs were also found to be positive for CD105, CD73 and CD90 and negative for CD45 and CD34 through immunofluorescence and to successfully differentiate into osteogenic, chondrogenic and adipogenic differentiation. According to the above results, the cells isolated from the NP tissues met the criteria stated by the ISCT.

H_2O_2 is commonly used to induce oxidative damage in mechanistic studies of IDD [20,22]. The inflammation, apoptosis and senescence of NP cells and the apoptosis and senescence of NPMSCs are important pathological models for exploring IDD, which can all be induced by H_2O_2 treatment[23-26]. In our previous study, we confirmed that H_2O_2 treatment could decrease the viability of NPMSCs in a dose- and concentration-dependent manner, and a concentration of 80 μM for 6 h could be used as a suitable concentration *in vitro*[20]. Cell senescence often exhibits the characteristics of irreversible cell cycle arrest, loss of proliferation capacity and reduced cell anabolic ability. We evaluated the senescence of NPMSCs induced by H_2O_2 through SA-β-Gal staining, cell cycle, cell proliferation ability and cytotoxicity. The results demonstrated

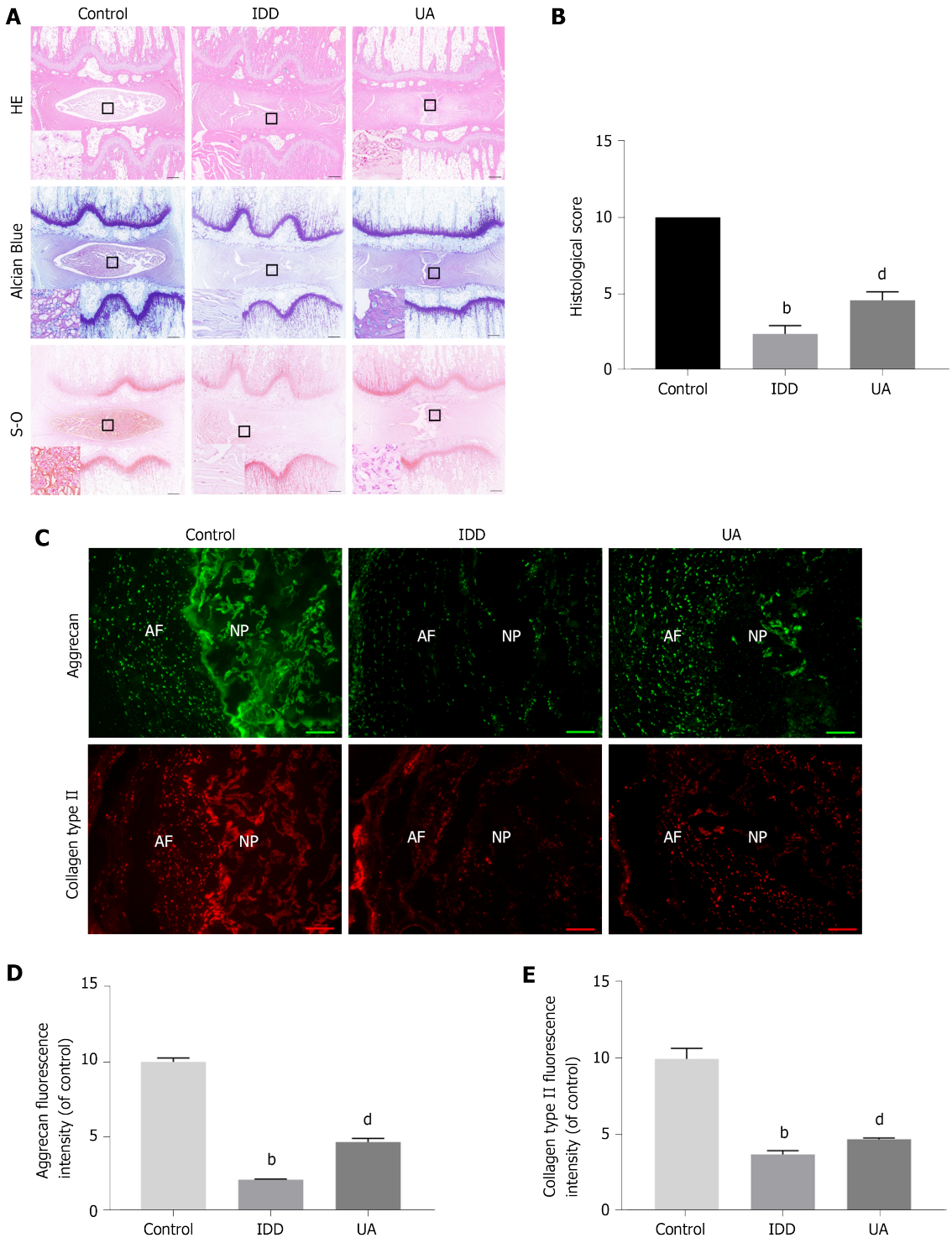

Figure 8 Hematoxylin-eosin, Safranin-O and Toluidine blue staining. A: Hematoxylin-eosin staining at 4 wk after puncture in different groups; B: Quantitative analysis of histological score in different groups (scale bar = 1 mm); C: The expression of aggrecan and collagen type II in different groups (scale bar = 200 μm); D-E: Quantitative analysis of aggrecan and collagen type II in different groups. All data are expressed as the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ compared with control group; $^{c}P < 0.05$, $^{d}P < 0.01$ compared with H_2O_2 group; $^{e}P < 0.05$, $^{f}P < 0.01$ compared with UA group. HE: Hematoxylin-eosin; NP: Nucleus pulposus; AF: Annulus fibrosus; IDD: Intervertebral disc degeneration; UA: Urolithin A.

that an increased positive rate of SA-β-Gal staining and cytotoxicity, arrested the cell cycle and weakened the cell proliferation ability were found after H_2O_2 treatment. As special markers, the expression of p16 and p21 is particularly important to reflect cell senescence and the cell cycle[27,28]. After H_2O_2 treatment, the decreased expression of p16 and p21 further confirmed that oxidative stress can induce NPMSCs senescence.

As a metabolite of ellagitannin and ellagic acid, UA shows anti-inflammatory, antioxidant and antiaging effects[10,11,13]. To explore the effect of UA on the sen-

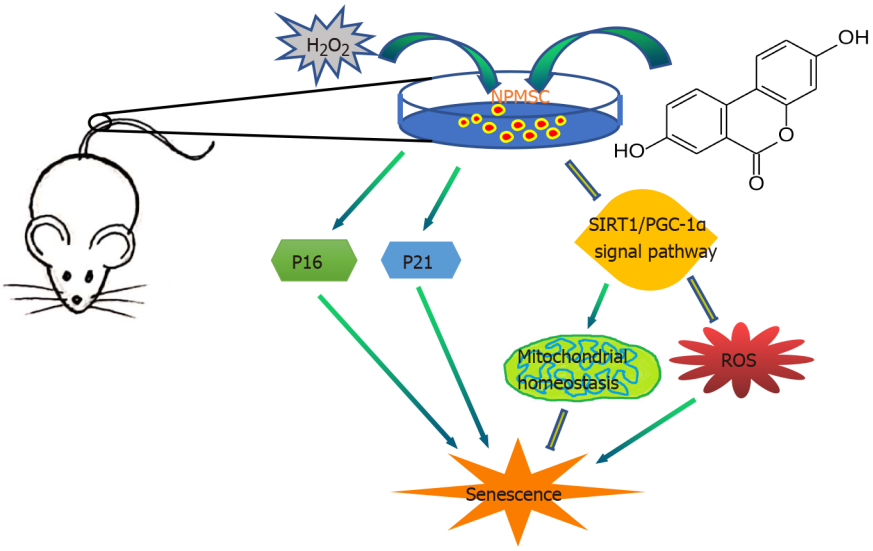

Figure 9 Schematic of protective effects of Urolithin A. Urolithin A activates the SIRT1/PGC-1α signaling pathway to protect mitochondrial function, alleviate nucleus pulposus-derived mesenchymal stem cells senescence *in vitro*, and delay intervertebral disc degeneration *in vivo*. SIRT1/PGC-1α: Silent information regulator of transcription 1/PPAR gamma coactivator-1α; NPMSCs: Nucleus pulposus-derived mesenchymal stem cells; UA: Urolithin A.

escence of NPMSCs, the effect of different concentrations and different time points of UA treatment on cell activity was assessed through CCK-8, and the results showed that 20 μM can be used as an appropriate intervention concentration. Then, NPMSCs were pretreated with UA before H_2O_2 intervention, and the results showed that the cell proliferation capacity was restored, the percentage of SA-β-Gal staining positive cells and the cytotoxicity induced by H_2O_2 was less than that of the H_2O_2 group, which indicated that UA might protect NPMSCs against oxidative stress damage. Moreover, the number of cells arrested in the G2/M phase decreased by 11.72% after UA pretreatment, allowing more cells to enter a new cell cycle.

A previous study found that UA potently prolongs the lifespan of C. elegans by activating mitochondrial biogenesis and mitochondrial functions[13]. Normal mitochondrial function is essential for maintaining intracellular energy metabolism [29]. During the process of senescence, cells show an increased number of mitochondria and decreased membrane potential of these mitochondria. Decreased mitochondrial function results in the release of mitochondrial enzymes and overdose production of ROS[30]. By detecting the MMP of NPMSCs, we found that the content of J-aggregates of MMP after H_2O_2 treatment was significantly reduced (red fluorescence downregulated), while the content of J-aggregates was partly recovered after UA treatment. Thus, UA might have an antiaging effect by regulating the function of mitochondria. Similarly, Cásedas *et al*[31] investigated the antioxidative and neuroprotective effects of UA on the murine Neuro-2a neuroblastoma cell line and found that UA could improve mitochondrial activity, decrease lipid peroxidation and enhance the activity of antioxidant-related enzymes in cells subjected to oxidative stress. Excessive accumulation of intracellular ROS is also an important factor in cell senescence[32]. Oxidative stress leads to excessive ROS production, which further leads to DNA damage, protein damage and mitochondrial dysfunction[33]. The intracellular ROS content of NPMSCs was also decreased after UA pretreatment. Therefore, regulating mitochondrial function and reducing ROS production may be the main mechanisms by which UA exerts antiaging effects on NPMSCs.

As a classic pathway regulating mitochondrial function, the SIRT1/PGC-1α pathway has been proven to be involved in the regulation of multiple pathological processes, such as antiaging and oxidative stress[34,35]. SIRT1, a member of the NAD+-dependent Sir2 histone deacetylase family, has been reported to regulate mitochondrial function and reduce oxidative stress[36]. PGC-1α is a key regulator of mitochondrial biogenesis and function that can be activated by SIRT1 through deacetylation[37]. SIRT1/PGC-1α pathway activation attenuates oxidative damage and protects against metabolic disease, whereas the decreased activation of the SIRT1/PGC-1α axis is often closely related to some diseases characterized by mitochondrial disorders[35,37,38]. To address whether UA regulates mitochondrial function by activating the SIRT1/PGC-1α pathway to delay the senescence of NPMSCs, NPMSCs were treated together with the PGC-1α pathway inhibitors SR-

18292 and UA. The results showed that the protective effect of UA was reversed by SR-18292. We also further evaluated the mRNA expression of SIRT1 and PGC-1α, and the results showed that the expression of SIRT1 and PGC-1α was significantly upregulated in the H_2O_2 + UA group compared with the H_2O_2 group. However, the mRNA expression levels of SIRT1 and PGC-1α were downregulated after SR-18292 treatment, which indicated that UA may have an antiaging effect by activating the SIRT1/PGC-1α pathway.

To evaluate the protective effect of UA more comprehensively, we also administered UA to IDD animal models. Delaying the loss of disc height and signal intensity of NP tissue also confirmed that UA can relieve IDD *in vivo*. Liu *et al*[15] found that UA treatment decreased matrix metalloproteinase production and the loss of collagen type II. We evaluated the expression of ECM at the histological level and found that UA can indeed delay the degradation of collagen type II and aggrecan, which further confirms that UA has a protective effect on degenerative IVD.

Admittedly, UA is reported to have pleiotropic properties, including the activation of signal pathways involving phosphatidylinositide 3-kinases, c-jun N-terminal kinase, nuclear factor-erythroid 2-related factor 2 and AMP-activated protein kinase[14,39,40]. However, in senescent NPMSCs induced by oxidative stress, we observed expression changes in pathway-related genes. Since we have not evaluated additional signaling pathways involved in the regulation of NPMSCs senescence, it is difficult to determine whether SIRT1/PGC-1α is the only pathway that regulates oxidative stress-induced NPMSCs senescence. Therefore, more signaling pathways that regulate the senescence of NPMSCs are worth exploring and discovering.

CONCLUSION

In summary, as shown in Figure 9, this study evaluated the protective effect of UA on oxidative stress-induced senescence in NPMSCs for the first time. H_2O_2 exposure could induce NPMSCs senescence and mitochondrial dysfunction. UA could activate the SIRT1/PGC-1α signaling pathway to protect mitochondrial function and alleviate cell senescence *in vitro*. UA could also delay ECM degradation and IDD *in vivo*. The results provide the possibility of promoting endogenous repair and retarding IDD.

ARTICLE HIGHLIGHTS

Research background

Intervertebral disc degeneration (IDD) is the main pathogenic factor of low back pain, but its pathological mechanism has not yet been elucidated. The isolation and identification of nucleus pulposus-derived mesenchymal stem cells (NPMSCs) provided a basis for the endogenous repair of IDD.

Research motivation

An unfavorable microenvironment of degenerative intervertebral disc such as inflammation, oxidative stress, and increased catabolism leads to increased senescence NPMSCs, which seriously affects endogenous repair. Therefore, rescuing the activity of NPMSCs and delaying cell senescence is of great significance to alleviate IDD.

Research objectives

The present study investigated whether urolithin A (UA) could alleviate NPMSCs senescence induced by oxidative stress and the potential mechanism.

Research methods

The protective effects of UA against oxidative stress-induced senescence in NPMSCs were investigated by evaluating the senescence-associated β-Galactosidase (SA-β-Gal) activity, cell cycle, cell proliferation ability, mitochondrial function and reactive oxygen species (ROS). Additionally, the expression of senescence-related and the silent information regulator of transcription 1/PPAR gamma coactivator-1α (SIRT1/PGC-1α) pathway-related proteins and mRNA was also used to evaluate the protective effects of UA *in vitro*. *In vivo*, an animal model of IDD were constructed, and X-rays, magnetic resonance imaging, and histological analysis were used to assessed whether UA could alleviate IDD *in vivo*.

Research results

in vitro, UA could reduce SA-β-Gal activity and senescence-related proteins and mRNA (P16 and P21) expression, alleviate cell cycle arrest and ROS production, stimulate cell proliferation ability and mitochondrial function by activating the SIRT1/PGC-1α pathway. *In vivo*, UA could alleviate an animal model of IDD by assessed the disc height index, Pfirrmann grade and the histological score.

Research conclusions

UA could activate the SIRT1/PGC-1α signaling pathway to protect mitochondrial function and alleviate cell senescence, and further delay extracellular matrix degradation and IDD, which provide the possibility of promoting endogenous repair and retarding IDD.

Research perspectives

We demonstrated the positive role of UA in attenuating oxidative stress-induced NPMSCs senescence and delaying IDD. UA may be successfully applied to IDD endogenous repair.

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