World Journal of
Stem Cells

World Journal of Stem Cells

Contents

Contents

ABOUT COVER

Editorial Board Member of *World Journal of Stem Cells*, Wei Zhang, PhD, Associated Professor, School of Medicine, Southeast University, No. 87 Ding Jiaqiao, Nanjing 210009, Jiangsu Province, China. zhang.wei@seu.edu.cn

AIMS AND SCOPE

The primary aim of *World Journal of Stem Cells* (*WJSC*, *World J Stem Cells*) is to provide scholars and readers from various fields of stem cells with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. *WJSC* publishes articles reporting research results obtained in the field of stem cell biology and regenerative medicine, related to the wide range of stem cells including embryonic stem cells, germline stem cells, tissue-specific stem cells, adult stem cells, mesenchymal stromal cells, induced pluripotent stem cells, embryonal carcinoma stem cells, hemangioblasts, lymphoid progenitor cells, *etc*.

INDEXING/ABSTRACTING

The *WJSC* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Journal Citation Reports/Science Edition, PubMed, PubMed Central, Scopus, Biological Abstracts, BIOSIS Previews, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database. The 2022 Edition of Journal Citation Reports cites the 2021 impact factor (IF) for *WJSC* as 5.247; IF without journal self cites: 5.028; 5-year IF: 4.964; Journal Citation Indicator: 0.56; Ranking: 12 among 29 journals in cell and tissue engineering; Quartile category: Q2; Ranking: 86 among 194 journals in cell biology; and Quartile category: Q2. The *WJSC*'s CiteScore for 2021 is 5.1 and Scopus CiteScore rank 2021: Histology is 17/61; Genetics is 145/335; Genetics (clinical) is 42/86; Molecular Biology is 221/386; Cell Biology is 164/274.

RESPONSIBLE EDITORS FOR THIS ISSUE

Therapeutic potential of dental pulp stem cells and their derivatives: Insights from basic research toward clinical applications

Sheng-Meng Yuan, Xue-Ting Yang, Si-Yuan Zhang, Wei-Dong Tian, Bo Yang

Sheng-Meng Yuan, Xue-Ting Yang, Si-Yuan Zhang, Wei-Dong Tian, Bo Yang, State Key Laboratory of Oral Diseases, National Clinical Research Center for Oral Diseases, Engineering Research Center of Oral Translational Medicine, National Engineering Laboratory for Oral Regenerative Medicine, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, Sichuan Province, China

Sheng-Meng Yuan, Xue-Ting Yang, Wei-Dong Tian, Bo Yang, Department of Oral and Maxillofacial Surgery, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, Sichuan Province, China

Corresponding author: Bo Yang, DDS, PhD, Academic Fellow, Chief Doctor, State Key Laboratory of Oral Diseases, National Clinical Research Center for Oral Diseases, Engineering Research Center of Oral Translational Medicine, National Engineering Laboratory for Oral Regenerative Medicine, West China Hospital of Stomatology, Sichuan University, No. 14 3rd Section, Renmin South Road, Chengdu 610041, Sichuan Province, China. dr.yangbo@scu.edu.cn

Abstract

For more than 20 years, researchers have isolated and identified postnatal dental pulp stem cells (DPSCs) from different teeth, including natal teeth, exfoliated deciduous teeth, healthy teeth, and diseased teeth. Their mesenchymal stem cell (MSC)-like immunophenotypic characteristics, high proliferation rate, potential for multidirectional differentiation and biological features were demonstrated to be superior to those of bone marrow MSCs. In addition, several main application forms of DPSCs and their derivatives have been investigated, including stem cell injections, modified stem cells, stem cell sheets and stem cell spheroids. *In vitro* and *in vivo* administration of DPSCs and their derivatives exhibited beneficial effects in various disease models of different tissues and organs. Therefore, DPSCs and their derivatives are regarded as excellent candidates for stem cell-based tissue regeneration. In this review, we aim to provide an overview of the potential application of DPSCs and their derivatives in the field of regenerative medicine. We describe the similarities and differences of DPSCs isolated from donors of different ages and health conditions. The methodologies for therapeutic administration of DPSCs and their derivatives are introduced, including single injections and the transplantation of the cells with a support, as cell sheets, or as cell spheroids. We also summarize the underlying mechanisms of the regenerative potential of DPSCs.

Key Words: Dental pulp stem cells; Cell injections; Modified cells; Cell sheets; Cell spheroids; Regeneration

Core Tip: In this review, we aim to outline the present understanding of the potential application of dental pulp stem cells (DPSCs) and their derivatives in the field of regenerative medicine. DPSCs have different properties and regenerative potentials according to the age and health condition of the donor. For therapeutic applications, DPSCs can be administered through different methodologies, including by single injections and the transplantation of the cells and their derivatives with a support, as cell sheets or as cell spheroids. The underlying mechanisms of the regenerative potential of DPSCs and their derivatives may occur through direct regulation and immunomodulatory and paracrine effects.

INTRODUCTION

Dental pulp stem cells (DPSCs) have received major attention since they were first isolated in 2000 due to their easily accessible properties, lack of ethical problems, high proliferation ability and multidirectional differentiation potential[1-3]. Since then, numerous studies have emerged on the extraction and identification of DPSCs. Scholars have obtained DPSCs from a variety of dental sources and confirmed that they are superior to bone marrow mesenchymal stem cells (MSCs) in various characteristics, such as easier access, higher proliferation, and better neural differentiation[4-8].

In view of their excellent stem cell characteristics, DPSCs, as an important source of postnatal MSCs, have been widely studied in the field of regenerative medicine in the past two decades, including but not limited to cerebral ischemia[9], bone and dental loss defects[10,11], the nervous system[12], the digestive system[13] and the endocrine system[14], and many studies have achieved meaningful therapeutic effects. Meanwhile, in the application of regenerative medicine, with the continuous progress of cell culture and modification technologies, the application forms of MSCs continue to expand, such as the transformation from primitive cells to modified cells and the leap from single-cell preparations to multicell units[15-17]. These enhanced stem cell applications play an important role in improving the therapeutic effect of stem cells, which promotes the development of tissue engineering and regenerative medicine.

However, the mechanism by which DPSCs promote regeneration has not been fully revealed. At present, two main aspects of MSCs, immunomodulatory and paracrine effects, have been widely discussed[18-20]. MSCs have been suggested to possibly be involved in the process of immune regulation in the host by regulating the physiological functions of immune cells such as T cells, B cells, dendritic cells (DCs), and natural killer (NK) cells[21] or transmitting intercellular signals through paracrine pathways such as secretomes, exosomes and extracellular vesicles (EVs)[22-24], thereby inhibiting inflammation and promoting disease improvement. In addition, transplanted stem cells may directly promote the repair or regeneration of tissue injury by residing at the transplant site and differentiating into corresponding cells[25-29]. As a promising member of the MSC family, DPSCs may also play a role through the above mechanism in the process of promoting injury repair and reconstruction or disease improvement[18,30,31].

In this review, we introduce DPSCs from different sources and their characteristics and discuss several main applications of DPSCs in regenerative medicine in recent years, including cell injections (cell suspensions), modified cells, cell sheets and cell spheroids. We introduce the background, biological characteristics, representative examples and preliminary therapeutic effects of these various derivatives in regenerative medicine and briefly summarize the possible mechanisms of DPSCs in promoting regeneration.

SOURCES OF DPSCS

Since their discovery, DPSCs have garnered extensive attention due to their easily accessible features and lack of ethical issues. In 2000, Gronthos *et al*[1] isolated clonogenic and highly proliferative cells

from enzymatically disaggregated dental pulp tissue of normal human impacted third molars for the first time. The isolated cells were termed DPSCs[1]. Since then, many researchers have focused on DPSCs and successfully isolated these cells from dental pulp tissue of different ages and different states, as well as using cell sorting technologies to isolate subsets of cells with special phenotypes. These DPSCs have both the same characteristics and obvious differences.

DPSCs from teeth of different ages

To date, researchers have extracted DPSCs from teeth of different age groups, including children (deciduous teeth), adolescents, adults, aged (permanent teeth) and even infant donors (natal teeth)[1,32-34]. The most common are DPSCs from adult permanent teeth (generally less than 30 years old) and exfoliated deciduous teeth.

Adults (generally less than 30 years old): Adult human health dental pulp tissue derived dental pulp cells, as subsequently demonstrated by Gronthos *et al*[1], contained clonogenic cell populations within them that have the ability to form clones. Although colonies of dental pulp cells occurred at a similar frequency in comparison to bone marrow mesenchymal stem cells (BMSCs) from bone marrow aspirates flushed free of hematopoietic cells, DPSCs exhibited a higher proliferation rate than BMSCs *in vitro*, and they maintained their high rate of proliferation even after extensive subculturing[1]. In view of this phenomenon, the authors believe that DPSCs satisfy two of the criteria of a postnatal somatic stem cell: *ex vivo* expansion and clonogenicity[35]. Also, transplanted DPSCs can generate a dentin-pulp-like complex representative of the microenvironments from which they were derived *in vivo*, which underscores one of their stem cell natures again: Tissue reconstitution[1,35]. Later studies improved the immunophenotypic identification of DPSCs from adult healthy teeth and repeatedly confirmed their multidirectional differentiation ability, finding that DPSCs are positive for mesenchymal lineage markers (CD13, CD29, CD44, CD73, CD90 and CD105) and negative for monocytic (CD14) and hematopoietic lineage markers (CD34, and CD45). At the same time, DPSCs have the potential to differentiate into typical mesodermal cell lineages, such as osteogenic, chondrogenic, and adipogenic lineages [4-7]. In conclusion, DPSCs derived from adult healthy dental pulp have the phenotypic characteristics and multidirectional differentiation ability of MSCs and meet the criteria for postnatal somatic stem cells.

Children (deciduous teeth): In 2003, Miura *et al*[32], for the first time, isolated stem cells from exfoliated human deciduous teeth, named stem cells from human exfoliated deciduous teeth (SHED), which were also identified as a population of highly proliferative, clonogenic cells capable of differentiating into a variety of cell types, including odontoblasts, endothelia, neural cells and adipocytes[32,36,37]. They could also express MSC markers such as CD29, CD73, CD90, CD105, CD146, and STRO-1[32,38,39]. However, SHED showed higher expression of CD105 and CD146 than DPSCs, suggesting that SHED is a unique undifferentiated stem cell lineage and may have a higher capacity for differentiation[38]. Subsequent studies confirmed the differences between SHED and adult DPSCs, including that the SHED exhibited more colony forming units, shorter doubling time, higher proliferation rate, higher endothelial differentiation potential, stronger osteogenic and adipogenic differentiation ability *in vitro* and osteoinductive capacity *in vivo*[32,38,40,41] *etc*. Even under the adverse culture conditions of hypoxia, high glucose and low serum, the above characteristics of SHED were still better than the characteristics of DPSCs[42]. However, the neurogenic ability of SHED is lower than the neurogenic ability of DPSCs[43,44]. In short, from the current findings, SHED may be a better seed cell player than DPSCs in addition to neurogenic ability, which may be attributed to its younger physiological age.

Other ages: In addition to the above two common sources of DPSCs, another study reported that DPSCs obtained from natal teeth also have the immunophenotypic characteristics (expressed CD13, CD44, CD73, CD90, CD146, and CD166, but not CD3, CD8, CD10, CD11b, CD14, CD15, CD19, CD33, CD34, CD45, CD71, CD117, and HLA-DR) and multidirectional differentiation (adipogenic, osteogenic, chondrogenic, myogenic and neurogenic) potential of MSCs[45]. In addition, compared with the deciduous pulp, the expression of nestin and CD44 was stronger in the dental pulp of natal teeth. Positive immune expression of SOX2 (embryonic stem cell marker) was observed only in the dental pulp of natal teeth, which confirms the presence of a higher percentage of stem/progenitor cell population compared with the deciduous pulp[34]. In addition, Wu *et al*[33] specifically compared the growth and differentiation characteristics of DPSCs from patients of different ages, including children, adolescents, adults and aged donors. The results showed that although a large portion of cell surface markers was expressed in all DPSC lines, the expression of CD29 was downregulated in the DPSCs from aged teeth. At the same time, the doubling time of DPSCs from aged teeth was prolonged, and the number of apoptotic cells increased with propagation. Moreover, these DPSCs from aged teeth were completely or partially deprived of lineage differentiation capacity[33]. These results suggest that DPSCs from younger ages are more suitable as excellent candidates for regenerative medicine stem cell resource banks.

DPSCs from different pulp health statuses

Although the sources of healthy dental pulp are very extensive, the sources of unhealthy dental pulp are more abundant with the trend of frequent occurrence of caries, dental pulp diseases and periodontitis. Therefore, some researchers have turned their attention to the field of dental pulp in different healthy states to expand the potential source range of DPSCs.

Yu *et al*[46] reported that stem cells from inflamed pulp of deciduous teeth (SCIDs) were positive for cell surface markers, including CD105, CD90, and CD146, and had high proliferation ability and osteogenic, adipogenic, and chondrogenic differentiation potentials. Except for SCIDs that secreted more tumor necrosis factor-α (TNF-α) protein, there was no significant difference in proliferation and differentiation potentials between SCIDs and SHED[46]. Pereira *et al*[47] also supported that the morphology, proliferation rate and differentiation potential of inflamed and normal DPSCs are similar [47].

However, Kim *et al*[48] and Alongi *et al*[49] reported different results: The abilities of colony forming and osteogenic differentiation *in vitro* and *in vivo* of stem cells from inflamed pulp tissue were decreased compared with normal DPSCs[48,49]. Another study also confirmed that DPSCs from carious teeth (DPSCs-CT) are not as efficient as normal DPSCs in differentiating into dopaminergic-like cells[50]. The colony forming capacity of stem cells isolated from pulp polyps (chronic hyperplastic pulpitis) and dental pulp with irreversible pulpitis was also lower than the colony forming capacity of healthy DPSCs, but both cell types (normal and pulpitis) have demonstrated the ability to form pulp/dentin complexes when transplanted into immunocompromised mice[51,52].

Interestingly, strong staining for CD146 was observed in inflamed pulps during the initial inflammatory response, and short-term TNF-α treatment has also been confirmed to enhance dental pulp cell function, including the ability to form cell colonies, migrate, and differentiate into odontogenic and adipogenic lineages[53]. Moreover, researchers have also identified and studied DPSCs isolated from teeth with different stages of aggressive periodontitis (representing different degrees of dental pulp inflammation) to explore the impact of periodontal infection during the progression of periodontitis on the pluripotency and regenerative potential of DPSCs within periodontally compromised dental pulp. The results showed that periodontal inflammation had a negative impact on the colony forming ability and proliferation of DPSCs, but the relationship between the effect on their pluripotency and the severity of the disease was uncertain. Although DPSCs from the inflamed dental pulp of teeth extracted due to aggressive periodontitis appear to have undergone some changes in terms of their stem cell properties, they still possess the capacity to differentiate into odontoblastic and osteoblastic phenotypes *in vitro* and form pulp- and dentin-like tissues *in vivo*[54]. These data indicate that the stem cell characteristics of unhealthy dental pulp-derived stem cells are unstable, which may be related to the degree of inflammation in the diseased dental pulp. Further characterization is needed to determine whether they can serve as a source of therapeutic cells for future regenerative therapies.

Subpopulations of DPSCs

DPSCs showed heterogeneity in the earliest isolation and identification studies: In primary cultured DPSCs, many of the phenotypic markers were not uniformly expressed but were found in subsets of cells, which may represent different pulp cell lineages[1], indicating that there are different cell subpopulations in the primary DPSCs isolated and cultured directly, which may dominate the different cell fates of stem cells. The development and progress of cell sorting technologies make the separation of cell subsets possible.

Currently, DPSC subpopulations that have been isolated include but are not limited to side population cells with stem cell phenotypic characteristics[55], bromodeoxyuridine (BrdU)-labeled label-retaining cells for the localization of dental pulp stem/progenitor cells[56], ALDH1+ cell subpopulations reflecting DPSC niches[57], DPSCs mobilized by G-CSF (MDPCs) with high proliferation rates and stability[58], CD34−/CD34+ DPSCs with different neural differentiation potentials[59], thy-1-positive cells in the subodontoblastic layer with the ability to differentiate into hard tissue-forming cells [60], and CD271+ dental MSCs with high odontogenic potential[61]. These subsets reflect the origin or location of stem cells or have high potential in a particular direction of differentiation. For example, a group of unique multipotent stem cells were recently identified from mouse dental papilla called multipotent dental pulp regenerative stem cells, exhibiting enhanced osteogenic/odontogenic differentiation capabilities *in vitro* and *in vivo* and efficiently regenerating functional pulpo-dentinal complex-like tissues *in vivo*[62]. CD146+ human DPSCs have also been reported to be able to regenerate an increased area of dentin/pulp-like structures *in vivo* compared with their CD146- counterparts or mixtures of the two[63]. These results suggest that the use of DPSCs is flexible in tissue engineering, and taking advantage of a specific side group may be the pathway to achieve accurate and efficient tissue regeneration.

Regardless of the source of DPSCs, DPSCs are used in the later description of this article, and no special distinction is made when describing their applications in regenerative medicine.

DERIVATIVES OF DPSCS

Stem cell-based tissue engineering has been developed for many years, during which the application forms of stem cells have undergone major changes from primitive cells to genetically modified cells and from discrete stem cell suspensions to multicellular units, prompting regenerative medicine to take a big step forward. Here, we will discuss several main derivatives of DPSCs derived from long-term application, including cell injections, genetically modified cells, cell sheets and cell spheroids, and introduce their formation background, preparation techniques, biological characteristics and examples of their application in regenerative medicine.

DPSC injections

The transplantation of DPSCs has been investigated as a potential therapy for a variety of injuries and diseases, including but not limited to stroke[64], spinal cord injury[65], cerebellar ataxia[66], retinal degeneration[15], diabetic neuropathy[67], parotid gland injury[68], cystitis[69], Sjögren syndrome[70], *etc*. In the treatment of these diseases using DPSCs, injection is one of the more commonly used forms. Stem cell injections are the easiest cell therapy products to prepare, and the commonly used vehicles at present for resuspension of stem cells is phosphate-buffered saline[69-73], Hank's Balanced Salt Solution [15], normal saline[14,67,74-76], and modified medium[66,68,77-79]. These mediators have no therapeutic effect and only serve as transport vectors for stem cells, so it is possible to observe the monotherapy effect of transplanted stem cells and rule out the influence of carriers. In addition, DPSC injections are usually administered by local injection[15,66,69,80], intramuscular injection[67,76,81] and intravenous injection[67,68,72,82]. Different methods of administration lead to different systemic distributions and different efficacies.

A study by Kim *et al*[83] indicated that intravenously injected human DPSCs *via* the tail vein in nude mice were distributed mostly to the lungs and rarely detected in other organs at all observed time points [83]. Another study confirmed that intravenously administered DPSCs did not show liver and kidney migration in Sprague-Dawley rats[72]. However, another study suggested that SHED transplantation *via* the tail vein in nonobese diabetic mice was observed mainly in the liver and spleen[70]. The mode of administration by intravenous injection can be seen to possibly cause drug distribution in multiple organs. However, locally administered DPSCs rarely migrated to other organs over time, *e.g.*, DPSCs were transplanted into the pulp chamber[83]. This result indicated a differential distribution pattern of transplanted DPSCs between the intravenous and local injections. Both intravenously and locally injected DPSCs have been shown to improve symptoms of various lesions[15,72,76,80]. DPSCs have also been shown to migrate and integrate into the site of injuries[15,69,73,74] and differentiate into corresponding cells[68,72,75,79,84].

Shahani *et al*[71] traced the biodistribution of intramuscularly transplanted human DPSCs in immunocompetent healthy rats. The results showed that DPSCs started entering into the blood vessels adjacent to the muscle at hour 24 and gradually metastasized, but the signal intensity in the muscles at the injection site remained highest, serving as a repository for DPSCs in transplantation. Intramuscular injection also avoids the lung "first pass effect" compared with intravenous injection, prolonging the survival of transplanted stem cells in the body and thus providing a sustained delocalized benefit for systemic diseases[71]. Datta *et al*[67] compared the effects of intramuscular and intravenous injection of DPSCs on diabetic neuropathy, and a more rapid improvement in neuropathic symptoms was observed for DPSC intravenous transplantation. However, DPSC intramuscular injection, especially after repeated administration, maintained the improved inflammatory state[67]. The study of Hata *et al*[14] also confirmed that the therapeutic effects of DPSC transplantation with a single intramuscular injection lasted for prolonged periods[14], further demonstrating the continued efficacy of DPSC intramuscular administration. Another study demonstrated that the efficacies of DPSC intramuscular transplantation were limited to the administration site, but it was difficult to play a role on the opposite side[81], possibly related to the number of DPSCs that migrated.

In summary, intravenously administered DPSCs are distributed mainly in the lungs and can also be transferred to the injured area; however, locally injected DPSCs are less likely to migrate to other parts of the body, whereas intramuscularly injected DPSCs may enter into the blood vessels and metastasize but are distributed mainly in the local muscles and less in the lungs and persist longer *in vivo*. Intravenous administration works more quickly than intramuscular administration, but the effects of intramuscular transplantation last longer, while the effect of local injection is mostly limited to the site of administration. In conclusion, regardless of the injection mode, DPSC injections have shown certain therapeutic effects in the treatment of various diseases.

Modified DPSCs

The combination of cell therapy and genetic engineering has resulted in genetically engineered cells, which are considered to have greater prospects of therapeutic potential and efficient treatment than nonengineered cell therapy approaches[85]. Although there are still many factors to consider before genetically modified stem cells can be directly used in regenerative medicine[86], gene therapy, as an increasingly mature discipline, has profoundly influenced the development of regenerative medicine.

Genetically engineered cell therapy using primary cells that overexpress tissue-specific or therapeutic genes makes it possible to produce therapeutic proteins at sites of regeneration or to differentiate new cells into the desired cellular lineage and thus promote tissue regeneration[87]. Obtaining the carrier cells, genetically modifying and expanding the cells *in vitro* and then using the cells for disease therapeutic strategies *in vivo* is a common method of applying genetically engineered cell therapy[88]. Genetically engineered cell therapy often uses MSCs as gene delivery vectors due to their accessibility for genetic modification *in vitro* and their ability to be cultured and expanded *in vitro*[85,87-89]. DPSCs, a type of MSC, are characterized by self-renewal, multipotent differentiation potential and amplification *in vitro*, as well as easy access, low risk of immune rejection and fewer ethical issues; DPSCs are also considered ideal gene vehicles with wide application prospects[16].

Compared with unmodified DPSCs, genetically modified DPSCs have been shown to be more potent in treating various diseases. For instance, compared with DPSCs, DPSCs overexpressing hepatocyte growth factor have been shown to dramatically relieve the disease activity of dextran sulfate sodium-induced ulcerative colitis[16], promote improvements in postischemia/reperfusion brain injury[90], promote the grafted DPSC-induced hepatic functional recovery from liver cirrhosis in a rat model[91], significantly improve periodontal bone regeneration in swine[92] and have a stronger capacity to significantly reduce ovariectomy-induced bone loss in the trabecular bone of the distal femur metaphysis[93]. Similarly, Rizk *et al*[94] engineered sizable three-dimensional (3D) cartilage-like constructs using human DPSCs, and the results showed that constructs with transforming growth factor (TGF)-β3-DPSCs showed higher collagen type II and Sox9 mRNA expression than nontransduced DPSC constructs *in vivo*[94]. Gene therapy using Runx2-modified DPSCs has also been reported to be more effective in tibial distraction osteogenesis during bone deposition and new bone formation[95].

These studies indicated that genetically modified DPSCs can not only play the role of DPSCs themselves but also secrete specific therapeutic proteins to enhance their therapeutic effects, which is a major direction of innovative applications of DPSCs.

DPSC sheets

Recently, as a cell transplantation system that requires no scaffolds or carriers, cell sheet engineering has gradually become the research focus of regenerative medicine based on cell therapies and has been used for regenerative treatment of the esophagus, cornea, heart, *etc*[96-98]. In contrast to conventional tissue engineering approaches, cell sheet technology allows cell harvest as a continuous cell sheet with intact extracellular matrix (ECM) proteins and cell-cell junctions, which facilitates cell transplantation without any other artificial biomaterials[99]. Compared with the traditional tissue engineering of cell suspensions combined with bioscaffolds, cell-sheet transplantation can better fix the transplanted cells at the graft site without considering the negative effects of scaffold material degradation[100,101]. In this trend, DPSC sheets also emerged.

According to previous reports, the initial study used temperature-responsive cell culture dishes to prepare the DPSC sheet and used it to successfully reconstruct the corneal epithelium[102]. The dish responds to temperature changes, allowing the formed DPSC sheets to automatically shed. Subsequent studies also confirmed that DPSC sheets can be prepared within 3-4 d using temperature-responsive cell culture dishes[103,104]. Although this method is not time-consuming, it requires the use of special materials and complicated production procedures, which limit its expanded use. Therefore, some researchers have developed a method of using vitamin C (VC) to induce DPSCs to form cell sheets[105], and it has gradually become the mainstream preparation method.

VC is an essential micronutrient for humans, a potent antioxidant and a cofactor for a family of biosynthetic and gene regulatory enzymes, and VC plays an important role in supporting the function of the immune system[106]. VC also plays a key role in the biosynthesis of collagen and other ECM constituents[107,108] and promotes the proliferation of stem cells without affecting their differentiation potential[109]. Therefore, some researchers have predicted that VC alone may induce cell sheet formation, streamline production procedures or avoid using special materials, and confirmed this prediction with their studies, developing a simple and inexpensive VC-mediated procedure to obtain MSC sheets[105]. The authors also explored the mechanism of VC-induced cell sheet formation, and the optimal dose of VC showed that VC is capable of inducing telomerase activity in MSCs, leading to upregulated expression of ECM and stem cell markers. Meanwhile, VC induces MSCs to form cell sheets in a dose-dependent manner, and 20 μg/mL VC is the optimal concentration for complete cell sheets with a high level of success. However, in the existing reports, the concentration of VC alone to induce DPSCs to form sheets ranged from 10 μg/mL to 100 μg/mL[110-114], incubated continuously for 10-15 d or until the edge of the cell sheet became slightly rolled up or spontaneously detached from the bottom of the dish, all of which resulted in the formation of operable sheets consisting of 2-3 layers of cells. It has even been reported that the harvested whole DPSC sheet contained five or six layers of cells [17]. Although the use of VC has the above characteristics, the time-consuming disadvantage is also very prominent.

In addition, some other studies have seeded cells on the surface of the amniotic membrane (AM)[115, 116] or even directly cultured them in basic medium[117] to obtain DPSC sheets. For example, after DPSCs were seeded on the amniotic membrane and cultured for 2 wk, the cells became confluent and formed 1-3 layers of cell sheets that adhered to the basement membrane AM[115]. DPSC sheets have

also been reported to be composed of multilayer cells forming after 4 wk of culture in basal medium [117]. In addition to the above cell sheet preparation techniques, more recently, scholars have also used techniques such as near infrared triggering, light induction, and the combination of rough surfaces with thermoresponsive polymers to accelerate or improve the formation of cell sheets[118-120]. These techniques need to be further applied to the preparation of DPSC sheets.

To date, use of DPSC sheets has been reported for the regenerative treatment of a variety of injuries, including but not limited to dental pulp diseases[112], periodontal tissue diseases[121], bone defects [122], nerve injuries[123,124], *etc*., and all of these applications have achieved significant results. In one representative clinical study, autologous DPSC sheets from deciduous teeth were transplanted into injured young permanent teeth due to trauma. This transplantation was able to regenerate whole dental pulp, increase the length of the root and reduce the width of the apical foramen at 12 mo after treatment [125]. Furthermore, the evaluation of DPSC sheets in a rat facial nerve crush injury model *in vivo* established that in comparison to untreated controls, nerves treated with dental pulp cell sheets had greater axon regeneration through the injury site and superior functional recovery as quantitatively assessed by compound muscle action potential measurements, possibly because the DPSC sheets can highly express neurotrophic factor (NTF) and continuously deliver the NTF to sites of peripheral nerve injury[123]. Moreover, bioengineered teeth using human DPSC aggregates combined with decellularized tooth matrix or avulsed teeth after traumatic dental injuries can regenerate 3D dental pulp and periodontium equipped with vasculature and innervation in both a preclinical pig model and a pilot clinical trial for treating tooth avulsion[126].

In summary, in cell-based regenerative medicine, the application of cells into the injured site using cell sheets shows a significant increase in the therapeutic effect compared to dissociated cell injections, which may be related to the form of cell sheets being able to provide a large number of seed cells and improve the survival rate of transplanted cells[127,128]. DPSC sheets have also been reported to be more effective in repairing periodontal bone defects and regenerating soft tissue than pulp stem cell injections [17]. Moreover, the applications of decellularized cell sheets suggest that in addition to the function of seed cells in the sheets, extracellular matrix may also play a certain role in regeneration[129]. For example, a decellularized matrix of DPSC sheets can promote the proliferation and osteogenic differentiation of inoculated human periodontal ligament stem cells[130].

DPSC spheroids

Most studies in cell biology are performed on a two-dimensional (2D) culture basis, although these studies facilitate microscopic analysis and medium changes and sustain cell proliferation for most cell types. However, this is generally not considered the natural microenvironment of the cells[131]. A cell spheroid is a 3D aggregation of cells, which is considered to be closer to the microenvironment *in vivo* because its formation mode simulates the natural processes of cells undergoing biological self-assembly to form complex tissues with 3D architecture and intensive cell–cell contacts from the perspective of embryonic development[132]. Cell spheroids have been proven to be able to mimic the architectural and functional characteristics of native tissue. For example, liver spheroids constructed *in vitro* by liver cells and endothelial cells have an ultrastructure of liver tissue, such as bile canaliculus-like and Disse's space-like structures, and show stable albumin secretion and ammonia removal activity[133]. 3D lung spheroids of outgrowth cells from healthy lung tissue explants can be expanded to a large quantity and can form alveoli-like structures and acquire mature lung epithelial phenotypes *in vitro*[134]. The introduction of endothelial cells can form capillary networks in spheroids from different kinds of cells, which is conducive to anastomosing with the host vasculature after transplantation and prolonging the survival time of cell spheroids[135-137]. In view of the above characteristics, cell spheroids are widely believed to be able to be used as excellent candidates for basic units of 3D tissue engineering constructs, thus providing new strategies for tissue defect repair and reconstruction.

At present, techniques to form cell spheroids include mainly pellet culture, spinner culture, hanging drop (HD), liquid overlay, rotating wall vessel, external force, microfluidics, micromolded nonadhesive hydrogels[138], microwell culture, medium regulation, bioreactors[139] and bioactive materials such as cellulose hydrogel film[140,141]. There are also methods to generate cell spheroids by using cell sheets as prophase tissues[142] or culturing in specific charged culture dishes based on polyion complex nanoparticles[143]. Some of these methods are still in the research stage and are not ready to be adopted for large-scale manufacturing. The manufacturing methods of DPSC spheroids have also emerged in an endless series, including but not limited to serum-free medium culture[144,145], culturing on Matrigel [146], special 3D Petri dish culture[135,147], low or ultralow attachment culture plates[62,148], culturing on gelatin methacrylamine/poly(ethylene glycol) diacrylate (GelMA/PEGDA) composite hydrogels [149], coculturing with microparticles with a leaf-stacked structure based on polycaprolactone[150], HD or molded parafilm-based methods[151]. Most of these methods belong to the classification of the aforementioned technologies.

Regardless of the method, the prepared DPSC spheroids basically have spherical or spheroid-like shapes, ranging in diameter from microns to millimeters. Since the typical viable rim of cells in spheroids is approximately 100–300 μm, cells die in the center of large spheroids due to the lack of oxygen and/or nutrients, accumulation of waste products and low pH[146]. Therefore, a larger spheroid diameter is not better. Histological examination of cell spheroids at the micron level revealed

that spheroids were compact throughout with small single cells evenly distributed after 24 h of culture [147], while a diversity of nuclei in the spheroids after 1 wk of culture suggested that cells in the spheroid were multitype[145]. Real-time reverse transcriptase-polymerase chain reaction analysis also demonstrated that the stemness/pluripotency markers Oct4, Sox2, NANOG, TP63, and CD44 were expressed in 3D cultured DPSCs, and the expression level was significantly increased when compared to 2D cultured DPSCs[145,147]. In addition, compared to 2D cultured DPSCs, the osteogenic, adipogenic, odontogenic differentiation potential and migration ability of DPSC spheroids are also enhanced[145,147,152-154]. These results indicate that the stemness of 3D cultured DPSCs is maintained while the multilineage differentiation potential could be enhanced, which may be related to the improvement of signal transmissions between cells.

DPSC spheroids have been proved to be able to differentiate into specific phenotypic cells, or simulate the structural and functional characteristics of the corresponding tissues, and play a therapeutic role. For example, evidence that DPSC spheroids can differentiate into neuron-like cells with potential functions under neurogenic induction *in vitro* has been reported[155]. The expression of neuronal markers such as microtubule-associated protein 2 in DPSC spheroids was increased after culture in neurogenic maturation medium or with the addition of central nervous system mitogens such as EGF and bFGF[156,157]. These DPSC spheroids are able to differentiate into functional neuronal cells and stimulate neurogenesis in the adult mouse hippocampus through neurotrophic support *in vitro* [158]. Dissanayaka *et al*[135] also confirmed that DPSCs support the survival of the co-cultured endothelial cells, and they can self-assemble into microtissue spheroids within the microwells of an agarose mold. Combined with tooth-root slices, these prevascularized, scaffold-free, microtissue spheroids could successfully regenerate vascular dental pulp-like tissue in immunodeficient mice[135]. In addition to spherical cell spheroids, there are some irregular cell aggregates constructed by 3D cell culture technology, which are still multicellular units of DPSCs in nature and have corresponding regenerative therapeutic effects. For instance, some scholars obtained rod-like 3D DPSC constructs by shaping sheet-like aggregates of DPSCs with a thermoresponsive hydrogel, which could form blood vessel-rich pulp-like tissues in nude mice[159].

If the cell sheet is still a cell aggregate in a two-dimensional concept, cell spheroids are advancing the concept of 3D tissue engineering. In 3D cell spheroids, cells are in close contact with each other and surrounded by extracellular matrix, enabling the simulation of cell-to-cell interactions and cell-extracellular matrix interactions *in vitro*. These processes are very important for signal transmission between cells and guiding cell behaviors such as movement, proliferation and differentiation[138]. Current studies have confirmed that these characteristics can enhance the properties of DPSCs in 3D cell spheroids, more studies are needed to explore the therapeutic advantages of DPSC spheroids compared with other DPSC products.

The above derivatives based on DPSCs themselves can be used alone or in combination with bioscaffold materials for the treatment of diseases, which will not be discussed here. Figure 1 shows an overview of the main sources of DPSCs, their derivatives and examples of their applications in regenerative medicine.

MECHANISM OF REGENERATION PROMOTED BY DPSCS

Direct effects on the regeneration process

For years, scholars have failed to fully understand the fate of implanted injured stem cells and their role in regeneration. The results of some studies show that the implanted stem cells can stay *in situ* and differentiate into corresponding cells, thus directly participating in the process of regeneration[25-29]. Researchers have preliminarily revealed this process through stem cell labeling techniques, such as green fluorescent protein (GFP)/BrdU labeling[26], superparamagnetic iron oxide[160], and fluorescence-based tracing[161]. For example, BrdU-labeled MSCs migrated into the entire periodontal tissue, including the periodontal ligament, alveolar bone, cementum and blood vessels, and differentiated into periodontal ligament fibroblasts and osteoblasts 6 wk after implantation in periodontal defects, confirming that MSC transplantation has the potential to regenerate periodontal tissue and that transplanted MSCs are at least partially directly involved in the formation of new tissue[29]. The findings of Hasegawa *et al*[25] also supported this conclusion[25]. Another example is the combination of DiI (a fluorescent dye) or GFP-prelabeled hESC-MSCs with the simulated tendon complex to form tissue-engineered tendons, which were then ectopically transplanted into the back of nude mice or orthotopically transplanted into the impaired rat Achilles tendon. Four weeks after transplantation, the transplanted MSCs partially survived and differentiated into the tenocyte lineage, and functional tendons were regenerated successfully[27].

Similarly, transplanted DPSCs have been proven to be partially involved directly in the process of tissue regeneration. For instance, 5 wk after DPSCs stably transduced with GFP, GFP-DPSCs were seeded into tooth slices/scaffolds and transplanted into the subcutaneous space in the dorsum of immunodeficient mice. The DPSCs were observed to differentiate into endothelial cells and form neovascularization anastomosed with host vessels by immunohistochemistry and immunofluorescence
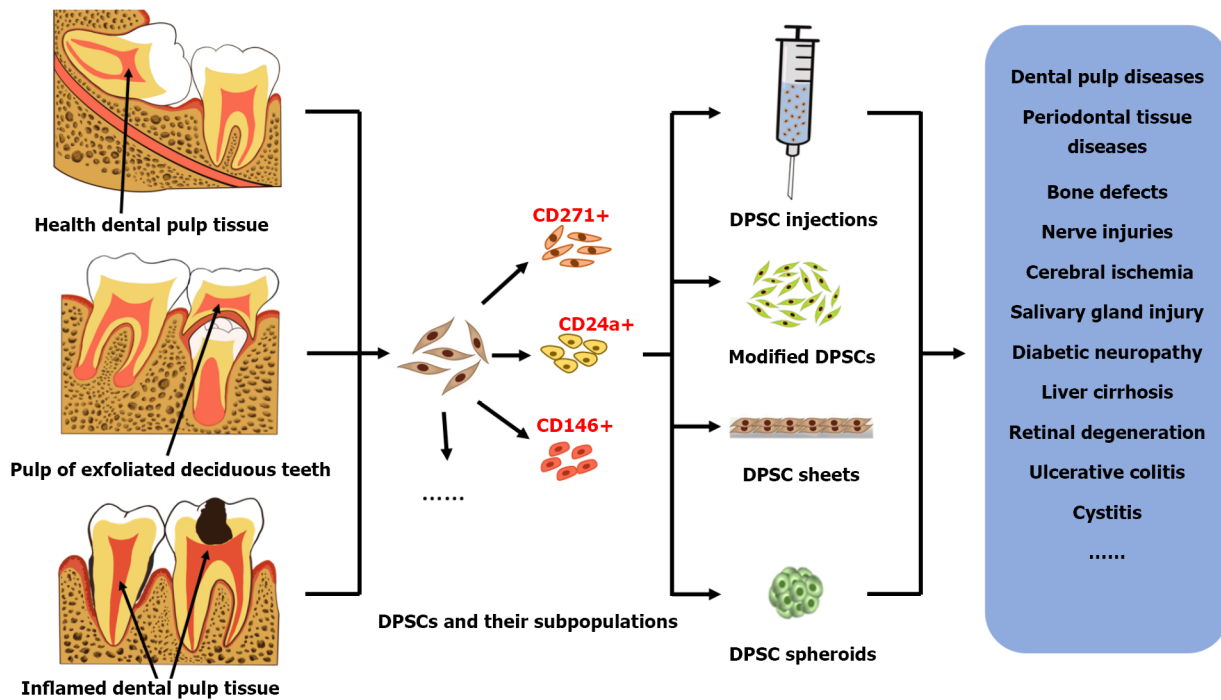
Figure 1 Overview of the main sources of dental pulp stem cells and their derivatives and examples of their applications in regenerative medicine. Dental pulp stem cells (DPSCs) can be isolated from dental pulp tissue of different ages and health statuses, such as the healthy dental pulp tissue of adult impacted teeth, the pulp of children's exfoliated deciduous teeth, and the inflamed dental pulp tissue of patients with caries or periodontitis. Using cell sorting technologies, dental pulp stem cells can be isolated into multiple cell subsets with specific phenotypes, such as CD271+ DPSCs, CD24a+ DPSCs, and CD146+ DPSCs. Several main derivatives of DPSCs derived from long-term applications, including cell injections, genetically modified cells, cell sheets and cell spheroids, which can be used in the treatment of various diseases such as dental pulp diseases, periodontal diseases, and bone defects. DPSC: Dental pulp stem cell.

staining. This process may be related to the activation of the Wnt/β-catenin signaling pathway by vascular endothelial growth factor (VEGF)[162]. Luzuriaga *et al*[163] also confirmed that human CD31+/CD146+ and Nestin + DPSC-derived cells can survive 1 mo after grafting into the brains of nude mice, expressing CD31 and VEGF, forming full blood vessels of human origin and integrating into the host brain vasculature[163]. In short, these results suggest that transplanted stem cells can partially survive and directly participate in the process of tissue repair and regeneration.

Immunomodulatory effects

In addition, stem cell transplantation has also shown promising results in the treatment of immune-related diseases[164,165], which suggests that stem cell transplantation may be involved in the process of immune regulation in the host. Previous studies have confirmed that MSCs have the characteristics of immunosuppression *in vivo* and *in vitro* and are capable of regulating immune cells, such as T cells, B cells, DCs, and NK cells[21]. MSCs can efficiently suppress the proliferation of T cells[166] and reduce the production and release of cytokines such as interferon (IFN)-γ and interleukin (IL)-17[167,168]. MSCs also efficiently inhibit the maturation of DCs and markedly impair a variety of functions of NK cells[169]. Furthermore, MSCs are able to inhibit the proliferation of B cells and their capacity to produce antibodies[20]. MSC-mediated immunosuppression may occur *via* the concerted action of chemokines and nitric oxide (NO). In the presence of IFN-γ, TNF-α, IL-1A, or IL-1β, MSCs can be stimulated to express several chemokines at high levels and inducible NO synthase. Chemokines drive T-cell migration into proximity with MSCs, where T-cell responsiveness is suppressed by NO, while blocking chemokine receptors could abolish immunosuppression[170]. However, there is a species variation in the mechanisms of MSC-mediated immunosuppression: Immunosuppression by cytokine-primed mouse MSCs is mediated by NO, whereas immunosuppression by cytokine-primed human MSCs is executed through indoleamine 2,3-dioxygenase (IDO). The similarity is that they both exert immunosuppression *via* the concerted action of chemokines and immune-inhibitory NO or IDO[19].

DPSCs have also been found to have immunomodulatory functions similar to those functions in MSCs. Therefore, DPSCs are considered promising candidates for cell therapy for a variety of immune- and inflammation-related diseases. Previous reports have demonstrated that DPSCs can suppress T cell proliferation and therefore might be suitable for preventing or treating T cell alloreactivity associated with hematopoietic or solid-organ allogeneic transplantation. The study also confirmed that DPSCs had a stronger inhibitory effect on the T cell response than BMSCs[171]. In addition, DPSCs can also inhibit acute allogeneic immune responses by their release of TGF-β as a result of allogeneic stimulation of T

lymphocytes[172] and inhibit the proliferation of peripheral blood mononuclear cells (PBMCs) *via* the expression of soluble factors partly induced by the secretion of IFN-γ by activated PBMCs[173]. In another study, Toll-like receptors, key molecules that bridge the innate and adaptive immune responses, were shown to trigger the immunosuppression of DPSCs by upregulating the expression of TGF-β and IL-6[174]. In addition, DPSCs could induce activated T cell apoptosis *in vitro* and ameliorate inflammation-related tissue injuries in mice with colitis, which was associated with the expression of the Fas ligand (FasL). Knockdown of FasL expression reduced the immunoregulatory properties of DPSCs in the context of inducing T cell apoptosis[21]. These studies indicate that DPSCs exert their immunoregulatory functions mainly by inhibiting immune cells, especially T cells, including inhibiting proliferation, reducing the release of cellular inflammatory factors, and inducing apoptosis.

Paracrine effects

However, the proposal of secretomes, exosomes or EVs provides a new direction for us to understand how stem cells promote regeneration: Paracrine cues and derives a cell-free tissue engineering strategy. Many studies have confirmed that conditioned media/secretomes/exosomes/EVs from BMSCs, DPSCs or other types of stem cells can perform functions similar to the functions of stem cells themselves, promote the regeneration of damaged tissue or improve the severity of the disease[18,31,175,176]. Exosomes from different MSCs have been reported for the treatment of liver disease, kidney disease, cardiovascular disease, neurological disease, immune disease, and skin wounds[24,177]. Exosomes from MSCs may accomplish changes in the cellular microenvironment and the behavior of their neighboring cells by transferring factors that modulate different metabolic and signaling pathways, such as the maintenance of a dynamic and homeostatic environment and the ability to activate angiogenesis, proliferation, migration and differentiation of the main cell types involved in regeneration, thereby restoring tissue homeostasis and enabling cells within the tissue to recover, repair and regenerate[24,177]. In addition, some scholars believe that paracrine signaling is mainly responsible for the involvement of MSCs in the modulation of immune responses and the progression of diseases. Through the release of secretomes consisting of a diverse range of cytokines, chemokines, and EVs, MSCs convey regulatory messages to recipient immune cells in the microenvironment[178]. DPSC-derived exosomes have also been shown to alleviate cerebral ischemia–reperfusion injury by suppressing inflammatory responses, such as reducing the protein expression of IL-6, IL-1β, and TNF-α[18], suggesting that the immunomodulatory effect of stem cells may also be achieved through the paracrine pathway.

In conclusion, DPSCs may promote tissue repair and regeneration by directly differentiating into corresponding cells, exerting immunomodulatory effects, or by releasing paracrine substances such as exosomes to maintain microenvironment homeostasis and activate the functions of adjacent cells.

CONCLUSION

According to published data, DPSCs have become one of the important seed cells of regenerative medicine. Younger DPSCs, such as DPSCs from natal teeth or SHED, are more suitable as excellent candidates for regenerative medicine stem cell resource banks. However, due to the lack of abundant sources of natal teeth, SHED may become the most powerful source of DPSCs in the future. The establishment of a stem cell bank for SHED is an urgent problem to be solved. In addition, taking advantage of a specific side group of DPSCs may be the pathway to achieve accurate and efficient tissue regeneration. However, this does not mean that the use of the total population of DPSCs is meaningless. The possible reason could be that the tissue is composed of a variety of cells with different functions, and a certain subpopulation currently reflects only one or a few of its dominant functions. The use of a subpopulation may not be sufficient to regenerate well-organized native-like tissue. Therefore, the combined use of several side groups may be more sensible DPSCs can be used in the treatment of diseases in the form of single injections, cell sheets and cell spheroids, and their therapeutic effect can be enhanced by gene modifications. DPSC injections may be a useful method for the treatment of systemic diseases, but for the treatment of localized damaged tissue, which requires *in situ* tissue regeneration, how to keep the implanted DPSCs *in situ* is worth considering; therefore, cell sheets and cell spheroids with supports are more suitable in this case. Current data show that DPSCs may promote the improvement, repair and regeneration of diseased and injured tissues by means of immune regulation, paracrine signaling and direct differentiation into corresponding cells to occupy the injured site. However, the mechanism by which DPSCs promote regeneration is complex, and the above discussion does not address all of the mechanism. We should continue to pay attention to the new applications of DPSCs and improve the mechanism by which DPSCs promote diseased tissue recovery.

FOOTNOTES

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Country/Territory of origin: China

ORCID number: Sheng-Meng Yuan 0000-0003-0585-514X; Xue-Ting Yang 0000-0002-0013-2893; Si-Yuan Zhang 0000-0002-6214-3459; Wei-Dong Tian 0000-0003-0175-1132; Bo Yang 0000-0003-0127-9632.

REFERENCES

1   **Gronthos S**, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) *in vitro* and in vivo. *Proc Natl Acad Sci U S A* 2000; **97**: 13625-13630 [PMID: 11087820 DOI: 10.1073/pnas.240309797]

2   **Zhang SY**, Ren JY, Yang B. Priming strategies for controlling stem cell fate: Applications and challenges in dental tissue regeneration. *World J Stem Cells* 2021; **13**: 1625-1646 [PMID: 34909115 DOI: 10.4252/wjsc.v13.i11.1625]

3   **Potdar PD**, Jethmalani YD. Human dental pulp stem cells: Applications in future regenerative medicine. *World J Stem Cells* 2015; **7**: 839-851 [PMID: 26131314 DOI: 10.4252/wjsc.v7.i5.839]

4   **Yamada Y**, Fujimoto A, Ito A, Yoshimi R, Ueda M. Cluster analysis and gene expression profiles: a cDNA microarray system-based comparison between human dental pulp stem cells (hDPSCs) and human mesenchymal stem cells (hMSCs) for tissue engineering cell therapy. *Biomaterials* 2006; **27**: 3766-3781 [PMID: 16563496 DOI: 10.1016/j.biomaterials.2006.02.009]

5   **Patil VR**, Kharat AH, Kulkarni DG, Kheur SM, Bhonde RR. Long term explant culture for harvesting homogeneous population of human dental pulp stem cells. *Cell Biol Int* 2018; **42**: 1602-1610 [PMID: 30353965 DOI: 10.1002/cbin.11065]

6   **Agha-Hosseini F**, Jahani MA, Jahani M, Mirzaii-Dizgah I, Ali-Moghaddam K. In vitro isolation of stem cells derived from human dental pulp. *Clin Transplant* 2010; **24**: E23-E28 [PMID: 20030679 DOI: 10.1111/j.1399-0012.2009.01137.x]

7   **Anitua E**, Zalduendo M, Troya M. Autologous plasma rich in growth factors technology for isolation and *ex vivo* expansion of human dental pulp stem cells for clinical translation. *Regen Med* 2019; **14**: 97-111 [PMID: 30767653 DOI: 10.2217/rme-2018-0066]

8   **Kumar A**, Kumar V, Rattan V, Jha V, Bhattacharyya S. Secretome Cues Modulate the Neurogenic Potential of Bone Marrow and Dental Stem Cells. *Mol Neurobiol* 2017; **54**: 4672-4682 [PMID: 27422132 DOI: 10.1007/s12035-016-0011-3]

9   **Sugiyama M**, Iohara K, Wakita H, Hattori H, Ueda M, Matsushita K, Nakashima M. Dental pulp-derived CD31$^-$/CD146$^-$ side population stem/progenitor cells enhance recovery of focal cerebral ischemia in rats. *Tissue Eng Part A* 2011; **17**: 1303-1311 [PMID: 21226624 DOI: 10.1089/ten.TEA.2010.0306]

10   **Cristaldi M**, Mauceri R, Tomasello L, Pizzo G, Pizzolanti G, Giordano C, Campisi G. Dental pulp stem cells for bone tissue engineering: a review of the current literature and a look to the future. *Regen Med* 2018 [PMID: 29553875 DOI: 10.2217/rme-2017-0112]

11   **Capparè P**, Tetè G, Sberna MT, Panina-Bordignon P. The Emerging Role of Stem Cells in Regenerative Dentistry. *Curr Gene Ther* 2020; **20**: 259-268 [PMID: 32811413 DOI: 10.2174/1566523220999200818115803]

12   **Wang DR**, Wang YH, Pan J, Tian WD. Neurotrophic effects of dental pulp stem cells in repair of peripheral nerve after crush injury. *World J Stem Cells* 2020; **12**: 1196-1213 [PMID: 33178401 DOI: 10.4252/wjsc.v12.i10.1196]

13   **Ikeda E**, Yagi K, Kojima M, Yagyuu T, Ohshima A, Sobajima S, Tadokoro M, Katsube Y, Isoda K, Kondoh M, Kawase M, Go MJ, Adachi H, Yokota Y, Kirita T, Ohgushi H. Multipotent cells from the human third molar: feasibility of cell-based therapy for liver disease. *Differentiation* 2008; **76**: 495-505 [PMID: 18093227 DOI: 10.1111/j.1432-0436.2007.00245.x]

14   **Hata M**, Omi M, Kobayashi Y, Nakamura N, Miyabe M, Ito M, Ohno T, Imanishi Y, Himeno T, Kamiya H, Nakamura J, Miyachi H, Ozawa S, Miyazawa K, Mitani A, Nagao T, Goto S, Takebe J, Matsubara T, Naruse K. Sustainable Effects of Human Dental Pulp Stem Cell Transplantation on Diabetic Polyneuropathy in Streptozotocine-Induced Type 1 Diabetes Model Mice. *Cells* 2021; **10** [PMID: 34572120 DOI: 10.3390/cells10092473]

15   **Alsaeedi HA**, Koh AE, Lam C, Rashid MBA, Harun MHN, Saleh MFBM, Teh SW, Luu CD, Ng MH, Isa HM, Leow SN, Then KY, Bastion MC, Mok PL, Muthuvenkatachalam BS, Samrot AV, Swamy KB, Nandakumar J, Kumar SS. Dental pulp stem cells therapy overcome photoreceptor cell death and protects the retina in a rat model of sodium iodate-induced

retinal degeneration. *J Photochem Photobiol B* 2019; **198**: 111561 [PMID: 31352000 DOI: 10.1016/j.jphotobiol.2019.111561]

16   **Li N**, Zhang Y, Nepal N, Li G, Yang N, Chen H, Lin Q, Ji X, Zhang S, Jin S. Dental pulp stem cells overexpressing hepatocyte growth factor facilitate the repair of DSS-induced ulcerative colitis. *Stem Cell Res Ther* 2021; **12**: 30 [PMID: 33413675 DOI: 10.1186/s13287-020-02098-4]

17   **Hu J**, Cao Y, Xie Y, Wang H, Fan Z, Wang J, Zhang C, Wu CT, Wang S. Periodontal regeneration in swine after cell injection and cell sheet transplantation of human dental pulp stem cells following good manufacturing practice. *Stem Cell Res Ther* 2016; **7**: 130 [PMID: 27613503 DOI: 10.1186/s13287-016-0362-8]

18   **Li S**, Luo L, He Y, Li R, Xiang Y, Xing Z, Li Y, Albashari AA, Liao X, Zhang K, Gao L, Ye Q. Dental pulp stem cell-derived exosomes alleviate cerebral ischaemia-reperfusion injury through suppressing inflammatory response. *Cell Prolif* 2021; **54**: e13093 [PMID: 34231932 DOI: 10.1111/cpr.13093]

19   **Shi Y**, Hu G, Su J, Li W, Chen Q, Shou P, Xu C, Chen X, Huang Y, Zhu Z, Huang X, Han X, Xie N, Ren G. Mesenchymal stem cells: a new strategy for immunosuppression and tissue repair. *Cell Res* 2010; **20**: 510-518 [PMID: 20368733 DOI: 10.1038/cr.2010.44]

20   **Zhao S**, Wehner R, Bornhäuser M, Wassmuth R, Bachmann M, Schmitz M. Immunomodulatory properties of mesenchymal stromal cells and their therapeutic consequences for immune-mediated disorders. *Stem Cells Dev* 2010; **19**: 607-614 [PMID: 19824807 DOI: 10.1089/scd.2009.0345]

21   **Zhao Y**, Wang L, Jin Y, Shi S. Fas ligand regulates the immunomodulatory properties of dental pulp stem cells. *J Dent Res* 2012; **91**: 948-954 [PMID: 22904205 DOI: 10.1177/0022034512458690]

22   **Scott SR**, March KL, Wang IW, Singh K, Liu J, Turrentine M, Sen CK, Wang M. Bone marrow- or adipose-mesenchymal stromal cell secretome preserves myocardial transcriptome profile and ameliorates cardiac damage following *ex vivo* cold storage. *J Mol Cell Cardiol* 2022; **164**: 1-12 [PMID: 34774548 DOI: 10.1016/j.yjmcc.2021.11.002]

23   **Fan XL**, Zhang Y, Li X, Fu QL. Mechanisms underlying the protective effects of mesenchymal stem cell-based therapy. *Cell Mol Life Sci* 2020; **77**: 2771-2794 [PMID: 31965214 DOI: 10.1007/s00018-020-03454-6]

24   **Casado-Díaz A**, Quesada-Gómez JM, Dorado G. Extracellular Vesicles Derived From Mesenchymal Stem Cells (MSC) in Regenerative Medicine: Applications in Skin Wound Healing. *Front Bioeng Biotechnol* 2020; **8**: 146 [PMID: 32195233 DOI: 10.3389/fbioe.2020.00146]

25   **Hasegawa N**, Kawaguchi H, Hirachi A, Takeda K, Mizuno N, Nishimura M, Koike C, Tsuji K, Iba H, Kato Y, Kurihara H. Behavior of transplanted bone marrow-derived mesenchymal stem cells in periodontal defects. *J Periodontol* 2006; **77**: 1003-1007 [PMID: 16734575 DOI: 10.1902/jop.2006.050341]

26   **Demeter K**, Herberth B, Duda E, Domonkos A, Jaffredo T, Herman JP, Madarász E. Fate of cloned embryonic neuroectodermal cells implanted into the adult, newborn and embryonic forebrain. *Exp Neurol* 2004; **188**: 254-267 [PMID: 15246825 DOI: 10.1016/j.expneurol.2004.04.011]

27   **Chen JL**, Yin Z, Shen WL, Chen X, Heng BC, Zou XH, Ouyang HW. Efficacy of hESC-MSCs in knitted silk-collagen scaffold for tendon tissue engineering and their roles. *Biomaterials* 2010; **31**: 9438-9451 [PMID: 20870282 DOI: 10.1016/j.biomaterials.2010.08.011]

28   **Jendelová P**, Herynek V, Urdzíková L, Glogarová K, Kroupová J, Andersson B, Bryja V, Burian M, Hájek M, Syková E. Magnetic resonance tracking of transplanted bone marrow and embryonic stem cells labeled by iron oxide nanoparticles in rat brain and spinal cord. *J Neurosci Res* 2004; **76**: 232-243 [PMID: 15048921 DOI: 10.1002/jnr.20041]

29   **Wei N**, Gong P, Liao D, Yang X, Li X, Liu Y, Yuan Q, Tan Z. Auto-transplanted mesenchymal stromal cell fate in periodontal tissue of beagle dogs. *Cytotherapy* 2010; **12**: 514-521 [PMID: 20370352 DOI: 10.3109/14653241003709702]

30   **Hossein-Khannazer N**, Hashemi SM, Namaki S, Ghanbarian H, Sattari M, Khojasteh A. Study of the immunomodulatory effects of osteogenic differentiated human dental pulp stem cells. *Life Sci* 2019; **216**: 111-118 [PMID: 30465790 DOI: 10.1016/j.lfs.2018.11.040]

31   **Makino E**, Nakamura N, Miyabe M, Ito M, Kanada S, Hata M, Saiki T, Sango K, Kamiya H, Nakamura J, Miyazawa K, Goto S, Matsubara T, Naruse K. Conditioned media from dental pulp stem cells improved diabetic polyneuropathy through anti-inflammatory, neuroprotective and angiogenic actions: Cell-free regenerative medicine for diabetic polyneuropathy. *J Diabetes Investig* 2019; **10**: 1199-1208 [PMID: 30892819 DOI: 10.1111/jdi.13045]

32   **Miura M**, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci U S A* 2003; **100**: 5807-5812 [PMID: 12716973 DOI: 10.1073/pnas.0937635100]

33   **Wu W**, Zhou J, Xu CT, Zhang J, Jin YJ, Sun GL. Derivation and growth characteristics of dental pulp stem cells from patients of different ages. *Mol Med Rep* 2015; **12**: 5127-5134 [PMID: 26239849 DOI: 10.3892/mmr.2015.4106]

34   **Shetty H**, Kakade A, Shetty S, Neelakantan P, Nagar S, Desai RS, Beri K. Immunohistochemical characterization of stem cell and differentiation markers of the dental pulp of human natal teeth. *Future Sci OA* 2018; **4**: FSO342 [PMID: 30450229 DOI: 10.4155/fsoa-2018-0062]

35   **Robey PG**. Stem cells near the century mark. *J Clin Invest* 2000; **105**: 1489-1491 [PMID: 10841501 DOI: 10.1172/JCI10256]

36   **Sakai VT**, Zhang Z, Dong Z, Neiva KG, Machado MA, Shi S, Santos CF, Nör JE. SHED differentiate into functional odontoblasts and endothelium. *J Dent Res* 2010; **89**: 791-796 [PMID: 20395410 DOI: 10.1177/0022034510368647]

37   **Gonmanee T**, Thonabulsombat C, Vongsavan K, Sritanaudomchai H. Differentiation of stem cells from human deciduous and permanent teeth into spiral ganglion neuron-like cells. *Arch Oral Biol* 2018; **88**: 34-41 [PMID: 29407749 DOI: 10.1016/j.archoralbio.2018.01.011]

38   **Wang X**, Sha XJ, Li GH, Yang FS, Ji K, Wen LY, Liu SY, Chen L, Ding Y, Xuan K. Comparative characterization of stem cells from human exfoliated deciduous teeth and dental pulp stem cells. *Arch Oral Biol* 2012; **57**: 1231-1240 [PMID: 22455989 DOI: 10.1016/j.archoralbio.2012.02.014]

39   **Zhu SY**, Yuan CY, Lin YF, Liu H, Yang YQ, Wong HM, Zhang CF, Wang PL, Gu M. Stem Cells from Human Exfoliated Deciduous Teeth (SHEDs) and Dental Pulp Stem Cells (DPSCs) Display a Similar Profile with Pericytes. *Stem Cells Int* 2021; **2021**: 8859902 [PMID: 34349804 DOI: 10.1155/2021/8859902]

40   **Xu JG**, Gong T, Wang YY, Zou T, Heng BC, Yang YQ, Zhang CF. Inhibition of TGF-β Signaling in SHED Enhances

Endothelial Differentiation. *J Dent Res* 2018; **97**: 218-225 [PMID: 28972822 DOI: 10.1177/0022034517733741]

41 **Hagar MN**, Yazid F, Luchman NA, Ariffin SHZ, Wahab RMA. Comparative evaluation of osteogenic differentiation potential of stem cells derived from dental pulp and exfoliated deciduous teeth cultured over granular hydroxyapatite based scaffold. *BMC Oral Health* 2021; **21**: 263 [PMID: 33992115 DOI: 10.1186/s12903-021-01621-0]

42 **Kanafi MM**, Ramesh A, Gupta PK, Bhonde RR. Influence of hypoxia, high glucose, and low serum on the growth kinetics of mesenchymal stem cells from deciduous and permanent teeth. *Cells Tissues Organs* 2013; **198**: 198-208 [PMID: 24192068 DOI: 10.1159/000354901]

43 **Govindasamy V**, Abdullah AN, Ronald VS, Musa S, Ab Aziz ZA, Zain RB, Totey S, Bhonde RR, Abu Kasim NH. Inherent differential propensity of dental pulp stem cells derived from human deciduous and permanent teeth. *J Endod* 2010; **36**: 1504-1515 [PMID: 20728718 DOI: 10.1016/j.joen.2010.05.006]

44 **Hollands P**, Aboyeji D, Orcharton M. Dental pulp stem cells in regenerative medicine. *Br Dent J* 2018 [PMID: 29725075 DOI: 10.1038/sj.bdj.2018.348]

45 **Karaöz E**, Doğan BN, Aksoy A, Gacar G, Akyüz S, Ayhan S, Genç ZS, Yürüker S, Duruksu G, Demircan PC, Sariboyaci AE. Isolation and *in vitro* characterisation of dental pulp stem cells from natal teeth. *Histochem Cell Biol* 2010; **133**: 95-112 [PMID: 19816704 DOI: 10.1007/s00418-009-0646-5]

46 **Yu S**, Diao S, Wang J, Ding G, Yang D, Fan Z. Comparative analysis of proliferation and differentiation potentials of stem cells from inflamed pulp of deciduous teeth and stem cells from exfoliated deciduous teeth. *Biomed Res Int* 2014; **2014**: 930907 [PMID: 25045714 DOI: 10.1155/2014/930907]

47 **Pereira LO**, Rubini MR, Silva JR, Oliveira DM, Silva IC, Poças-Fonseca MJ, Azevedo RB. Comparison of stem cell properties of cells isolated from normal and inflamed dental pulps. *Int Endod J* 2012; **45**: 1080-1090 [PMID: 22747502 DOI: 10.1111/j.1365-2591.2012.02068.x]

48 **Kim J**, Park JC, Kim SH, Im GI, Kim BS, Lee JB, Choi EY, Song JS, Cho KS, Kim CS. Treatment of FGF-2 on stem cells from inflamed dental pulp tissue from human deciduous teeth. *Oral Dis* 2014; **20**: 191-204 [PMID: 23496287 DOI: 10.1111/odi.12089]

49 **Alongi DJ**, Yamaza T, Song Y, Fouad AF, Romberg EE, Shi S, Tuan RS, Huang GT. Stem/progenitor cells from inflamed human dental pulp retain tissue regeneration potential. *Regen Med* 2010; **5**: 617-631 [PMID: 20465527 DOI: 10.2217/rme.10.30]

50 **Gnanasegaran N**, Govindasamy V, Abu Kasim NH. Differentiation of stem cells derived from carious teeth into dopaminergic-like cells. *Int Endod J* 2016; **49**: 937-949 [PMID: 26354006 DOI: 10.1111/iej.12545]

51 **Attar A**, Eslaminejad MB, Tavangar MS, Karamzadeh R, Dehghani-Nazhvani A, Ghahramani Y, Malekmohammadi F, Hosseini SM. Dental pulp polyps contain stem cells comparable to the normal dental pulps. *J Clin Exp Dent* 2014; **6**: e53-e59 [PMID: 24596636 DOI: 10.4317/jced.51305]

52 **Wang Z**, Pan J, Wright JT, Bencharit S, Zhang S, Everett ET, Teixeira FB, Preisser JS. Putative stem cells in human dental pulp with irreversible pulpitis: an exploratory study. *J Endod* 2010; **36**: 820-825 [PMID: 20416426 DOI: 10.1016/j.joen.2010.02.003]

53 **Ueda M**, Fujisawa T, Ono M, Hara ES, Pham HT, Nakajima R, Sonoyama W, Kuboki T. A short-term treatment with tumor necrosis factor-alpha enhances stem cell phenotype of human dental pulp cells. *Stem Cell Res Ther* 2014; **5**: 31 [PMID: 24580841 DOI: 10.1186/scrt420]

54 **Sun HH**, Chen B, Zhu QL, Kong H, Li QH, Gao LN, Xiao M, Chen FM, Yu Q. Investigation of dental pulp stem cells isolated from discarded human teeth extracted due to aggressive periodontitis. *Biomaterials* 2014; **35**: 9459-9472 [PMID: 25172527 DOI: 10.1016/j.biomaterials.2014.08.003]

55 **Wang J**, Wei X, Ling J, Huang Y, Gong Q, Huo Y. Identification and characterization of side population cells from adult human dental pulp after ischemic culture. *J Endod* 2012; **38**: 1489-1497 [PMID: 23063223 DOI: 10.1016/j.joen.2012.08.004]

56 **Ishikawa Y**, Ida-Yonemochi H, Nakakura-Ohshima K, Ohshima H. The relationship between cell proliferation and differentiation and mapping of putative dental pulp stem/progenitor cells during mouse molar development by chasing BrdU-labeling. *Cell Tissue Res* 2012; **348**: 95-107 [PMID: 22370596 DOI: 10.1007/s00441-012-1347-2]

57 **Machado CV**, Passos ST, Campos TM, Bernardi L, Vilas-Bôas DS, Nör JE, Telles PD, Nascimento IL. The dental pulp stem cell niche based on aldehyde dehydrogenase 1 expression. *Int Endod J* 2016; **49**: 755-763 [PMID: 26198909 DOI: 10.1111/iej.12511]

58 **Murakami M**, Horibe H, Iohara K, Hayashi Y, Osako Y, Takei Y, Nakata K, Motoyama N, Kurita K, Nakashima M. The use of granulocyte-colony stimulating factor induced mobilization for isolation of dental pulp stem cells with high regenerative potential. *Biomaterials* 2013; **34**: 9036-9047 [PMID: 23988014 DOI: 10.1016/j.biomaterials.2013.08.011]

59 **Pisciotta A**, Carnevale G, Meloni S, Riccio M, De Biasi S, Gibellini L, Ferrari A, Bruzzesi G, De Pol A. Human dental pulp stem cells (hDPSCs): isolation, enrichment and comparative differentiation of two sub-populations. *BMC Dev Biol* 2015; **15**: 14 [PMID: 25879198 DOI: 10.1186/s12861-015-0065-x]

60 **Hosoya A**, Hiraga T, Ninomiya T, Yukita A, Yoshiba K, Yoshiba N, Takahashi M, Ito S, Nakamura H. Thy-1-positive cells in the subodontoblastic layer possess high potential to differentiate into hard tissue-forming cells. *Histochem Cell Biol* 2012; **137**: 733-742 [PMID: 22327831 DOI: 10.1007/s00418-012-0928-1]

61 **Alvarez R**, Lee HL, Hong C, Wang CY. Single CD271 marker isolates mesenchymal stem cells from human dental pulp. *Int J Oral Sci* 2015; **7**: 205-212 [PMID: 26674422 DOI: 10.1038/ijos.2015.29]

62 **Chen H**, Fu H, Wu X, Duan Y, Zhang S, Hu H, Liao Y, Wang T, Yang Y, Chen G, Li Z, Tian W. Regeneration of pulpo-dentinal-like complex by a group of unique multipotent CD24a$^+$ stem cells. *Sci Adv* 2020; **6**: eaay1514 [PMID: 32284993 DOI: 10.1126/sciadv.aay1514]

63 **Matsui M**, Kobayashi T, Tsutsui TW. CD146 positive human dental pulp stem cells promote regeneration of dentin/pulp-like structures. *Hum Cell* 2018; **31**: 127-138 [PMID: 29313241 DOI: 10.1007/s13577-017-0198-2]

64 **Nagpal A**, Kremer KL, Hamilton-Bruce MA, Kaidonis X, Milton AG, Levi C, Shi S, Carey L, Hillier S, Rose M, Zacest A, Takhar P, Koblar SA. TOOTH (The Open study Of dental pulp stem cell Therapy in Humans): Study protocol for evaluating safety and feasibility of autologous human adult dental pulp stem cell therapy in patients with chronic disability

after stroke. *Int J Stroke* 2016; **11**: 575-585 [PMID: 27030504 DOI: 10.1177/1747493016641111]

65   **Bonaventura G**, Incontro S, Iemmolo R, La Cognata V, Barbagallo I, Costanzo E, Barcellona ML, Pellitteri R, Cavallaro S. Dental mesenchymal stem cells and neuro-regeneration: a focus on spinal cord injury. *Cell Tissue Res* 2020; **379**: 421-428 [PMID: 31776822 DOI: 10.1007/s00441-019-03109-4]

66   **Aliaghaei A**, Boroujeni ME, Ahmadi H, Bayat AH, Tavirani MR, Abdollahifar MA, Pooyafar MH, Mansouri V. Dental pulp stem cell transplantation ameliorates motor function and prevents cerebellar atrophy in rat model of cerebellar ataxia. *Cell Tissue Res* 2019; **376**: 179-187 [PMID: 30635776 DOI: 10.1007/s00441-018-02980-x]

67   **Datta I**, Bhadri N, Shahani P, Majumdar D, Sowmithra S, Razdan R, Bhonde R. Functional recovery upon human dental pulp stem cell transplantation in a diabetic neuropathy rat model. *Cytotherapy* 2017; **19**: 1208-1224 [PMID: 28864291 DOI: 10.1016/j.jcyt.2017.07.009]

68   **Al-Serwi RH**, El-Kersh AOFO, El-Akabawy G. Human dental pulp stem cells attenuate streptozotocin-induced parotid gland injury in rats. *Stem Cell Res Ther* 2021; **12**: 577 [PMID: 34775989 DOI: 10.1186/s13287-021-02646-6]

69   **Hirose Y**, Yamamoto T, Nakashima M, Funahashi Y, Matsukawa Y, Yamaguchi M, Kawabata S, Gotoh M. Injection of Dental Pulp Stem Cells Promotes Healing of Damaged Bladder Tissue in a Rat Model of Chemically Induced Cystitis. *Cell Transplant* 2016; **25**: 425-436 [PMID: 26395427 DOI: 10.3727/096368915X689523]

70   **Du ZH**, Ding C, Zhang Q, Zhang Y, Ge XY, Li SL, Yu GY. Stem cells from exfoliated deciduous teeth alleviate hyposalivation caused by Sjögren syndrome. *Oral Dis* 2019; **25**: 1530-1544 [PMID: 31046162 DOI: 10.1111/odi.13113]

71   **Shahani P**, Kaushal A, Waghmare G, Datta I. Biodistribution of Intramuscularly-Transplanted Human Dental Pulp Stem Cells in Immunocompetent Healthy Rats through NIR Imaging. *Cells Tissues Organs* 2020; **209**: 215-226 [PMID: 33333518 DOI: 10.1159/000511569]

72   **Song M**, Lee JH, Bae J, Bu Y, Kim EC. Human Dental Pulp Stem Cells Are More Effective Than Human Bone Marrow-Derived Mesenchymal Stem Cells in Cerebral Ischemic Injury. *Cell Transplant* 2017; **26**: 1001-1016 [PMID: 28105979 DOI: 10.3727/096368916X694391]

73   **Zordani A**, Pisciotta A, Bertoni L, Bertani G, Vallarola A, Giuliani D, Puliatti S, Mecugni D, Bianchi G, de Pol A, Carnevale G. Regenerative potential of human dental pulp stem cells in the treatment of stress urinary incontinence: In vitro and *in vivo* study. *Cell Prolif* 2019; **52**: e12675 [PMID: 31553127 DOI: 10.1111/cpr.12675]

74   **Sanches EF**, Valentim L, de Almeida Sassi F, Bernardi L, Arteni N, Weis SN, Odorcyk FK, Pranke P, Netto CA. Intracardiac Injection of Dental Pulp Stem Cells After Neonatal Hypoxia-Ischemia Prevents Cognitive Deficits in Rats. *Neurochem Res* 2018; **43**: 2268-2276 [PMID: 30255215 DOI: 10.1007/s11064-018-2647-z]

75   **Zhang X**, Zhou Y, Li H, Wang R, Yang D, Li B, Fu J. Intravenous administration of DPSCs and BDNF improves neurological performance in rats with focal cerebral ischemia. *Int J Mol Med* 2018; **41**: 3185-3194 [PMID: 29512704 DOI: 10.3892/ijmm.2018.3517]

76   **Omi M**, Hata M, Nakamura N, Miyabe M, Ozawa S, Nukada H, Tsukamoto M, Sango K, Himeno T, Kamiya H, Nakamura J, Takebe J, Matsubara T, Naruse K. Transplantation of dental pulp stem cells improves long-term diabetic polyneuropathy together with improvement of nerve morphometrical evaluation. *Stem Cell Res Ther* 2017; **8**: 279 [PMID: 29237486 DOI: 10.1186/s13287-017-0729-5]

77   **Merckx G**, Lo Monaco M, Lambrichts I, Himmelreich U, Bronckaers A, Wolfs E. Safety and Homing of Human Dental Pulp Stromal Cells in Head and Neck Cancer. *Stem Cell Rev Rep* 2021; **17**: 1619-1634 [PMID: 33822326 DOI: 10.1007/s12015-021-10159-1]

78   **Kremer KL**, Smith AE, Sandeman L, Inglis JM, Ridding MC, Koblar SA. Transcranial Magnetic Stimulation of Human Adult Stem Cells in the Mammalian Brain. *Front Neural Circuits* 2016; **10**: 17 [PMID: 27013982 DOI: 10.3389/fncir.2016.00017]

79   **El-Kersh AOFO**, El-Akabawy G, Al-Serwi RH. Transplantation of human dental pulp stem cells in streptozotocin-induced diabetic rats. *Anat Sci Int* 2020; **95**: 523-539 [PMID: 32476103 DOI: 10.1007/s12565-020-00550-2]

80   **Lam C**, Alsaeedi HA, Koh AE, Harun MHN, Hwei ANM, Mok PL, Luu CD, Yong TK, Subbiah SK, Bastion MC. Human Dental Pulp Stem Cells (DPSCs) Therapy in Rescuing Photoreceptors and Establishing a Sodium Iodate-Induced Retinal Degeneration Rat Model. *Tissue Eng Regen Med* 2021; **18**: 143-154 [PMID: 33415670 DOI: 10.1007/s13770-020-00312-1]

81   **Kanada S**, Makino E, Nakamura N, Miyabe M, Ito M, Hata M, Yamauchi T, Sawada N, Kondo S, Saiki T, Minato T, Miyazawa K, Goto S, Matsubara T, Naruse K. Direct Comparison of Therapeutic Effects on Diabetic Polyneuropathy between Transplantation of Dental Pulp Stem Cells and Administration of Dental Pulp Stem Cell-Secreted Factors. *Int J Mol Sci* 2020; **21** [PMID: 32842469 DOI: 10.3390/ijms21176064]

82   **Nitahara-Kasahara Y**, Kuraoka M, Guillermo PH, Hayashita-Kinoh H, Maruoka Y, Nakamura-Takahasi A, Kimura K, Takeda S, Okada T. Dental pulp stem cells can improve muscle dysfunction in animal models of Duchenne muscular dystrophy. *Stem Cell Res Ther* 2021; **12**: 78 [PMID: 33494794 DOI: 10.1186/s13287-020-02099-3]

83   **Kim S**, Lee S, Jung HS, Kim SY, Shin SJ, Kang MK, Kim E. Evaluation of the Biodistribution of Human Dental Pulp Stem Cells Transplanted into Mice. *J Endod* 2018; **44**: 592-598 [PMID: 29370943 DOI: 10.1016/j.joen.2017.12.007]

84   **Fang CZ**, Yang YJ, Wang QH, Yao Y, Zhang XY, He XH. Intraventricular injection of human dental pulp stem cells improves hypoxic-ischemic brain damage in neonatal rats. *PLoS One* 2013; **8**: e66748 [PMID: 23799131 DOI: 10.1371/journal.pone.0066748]

85   **Sheyn D**, Mizrahi O, Benjamin S, Gazit Z, Pelled G, Gazit D. Genetically modified cells in regenerative medicine and tissue engineering. *Adv Drug Deliv Rev* 2010; **62**: 683-698 [PMID: 20114067 DOI: 10.1016/j.addr.2010.01.002]

86   **Porteus M**. Translating the lessons from gene therapy to the development of regenerative medicine. *Mol Ther* 2011; **19**: 439-441 [PMID: 21358706 DOI: 10.1038/mt.2011.14]

87   **Kimelman N**, Pelled G, Helm GA, Huard J, Schwarz EM, Gazit D. Review: gene- and stem cell-based therapeutics for bone regeneration and repair. *Tissue Eng* 2007; **13**: 1135-1150 [PMID: 17516852 DOI: 10.1089/ten.2007.0096]

88   **Bao Q**, Zhao Y, Niess H, Conrad C, Schwarz B, Jauch KW, Huss R, Nelson PJ, Bruns CJ. Mesenchymal stem cell-based tumor-targeted gene therapy in gastrointestinal cancer. *Stem Cells Dev* 2012; **21**: 2355-2363 [PMID: 22530882 DOI: 10.1089/scd.2012.0060]

89   **Hodgkinson CP**, Gomez JA, Mirotsou M, Dzau VJ. Genetic engineering of mesenchymal stem cells and its application in human disease therapy. *Hum Gene Ther* 2010; **21**: 1513-1526 [PMID: 20825283 DOI: 10.1089/hum.2010.165]

90   **Sowa K**, Nito C, Nakajima M, Suda S, Nishiyama Y, Sakamoto Y, Nitahara-Kasahara Y, Nakamura-Takahashi A, Ueda M, Kimura K, Okada T. Impact of Dental Pulp Stem Cells Overexpressing Hepatocyte Growth Factor after Cerebral Ischemia/Reperfusion in Rats. *Mol Ther Methods Clin Dev* 2018; **10**: 281-290 [PMID: 30151417 DOI: 10.1016/j.omtm.2018.07.009]

91   **Cao XF**, Jin SZ, Sun L, Zhan YB, Lin F, Li Y, Zhou YL, Wang XM, Gao L, Zhang B. Therapeutic effects of hepatocyte growth factor-overexpressing dental pulp stem cells on liver cirrhosis in a rat model. *Sci Rep* 2017; **7**: 15812 [PMID: 29150644 DOI: 10.1038/s41598-017-14995-5]

92   **Cao Y**, Liu Z, Xie Y, Hu J, Wang H, Fan Z, Zhang C, Wang J, Wu CT, Wang S. Adenovirus-mediated transfer of hepatocyte growth factor gene to human dental pulp stem cells under good manufacturing practice improves their potential for periodontal regeneration in swine. *Stem Cell Res Ther* 2015; **6**: 249 [PMID: 26670567 DOI: 10.1186/s13287-015-0244-5]

93   **Kong F**, Shi X, Xiao F, Yang Y, Zhang X, Wang LS, Wu CT, Wang H. Transplantation of Hepatocyte Growth Factor-Modified Dental Pulp Stem Cells Prevents Bone Loss in the Early Phase of Ovariectomy-Induced Osteoporosis. *Hum Gene Ther* 2018; **29**: 271-282 [PMID: 28950723 DOI: 10.1089/hum.2017.091]

94   **Rizk A**, Rabie AB. Human dental pulp stem cells expressing transforming growth factor β3 transgene for cartilage-like tissue engineering. *Cytotherapy* 2013; **15**: 712-725 [PMID: 23474328 DOI: 10.1016/j.jcyt.2013.01.012]

95   **Feng G**, Zhang J, Feng X, Wu S, Huang D, Hu J, Zhu S, Song D. Runx2 modified dental pulp stem cells (DPSCs) enhance new bone formation during rapid distraction osteogenesis (DO). *Differentiation* 2016; **92**: 195-203 [PMID: 27313006 DOI: 10.1016/j.diff.2016.06.001]

96   **Kanai N**, Yamato M, Okano T. Cell sheets engineering for esophageal regenerative medicine. *Ann Transl Med* 2014; **2**: 28 [PMID: 25333004 DOI: 10.3978/j.issn.2305-5839.2014.03.06]

97   **Sakaguchi K**, Shimizu T, Okano T. Construction of three-dimensional vascularized cardiac tissue with cell sheet engineering. *J Control Release* 2015; **205**: 83-88 [PMID: 25523520 DOI: 10.1016/j.jconrel.2014.12.016]

98   **Hayashi R**, Yamato M, Takayanagi H, Oie Y, Kubota A, Hori Y, Okano T, Nishida K. Validation system of tissue-engineered epithelial cell sheets for corneal regenerative medicine. *Tissue Eng Part C Methods* 2010; **16**: 553-560 [PMID: 19722828 DOI: 10.1089/ten.TEC.2009.0277]

99   **Baek J**, Cho Y, Park HJ, Choi G, Lee JS, Lee M, Yu SJ, Cho SW, Lee E, Im SG. A Surface-Tailoring Method for Rapid Non-Thermosensitive Cell-Sheet Engineering *via* Functional Polymer Coatings. *Adv Mater* 2020; **32**: e1907225 [PMID: 32157771 DOI: 10.1002/adma.201907225]

100   **Guo R**, Morimatsu M, Feng T, Lan F, Chang D, Wan F, Ling Y. Stem cell-derived cell sheet transplantation for heart tissue repair in myocardial infarction. *Stem Cell Res Ther* 2020; **11**: 19 [PMID: 31915074 DOI: 10.1186/s13287-019-1536-y]

101   **Yang J**, Yamato M, Kohno C, Nishimoto A, Sekine H, Fukai F, Okano T. Cell sheet engineering: recreating tissues without biodegradable scaffolds. *Biomaterials* 2005; **26**: 6415-6422 [PMID: 16011847 DOI: 10.1016/j.biomaterials.2005.04.061]

102   **Gomes JA**, Geraldes Monteiro B, Melo GB, Smith RL, Cavenaghi Pereira da Silva M, Lizier NF, Kerkis A, Cerruti H, Kerkis I. Corneal reconstruction with tissue-engineered cell sheets composed of human immature dental pulp stem cells. *Invest Ophthalmol Vis Sci* 2010; **51**: 1408-1414 [PMID: 19892864 DOI: 10.1167/iovs.09-4029]

103   **Lee JM**, Kim HY, Park JS, Lee DJ, Zhang S, Green DW, Okano T, Hong JH, Jung HS. Developing palatal bone using human mesenchymal stem cell and stem cells from exfoliated deciduous teeth cell sheets. *J Tissue Eng Regen Med* 2019; **13**: 319-327 [PMID: 30644640 DOI: 10.1002/term.2811]

104   **Watanabe M**, Ohyama A, Ishikawa H, Tanaka A. Three-dimensional bone formation including vascular networks derived from dental pulp stem cells in vitro. *Hum Cell* 2019; **32**: 114-124 [PMID: 30523537 DOI: 10.1007/s13577-018-00228-y]

105   **Wei F**, Qu C, Song T, Ding G, Fan Z, Liu D, Liu Y, Zhang C, Shi S, Wang S. Vitamin C treatment promotes mesenchymal stem cell sheet formation and tissue regeneration by elevating telomerase activity. *J Cell Physiol* 2012; **227**: 3216-3224 [PMID: 22105792 DOI: 10.1002/jcp.24012]

106   **Carr AC**, Maggini S. Vitamin C and Immune Function. *Nutrients* 2017; **9** [PMID: 29099763 DOI: 10.3390/nu9111211]

107   **Franceschi RT**. The role of ascorbic acid in mesenchymal differentiation. *Nutr Rev* 1992; **50**: 65-70 [PMID: 1565288 DOI: 10.1111/j.1753-4887.1992.tb01271.x]

108   **Villacorta L**, Azzi A, Zingg JM. Regulatory role of vitamins E and C on extracellular matrix components of the vascular system. *Mol Aspects Med* 2007; **28**: 507-537 [PMID: 17624419 DOI: 10.1016/j.mam.2007.05.002]

109   **Mekala NK**, Baadhe RR, Rao Parcha S, Prameela Devi Y. Enhanced proliferation and osteogenic differentiation of human umbilical cord blood stem cells by L-ascorbic acid, in vitro. *Curr Stem Cell Res Ther* 2013; **8**: 156-162 [PMID: 23140501 DOI: 10.2174/1574888x11308020006]

110   **Yang X**, Ma Y, Guo W, Yang B, Tian W. Stem cells from human exfoliated deciduous teeth as an alternative cell source in bio-root regeneration. *Theranostics* 2019; **9**: 2694-2711 [PMID: 31131062 DOI: 10.7150/thno.31801]

111   **Pedroni ACF**, Diniz IMA, Abe GL, Moreira MS, Sipert CR, Marques MM. Photobiomodulation therapy and vitamin C on longevity of cell sheets of human dental pulp stem cells. *J Cell Physiol* 2018; **233**: 7026-7035 [PMID: 29744863 DOI: 10.1002/jcp.26626]

112   **Chen YJ**, Zhao YH, Zhao YJ, Liu NX, Lv X, Li Q, Chen FM, Zhang M. Potential dental pulp revascularization and odonto-/osteogenic capacity of a novel transplant combined with dental pulp stem cells and platelet-rich fibrin. *Cell Tissue Res* 2015; **361**: 439-455 [PMID: 25797716 DOI: 10.1007/s00441-015-2125-8]

113   **Meng H**, Hu L, Zhou Y, Ge Z, Wang H, Wu CT, Jin J. A Sandwich Structure of Human Dental Pulp Stem Cell Sheet, Treated Dentin Matrix, and Matrigel for Tooth Root Regeneration. *Stem Cells Dev* 2020; **29**: 521-532 [PMID: 32089088 DOI: 10.1089/scd.2019.0162]

114   **Garrido PR**, Pedroni ACF, Cury DP, Moreira MS, Rosin F, Sarra G, Marques MM. Effects of photobiomodulation therapy on the extracellular matrix of human dental pulp cell sheets. *J Photochem Photobiol B* 2019; **194**: 149-157

[PMID: 30954874 DOI: 10.1016/j.jphotobiol.2019.03.017]

115   **Honjo K**, Yamamoto T, Adachi T, Amemiya T, Mazda O, Kanamura N, Kita M. Evaluation of a dental pulp-derived cell sheet cultured on amniotic membrane substrate. *Biomed Mater Eng* 2015; **25**: 203-212 [PMID: 25813958 DOI: 10.3233/BME-151270]

116   **Takizawa S**, Yamamoto T, Honjo KI, Sato Y, Nakamura K, Yamamoto K, Adachi T, Uenishi T, Oseko F, Amemiya T, Yamamoto Y, Kumagai W, Kita M, Kanamura N. Transplantation of dental pulp-derived cell sheets cultured on human amniotic membrane induced to differentiate into bone. *Oral Dis* 2019; **25**: 1352-1362 [PMID: 30912198 DOI: 10.1111/odi.13096]

117   **Tatsuhiro F**, Seiko T, Yusuke T, Reiko TT, Kazuhito S. Dental Pulp Stem Cell-Derived, Scaffold-Free Constructs for Bone Regeneration. *Int J Mol Sci* 2018; **19** [PMID: 29932167 DOI: 10.3390/ijms19071846]

118   **Xu W**, Chen S, Yao M, Jiang X, Lu Q. A Near-Infrared-Triggered Dynamic Wrinkling Biointerface for Noninvasive Harvesting of Practical Cell Sheets. *ACS Appl Mater Interfaces* 2021; **13**: 32790-32798 [PMID: 34232024 DOI: 10.1021/acsami.1c07962]

119   **Stanton MM**, Lambert CR. A thermoresponsive, micro-roughened cell culture surface. *Acta Biomater* 2015; **15**: 11-19 [PMID: 25523874 DOI: 10.1016/j.actbio.2014.12.006]

120   **Jiang Z**, Xi Y, Lai K, Wang Y, Wang H, Yang G. Laminin-521 Promotes Rat Bone Marrow Mesenchymal Stem Cell Sheet Formation on Light-Induced Cell Sheet Technology. *Biomed Res Int* 2017; **2017**: 9474573 [PMID: 28164129 DOI: 10.1155/2017/9474573]

121   **Magalhães FD**, Sarra G, Carvalho GL, Pedroni ACF, Marques MM, Chambrone L, Gimenez T, Moreira MS. Dental tissue-derived stem cell sheet biotechnology for periodontal tissue regeneration: A systematic review. *Arch Oral Biol* 2021; **129**: 105182 [PMID: 34098416 DOI: 10.1016/j.archoralbio.2021.105182]

122   **Pedroni ACF**, Sarra G, de Oliveira NK, Moreira MS, Deboni MCZ, Marques MM. Cell sheets of human dental pulp stem cells for future application in bone replacement. *Clin Oral Investig* 2019; **23**: 2713-2721 [PMID: 30357480 DOI: 10.1007/s00784-018-2630-8]

123   **Ahmed MN**, Shi D, Dailey MT, Rothermund K, Drewry MD, Calabrese TC, Cui XT, Syed-Picard FN. Dental Pulp Cell Sheets Enhance Facial Nerve Regeneration *via* Local Neurotrophic Factor Delivery. *Tissue Eng Part A* 2021; **27**: 1128-1139 [PMID: 33164704 DOI: 10.1089/ten.TEA.2020.0265]

124   **Drewry MD**, Dailey MT, Rothermund K, Backman C, Dahl KN, Syed-Picard FN. Promoting and Orienting Axon Extension Using Scaffold-Free Dental Pulp Stem Cell Sheets. *ACS Biomater Sci Eng* 2022; **8**: 814-825 [PMID: 34982537 DOI: 10.1021/acsbiomaterials.1c01517]

125   **Xuan K**, Li B, Guo H, Sun W, Kou X, He X, Zhang Y, Sun J, Liu A, Liao L, Liu S, Liu W, Hu C, Shi S, Jin Y. Deciduous autologous tooth stem cells regenerate dental pulp after implantation into injured teeth. *Sci Transl Med* 2018; **10** [PMID: 30135248 DOI: 10.1126/scitranslmed.aaf3227]

126   **Guo H**, Li B, Wu M, Zhao W, He X, Sui B, Dong Z, Wang L, Shi S, Huang X, Liu X, Li Z, Guo X, Xuan K, Jin Y. Odontogenesis-related developmental microenvironment facilitates deciduous dental pulp stem cell aggregates to revitalize an avulsed tooth. *Biomaterials* 2021; **279**: 121223 [PMID: 34736149 DOI: 10.1016/j.biomaterials.2021.121223]

127   **Sekine H**, Shimizu T, Dobashi I, Matsuura K, Hagiwara N, Takahashi M, Kobayashi E, Yamato M, Okano T. Cardiac cell sheet transplantation improves damaged heart function *via* superior cell survival in comparison with dissociated cell injection. *Tissue Eng Part A* 2011; **17**: 2973-2980 [PMID: 21875331 DOI: 10.1089/ten.tea.2010.0659]

128   **Takeuchi R**, Kuruma Y, Sekine H, Dobashi I, Yamato M, Umezu M, Shimizu T, Okano T. In vivo vascularization of cell sheets provided better long-term tissue survival than injection of cell suspension. *J Tissue Eng Regen Med* 2016; **10**: 700-710 [PMID: 24470393 DOI: 10.1002/term.1854]

129   **Gilpin A**, Yang Y. Decellularization Strategies for Regenerative Medicine: From Processing Techniques to Applications. *Biomed Res Int* 2017; **2017**: 9831534 [PMID: 28540307 DOI: 10.1155/2017/9831534]

130   **Huang JP**, Wu YM, Liu JM, Zhang L, Li BX, Chen LL, Ding PH, Tan JY. Decellularized matrix could affect the proliferation and differentiation of periodontal ligament stem cells in vitro. *J Periodontal Res* 2021; **56**: 929-939 [PMID: 34173232 DOI: 10.1111/jre.12889]

131   **Fennema E**, Rivron N, Rouwkema J, van Blitterswijk C, de Boer J. Spheroid culture as a tool for creating 3D complex tissues. *Trends Biotechnol* 2013; **31**: 108-115 [PMID: 23336996 DOI: 10.1016/j.tibtech.2012.12.003]

132   **Laschke MW**, Menger MD. Life is 3D: Boosting Spheroid Function for Tissue Engineering. *Trends Biotechnol* 2017; **35**: 133-144 [PMID: 27634310 DOI: 10.1016/j.tibtech.2016.08.004]

133   **Lee D**, Yoon H, Lee J, Lee K, Lee S, Kim S, Choi J, Kim Y, Park J. Enhanced liver-specific functions of endothelial cell-covered hepatocyte hetero-spheroids. *Biochem Eng J* 2004; **20**: 181-187 [DOI: 10.1016/j.bej.2003.07.005]

134   **Henry E**, Cores J, Hensley MT, Anthony S, Vandergriff A, de Andrade JB, Allen T, Caranasos TG, Lobo LJ, Cheng K. Adult Lung Spheroid Cells Contain Progenitor Cells and Mediate Regeneration in Rodents With Bleomycin-Induced Pulmonary Fibrosis. *Stem Cells Transl Med* 2015; **4**: 1265-1274 [PMID: 26359426 DOI: 10.5966/sctm.2015-0062]

135   **Dissanayaka WL**, Zhu L, Hargreaves KM, Jin L, Zhang C. Scaffold-free Prevascularized Microtissue Spheroids for Pulp Regeneration. *J Dent Res* 2014; **93**: 1296-1303 [PMID: 25201919 DOI: 10.1177/0022034514550040]

136   **Kim EM**, Lee YB, Kim SJ, Park J, Lee J, Kim SW, Park H, Shin H. Fabrication of core-shell spheroids as building blocks for engineering 3D complex vascularized tissue. *Acta Biomater* 2019; **100**: 158-172 [PMID: 31542503 DOI: 10.1016/j.actbio.2019.09.028]

137   **Inamori M**, Mizumoto H, Kajiwara T. An approach for formation of vascularized liver tissue by endothelial cell-covered hepatocyte spheroid integration. *Tissue Eng Part A* 2009; **15**: 2029-2037 [PMID: 19320555 DOI: 10.1089/ten.tea.2008.0403]

138   **Achilli TM**, Meyer J, Morgan JR. Advances in the formation, use and understanding of multi-cellular spheroids. *Expert Opin Biol Ther* 2012; **12**: 1347-1360 [PMID: 22784238 DOI: 10.1517/14712598.2012.707181]

139   **Liu D**, Chen S, Win Naing M. A review of manufacturing capabilities of cell spheroid generation technologies and future development. *Biotechnol Bioeng* 2021; **118**: 542-554 [PMID: 33146407 DOI: 10.1002/bit.27620]

140   **Tseng TC**, Wong CW, Hsieh FY, Hsu SH. Biomaterial Substrate-Mediated Multicellular Spheroid Formation and Their

Applications in Tissue Engineering. *Biotechnol J* 2017; **12** [PMID: 28925549 DOI: 10.1002/biot.201700064]

141   **Kim HJ**, Castañeda R, Kang TH, Kimura S, Wada M, Kim U. Cellulose hydrogel film for spheroid formation of human adipose-derived stemcells. *Cellulose* 2018; **25**: 2589-2598 [DOI: 10.1007/s10570-018-1732-4]

142   **Byun H**, Bin Lee Y, Kim EM, Shin H. Fabrication of size-controllable human mesenchymal stromal cell spheroids from micro-scaled cell sheets. *Biofabrication* 2019; **11**: 035025 [PMID: 31096204 DOI: 10.1088/1758-5090/ab21f6]

143   **Iwai R**, Nemoto Y, Nakayama Y. Preparation and characterization of directed, one-day-self-assembled millimeter-size spheroids of adipose-derived mesenchymal stem cells. *J Biomed Mater Res A* 2016; **104**: 305-312 [PMID: 26386244 DOI: 10.1002/jbm.a.35568]

144   **Zheng Y**, Jiang LI, Yan M, Gosau M, Smeets R, Kluwe L, Friedrich RE. Optimizing Conditions for Spheroid Formation of Dental Pulp Cells in Cell Culture. *In Vivo* 2021; **35**: 1965-1972 [PMID: 34182470 DOI: 10.21873/invivo.12464]

145   **Xiao L**, Tsutsui T. Characterization of human dental pulp cells-derived spheroids in serum-free medium: stem cells in the core. *J Cell Biochem* 2013; **114**: 2624-2636 [PMID: 23794488 DOI: 10.1002/jcb.24610]

146   **Xiao L**, Kumazawa Y, Okamura H. Cell death, cavitation and spontaneous multi-differentiation of dental pulp stem cells-derived spheroids in vitro: a journey to survival and organogenesis. *Biol Cell* 2014; **106**: 405-419 [PMID: 25175801 DOI: 10.1111/boc.201400024]

147   **Son YB**, Bharti D, Kim SB, Jo CH, Bok EY, Lee SL, Kang YH, Rho GJ. Comparison of Pluripotency, Differentiation, and Mitochondrial Metabolism Capacity in Three-Dimensional Spheroid Formation of Dental Pulp-Derived Mesenchymal Stem Cells. *Biomed Res Int* 2021; **2021**: 5540877 [PMID: 34337022 DOI: 10.1155/2021/5540877]

148   **Kawashima N**, Noda S, Yamamoto M, Okiji T. Properties of Dental Pulp-derived Mesenchymal Stem Cells and the Effects of Culture Conditions. *J Endod* 2017; **43**: S31-S34 [PMID: 28781092 DOI: 10.1016/j.joen.2017.06.004]

149   **Han X**, Tang S, Wang L, Xu X, Yan R, Yan S, Guo Z, Hu K, Yu T, Li M, Li Y, Zhang F, Gu N. Multicellular Spheroids Formation on Hydrogel Enhances Osteogenic/Odontogenic Differentiation of Dental Pulp Stem Cells Under Magnetic Nanoparticles Induction. *Int J Nanomedicine* 2021; **16**: 5101-5115 [PMID: 34349510 DOI: 10.2147/IJN.S318991]

150   **Min TJ**, Kim MJ, Kang KJ, Jeoung YJ, Oh SH, Jang YJ. 3D Spheroid Formation Using BMP-Loaded Microparticles Enhances Odontoblastic Differentiation of Human Dental Pulp Stem Cells. *Stem Cells Int* 2021; **2021**: 9326298 [PMID: 34512768 DOI: 10.1155/2021/9326298]

151   **Kim H**, Han Y, Suhito IR, Choi Y, Kwon M, Son H, Kim HR, Kim TH. Raman Spectroscopy-Based 3D Analysis of Odontogenic Differentiation of Human Dental Pulp Stem Cell Spheroids. *Anal Chem* 2021; **93**: 9995-10004 [PMID: 34241992 DOI: 10.1021/acs.analchem.0c05165]

152   **Yamamoto M**, Kawashima N, Takashino N, Koizumi Y, Takimoto K, Suzuki N, Saito M, Suda H. Three-dimensional spheroid culture promotes odonto/osteoblastic differentiation of dental pulp cells. *Arch Oral Biol* 2014; **59**: 310-317 [PMID: 24581854 DOI: 10.1016/j.archoralbio.2013.12.006]

153   **Zhang S**, Buttler-Buecher P, Denecke B, Arana-Chavez VE, Apel C. A comprehensive analysis of human dental pulp cell spheroids in a three-dimensional pellet culture system. *Arch Oral Biol* 2018; **91**: 1-8 [PMID: 29621667 DOI: 10.1016/j.archoralbio.2018.02.008]

154   **Hsieh HY**, Young TH, Yao CC, Chen YJ. Aggregation of human dental pulp cells into 3D spheroids enhances their migration ability after reseeding. *J Cell Physiol* 2018; **234**: 976-986 [PMID: 30132855 DOI: 10.1002/jcp.26927]

155   **Li D**, Zou XY, El-Ayachi I, Romero LO, Yu Z, Iglesias-Linares A, Cordero-Morales JF, Huang GT. Human Dental Pulp Stem Cells and Gingival Mesenchymal Stem Cells Display Action Potential Capacity In Vitro after Neuronogenic Differentiation. *Stem Cell Rev Rep* 2019; **15**: 67-81 [PMID: 30324358 DOI: 10.1007/s12015-018-9854-5]

156   **Gervois P**, Struys T, Hilkens P, Bronckaers A, Ratajczak J, Politis C, Brône B, Lambrichts I, Martens W. Neurogenic maturation of human dental pulp stem cells following neurosphere generation induces morphological and electrophysiological characteristics of functional neurons. *Stem Cells Dev* 2015; **24**: 296-311 [PMID: 25203005 DOI: 10.1089/scd.2014.0117]

157   **Rafiee F**, Pourteymourfard-Tabrizi Z, Mahmoudian-Sani MR, Mehri-Ghahfarrokhi A, Soltani A, Hashemzadeh-Chaleshtori M, Jami MS. Differentiation of dental pulp stem cells into neuron-like cells. *Int J Neurosci* 2020; **130**: 107-116 [PMID: 31599165 DOI: 10.1080/00207454.2019.1664518]

158   **Xiao L**, Ide R, Saiki C, Kumazawa Y, Okamura H. Human Dental Pulp Cells Differentiate toward Neuronal Cells and Promote Neuroregeneration in Adult Organotypic Hippocampal Slices In Vitro. *Int J Mol Sci* 2017; **18** [PMID: 28800076 DOI: 10.3390/ijms18081745]

159   **Itoh Y**, Sasaki JI, Hashimoto M, Katata C, Hayashi M, Imazato S. Pulp Regeneration by 3-dimensional Dental Pulp Stem Cell Constructs. *J Dent Res* 2018; **97**: 1137-1143 [PMID: 29702010 DOI: 10.1177/0022034518772260]

160   **Farrell E**, Wielopolski P, Pavljasevic P, Kops N, Weinans H, Bernsen MR, van Osch GJ. Cell labelling with superparamagnetic iron oxide has no effect on chondrocyte behaviour. *Osteoarthritis Cartilage* 2009; **17**: 961-967 [PMID: 19147376 DOI: 10.1016/j.joca.2008.11.016]

161   **Bergenheim F**, Seidelin JB, Pedersen MT, Mead BE, Jensen KB, Karp JM, Nielsen OH. Fluorescence-based tracing of transplanted intestinal epithelial cells using confocal laser endomicroscopy. *Stem Cell Res Ther* 2019; **10**: 148 [PMID: 31133056 DOI: 10.1186/s13287-019-1246-5]

162   **Zhang Z**, Nör F, Oh M, Cucco C, Shi S, Nör JE. Wnt/β-Catenin Signaling Determines the Vasculogenic Fate of Postnatal Mesenchymal Stem Cells. *Stem Cells* 2016; **34**: 1576-1587 [PMID: 26866635 DOI: 10.1002/stem.2334]

163   **Luzuriaga J**, Pastor-Alonso O, Encinas JM, Unda F, Ibarretxe G, Pineda JR. Human Dental Pulp Stem Cells Grown in Neurogenic Media Differentiate Into Endothelial Cells and Promote Neovasculogenesis in the Mouse Brain. *Front Physiol* 2019; **10**: 347 [PMID: 30984027 DOI: 10.3389/fphys.2019.00347]

164   **Sarsenova M**, Issabekova A, Abisheva S, Rutskaya-Moroshan K, Ogay V, Saparov A. Mesenchymal Stem Cell-Based Therapy for Rheumatoid Arthritis. *Int J Mol Sci* 2021; **22** [PMID: 34769021 DOI: 10.3390/ijms222111592]

165   **Matheakakis A**, Batsali A, Papadaki HA, Pontikoglou CG. Therapeutic Implications of Mesenchymal Stromal Cells and Their Extracellular Vesicles in Autoimmune Diseases: From Biology to Clinical Applications. *Int J Mol Sci* 2021; **22** [PMID: 34576296 DOI: 10.3390/ijms221810132]

166   **Di Nicola M**, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM. Human bone

marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002; **99**: 3838-3843 [PMID: 11986244 DOI: 10.1182/blood.v99.10.3838]

167   **Rafei M**, Campeau PM, Aguilar-Mahecha A, Buchanan M, Williams P, Birman E, Yuan S, Young YK, Boivin MN, Forner K, Basik M, Galipeau J. Mesenchymal stromal cells ameliorate experimental autoimmune encephalomyelitis by inhibiting CD4 Th17 T cells in a CC chemokine ligand 2-dependent manner. *J Immunol* 2009; **182**: 5994-6002 [PMID: 19414750 DOI: 10.4049/jimmunol.0803962]

168   **Aggarwal S**, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005; **105**: 1815-1822 [PMID: 15494428 DOI: 10.1182/blood-2004-04-1559]

169   **Zhang W**, Ge W, Li C, You S, Liao L, Han Q, Deng W, Zhao RC. Effects of mesenchymal stem cells on differentiation, maturation, and function of human monocyte-derived dendritic cells. *Stem Cells Dev* 2004; **13**: 263-271 [PMID: 15186722 DOI: 10.1089/154732804323099190]

170   **Ren G**, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y. Mesenchymal stem cell-mediated immunosuppression occurs *via* concerted action of chemokines and nitric oxide. *Cell Stem Cell* 2008; **2**: 141-150 [PMID: 18371435 DOI: 10.1016/j.stem.2007.11.014]

171   **Pierdomenico L**, Bonsi L, Calvitti M, Rondelli D, Arpinati M, Chirumbolo G, Becchetti E, Marchionni C, Alviano F, Fossati V, Staffolani N, Franchina M, Grossi A, Bagnara GP. Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. *Transplantation* 2005; **80**: 836-842 [PMID: 16210973 DOI: 10.1097/01.tp.0000173794.72151.88]

172   **Kwack KH**, Lee JM, Park SH, Lee HW. Human Dental Pulp Stem Cells Suppress Alloantigen-induced Immunity by Stimulating T Cells to Release Transforming Growth Factor Beta. *J Endod* 2017; **43**: 100-108 [PMID: 27871783 DOI: 10.1016/j.joen.2016.09.005]

173   **Wada N**, Menicanin D, Shi S, Bartold PM, Gronthos S. Immunomodulatory properties of human periodontal ligament stem cells. *J Cell Physiol* 2009; **219**: 667-676 [PMID: 19160415 DOI: 10.1002/jcp.21710]

174   **Tomic S**, Djokic J, Vasilijic S, Vucevic D, Todorovic V, Supic G, Colic M. Immunomodulatory properties of mesenchymal stem cells derived from dental pulp and dental follicle are susceptible to activation by toll-like receptor agonists. *Stem Cells Dev* 2011; **20**: 695-708 [PMID: 20731536 DOI: 10.1089/scd.2010.0145]

175   **Fujio M**, Xing Z, Sharabi N, Xue Y, Yamamoto A, Hibi H, Ueda M, Fristad I, Mustafa K. Conditioned media from hypoxic-cultured human dental pulp cells promotes bone healing during distraction osteogenesis. *J Tissue Eng Regen Med* 2017; **11**: 2116-2126 [PMID: 26612624 DOI: 10.1002/term.2109]

176   **Mancuso P**, Raman S, Glynn A, Barry F, Murphy JM. Mesenchymal Stem Cell Therapy for Osteoarthritis: The Critical Role of the Cell Secretome. *Front Bioeng Biotechnol* 2019; **7**: 9 [PMID: 30761298 DOI: 10.3389/fbioe.2019.00009]

177   **Yin K**, Wang S, Zhao RC. Exosomes from mesenchymal stem/stromal cells: a new therapeutic paradigm. *Biomark Res* 2019; **7**: 8 [PMID: 30992990 DOI: 10.1186/s40364-019-0159-x]

178   **Zhou Y**, Yamamoto Y, Xiao Z, Ochiya T. The Immunomodulatory Functions of Mesenchymal Stromal/Stem Cells Mediated *via* Paracrine Activity. *J Clin Med* 2019; **8** [PMID: 31336889 DOI: 10.3390/jcm8071025]

Role of hypoxia preconditioning in therapeutic potential of mesenchymal stem-cell-derived extracellular vesicles

Victoria Pulido-Escribano, Bárbara Torrecillas-Baena, Marta Camacho-Cardenosa, Gabriel Dorado, María Ángeles Gálvez-Moreno, Antonio Casado-Díaz

Victoria Pulido-Escribano, Bárbara Torrecillas-Baena, Marta Camacho-Cardenosa, María Ángeles Gálvez-Moreno, Antonio Casado-Díaz, Unidad de Gestión Clínica de Endocrinología y Nutrición-GC17, Instituto Maimónides de Investigación Biomédica de Córdoba, Hospital Universitario Reina Sofía, Córdoba 14004, Spain

Gabriel Dorado, Dep. Bioquímica y Biología Molecular, Campus Rabanales C6-1-E17, Campus de Excelencia Internacional Agroalimentario (ceiA3), Universidad de Córdoba, CIBERFES, Córdoba 14071, Spain

Corresponding author: Antonio Casado-Díaz, PhD, Research Scientist, Unidad de Gestión Clínica de Endocrinología y Nutrición-GC17, Instituto Maimónides de Investigación Biomédica de Córdoba, Hospital Universitario Reina Sofía, Avda, Menéndez Pidal, s/n, Córdoba 14004, Spain. bb1cadia@uco.es

Abstract

The use of mesenchymal stem-cells (MSC) in cell therapy has received considerable attention because of their properties. These properties include high expansion and differentiation *in vitro*, low immunogenicity, and modulation of biological processes, such as inflammation, angiogenesis and hematopoiesis. Curiously, the regenerative effect of MSC is partly due to their paracrine activity. This has prompted numerous studies, to investigate the therapeutic potential of their secretome in general, and specifically their extracellular vesicles (EV). The latter contain proteins, lipids, nucleic acids, and other metabolites, which can cause physiological changes when released into recipient cells. Interestingly, contents of EV can be modulated by preconditioning MSC under different culture conditions. Among them, exposure to hypoxia stands out; these cells respond by activating hypoxia-inducible factor (HIF) at low O_2 concentrations. HIF has direct and indirect pleiotropic effects, modulating expression of hundreds of genes involved in processes such as inflammation, migration, proliferation, differentiation, angiogenesis, metabolism, and cell apoptosis. Expression of these genes is reflected in the contents of secreted EV. Interestingly, numerous studies show that MSC-derived EV conditioned under hypoxia have a higher regenerative capacity than those obtained under normoxia. In this review, we show the implications of hypoxia responses in relation to tissue regeneration. In addition, hypoxia preconditioning of MSC is being evaluated as a very attractive strategy for isolation of EV, with a high potential for clinical use in regenerative medicine that can be applied to different pathologies.

Core Tip: Mesenchymal stem-cells (MSC)-derived EV have a high therapeutic interest. The composition of extracellular vesicles (EV) depends on the state of source cells, generating physiological changes in recipient cells. MSC culture preconditioning affects the cargos of EV. Thus, hypoxia exposition leads to hypoxia-inducible factor induction and regulation of hundreds of genes involved in processes such as inflammation, migration, proliferation, differentiation, angiogenesis, metabolism, and apoptosis. This affects the contents of secreted EV. Accordingly, numerous studies have shown that EV from MSC under hypoxia have a higher regenerative capacity than those obtained under normoxia. Therefore, the former have a high clinical potential in different pathologies.

INTRODUCTION

Mesenchymal stem-cells or mesenchymal stromal-cells (MSC) derived from adult tissues are characterized by their low immunogenicity, high proliferation capacity, differentiation capabilities, and modulation of physiological processes such as inflammation, hematopoiesis, and angiogenesis[1-3]. MSC can be isolated from different tissues for their culture and expansion *in vitro*. Therefore, they are currently considered an important therapeutic tool in the field of regenerative medicine[4,5]. However, one of the main limitations of their use is the need to obtain and expand MSC *in vitro*, which cannot always be obtained from the same patient to be treated. Unfortunately, MSC manipulations may cause cell-functionality loss and genetic instability when performed outside their natural niches[6]. Moreover, one risk of the application of cell therapy in regenerative medicine is that MSC may remain undifferentiated and produce tumors[7].

Recently, numerous studies have shown that the regenerative capacity of MSC mainly depends on their paracrine functions. Therefore, an alternative or complement to cell therapy in regenerative medicine is the use of media enriched in cytokines and other factors secreted by *in vitro* cultures of progenitor cells[8]. The MSC secretome is composed by soluble factors and extracellular vesicles (EV) [9]. The main functions of EV are cell communications and interactions. Their contents depend on their cellular origin and the physiological conditions in which they are produced[10]. Therefore, preconditioning MSC under conditions that increase their regenerative power, like hypoxia, may induce the production of EV with enhanced regenerative potential. In the presence of damage, tissues normally undergo ischemic processes. These reduce the supply of O_2 and nutrients to damaged areas. This causes cellular responses that induce the release of factors, promoting vessel formation and tissue regeneration [11]. Indeed, MSC preconditioning in hypoxia can induce this response, leading to the production of EV rich in angiogenic factors and inducers of tissue regeneration. In this context, the main aim of this review is to describe the effects that preconditioning in hypoxia may have on MSC, mainly in the contents of their EV, and how this strategy has a great potential for regenerative medicine.

MSC AND REGENERATIVE MEDICINE

MSC are multipotent cells first discovered by Friedenstein *et al*[12] in 1970. They have fibroblast-like morphology, behaving as colony-forming units-fibroblasts. These cells originate in the mesoderm, having the ability to differentiate into different cell types including osteoblasts, adipocytes, and chondrocytes[13]. The minimum characteristics that a cell must have to be considered MSC, according to the International Society for Cellular Therapy, are: (1) Adhere to plastic under standard culture conditions; (2) Exhibit several clusters of differentiation (CD): CD-73, CD-90, and CD-105, lacking CD-11b, CD-14, CD-19, CD-34, CD-45, CD-79a, and human leukocyte antigen–D-related isotype; and (3) Differentiation potency into osteoblasts, adipocytes, or chondrocytes *in vitro*[14]. Isolated MSC may have different origins, such as adipose tissue, placenta, umbilical-cord blood or Wharton's jelly,

synovium, periodontal ligament, menstrual blood, and bone marrow, the latter being one of the essential sources of these cells for research and clinical applications[15-17].

MSC are involved in tissue regeneration, being necessary for maintaining vital functions and delaying aging. The application of MSC in regenerative therapies is gaining great interest due to their advantages. Thus, these cells can be isolated and cultured *in vitro*, have the capacity to undergo multilineage differentiation, and also possess anti-inflammatory and immunosuppressive properties[5]. Indeed, such cells have great potential to treat various pathologies, including those of the nervous system, bone, skin, myocardium, and liver, among others[4,18-20]. In this regard, multiple clinical trials related to these pathologies have demonstrated the potential of MSC in human clinical practice[21-23]. Nevertheless, despite the potential and good results obtained in cell therapy, the risks involved when using cells in regenerative medicine should be considered, as indicated above. Therefore, in the last few years, cell-free therapies have gained attention, becoming the preferred options in many instances.

MSC-DERIVED EV AS A NOVEL APPROACH TO CELL-FREE THERAPIES

It is well known that MSC-based cell therapies have beneficial therapeutic effects in different pathologies. Nevertheless, some studies suggest that these benefits may not be due to the cells themselves, but mainly linked to their paracrine effects. For instance, at the site of injury[13,24,25], the EV secreted by these cells are the key players[26]. Thus, the use of MSC-derived EV has been found to be beneficial to improve cartilage repair and regeneration, cardiac repair after myocardial infarction, wound healing, and lung repair, among other clinical applications[27-30].

According to their size, biogenesis, release pathway, function, and content, EV have been classified into microvesicles, exosomes, and apoptotic bodies. Microvesicles range between 100 to 1000 nm in diameter and are formed through outward outgrowth. Exosomes are vesicles generating after fusion of multivesicular bodies with plasma membranes, ranging between 40 to 100 nm[10,31]. They should not be confused with RNA-degrading complexes with the same name found in both archaea and eukaryotes. On the other hand, apoptotic bodies are released during early apoptosis and are larger than 1000 nm[32,33]. However, there is a lack of consensus about classification and biochemical markers characterizing the different EV types. Therefore, the International Society for EV stated the following in the "Minimal Information for Studies of Extracellular Vesicles 2018" (MISEV2018), in relation to the EV nomenclature: "EV is the preferred generic term for the subject of our investigations, and subtypes should be defined by physical and biochemical characteristics and/or conditions/sources. When other terms are used, careful definition is required"[34].

EV may contain proteins, nucleic acids (including coding and non-coding RNA), lipids, and other metabolites. Normally, the content is rich in cytoskeletal proteins (such as TSG10 or CD63 tetraspanins), integrins, and major histocompatibility complex molecules[35]. Depending on cell types and microenvironments in which they are secreted, contents of EV may change. Thus, EV reflect physiological states of cells generating them. For this reason, MSC growth under different conditions, such as hypoxia, presence of trophic and physical factors, or chemical and pharmacological agents, may stimulate secretion of EV enriched in certain cytokines, growth factors, or non-coding RNA like microRNA (miRNA)[36].

In relation to the functionality of EV, at first it was thought that they were a mechanism for cells to get rid of unwanted material. It was later demonstrated that EV play a fundamental role in cellular homeostasis, being key elements in cell-to-cell communications[33,37]. Thus, these vesicles regulate different physiological processes such as cell proliferation, differentiation, and migration[38].

EV can be isolated from various sources, including blood, urine, breast milk, amniotic fluid, and synovial fluid, among others, as well as supernatants from cell cultures such as endothelial, epithelial, cancer, MSC, *etc*[39]. There are different purification approaches such as differential and density-gradient ultracentrifugation, ultrafiltration, size-exclusion chromatography, precipitation, immunoaffinity, and microfluidic-based methods[33]. Likewise, isolated EV can be characterized by different techniques like electron microscopy, flow cytometry, nanoparticle tracking analysis, dynamic light scattering, tunable-resistive pulse sensing, and atomic-force microscopy, among others[38,40].

Using EV in regenerative medicine has some advantages in comparison with whole-cell therapies[41] including: (1) Can be easily stored, being immediately available for clinical applications; (2) Production of large quantities of cells is not required; (3) Can be evaluated for safety, dosage, and activity in a manner similar to conventional pharmaceutical agents; (4) Are stable, exhibiting a long half-life; indeed, the lipid bilayers of their membranes protect their contents from degradation *in vivo*; (5) Can be more easily applied for clinical purposes than proliferative cells; for example, they can be intravenously injected, circulate through the smallest capillaries, and cross the blood-brain barrier; (6) Risks of immune rejection, cellular dedifferentiation, or tumor formation are lower than in whole-cell therapies; and (7) EV can be manipulated for more precise effects as therapeutic agents[10,41]. Therefore, the use of EV in therapy has become a great tool for regenerative medicine in recent years.

ROLE OF HYPOXIA-INDUCIBLE FACTOR IN ADAPTATION TO HYPOXIA AND TISSUE REGENERATION

When oxygen concentrations decrease to less than 5% in tissues, cells have to adapt their metabolism and functions to such hypoxic conditions. Moderate (< 5% to > 2% O_2), severe (≤ 2% to ≥ 0.1% O_2), and anoxia (< 0.1% O_2) are hypoxia levels equal or below 5% oxygen concentration. Depending on O_2 concentration and hypoxia time, cells show different responses, as observed in human embryonic-derived MSC[42]. That occurs mainly through activation of hypoxia-inducible factor (HIF). This is a transcription factor consisting in a heterodimer of two basic helix-loop-helix proteins: Alpha (HIFA) and beta (HIFB)[43,44]. While expression of the gene encoding alpha subunits is induced by hypoxia, the gene encoding HIFB, also known as aryl hydrocarbon-receptor nuclear translocator, is constitutively expressed[45]. There are three alpha subunits (HIF1A, HIF2A, and HIF3A),with the first two well-known. HIF1A and HIF2A have 48% amino acid sequence identity and similar protein structures. Although they share functions, they can regulate the expression of different genes[46].

HIF2A, which is also known as endothelial PAS (Period, Aryl-hydrocarbon-receptor, Single minded) domain protein-1, was originally associated with endothelial development and regulation. Its encoding gene exhibits a more restricted expression relative to the one of *HIF1A*[47]. Furthermore, whereas HIF1A requires very low O_2 concentrations for stabilization, H2FA can be activated at less severe levels of hypoxia (approximately 5%). Therefore, HIF1A would act in the initial response, whereas HIF2A would regulate the response to long periods of hypoxia[48,49]. On the other hand, HIF3A has three isoforms (HIF3A, neonatal and embryonic PAS, and inhibitory PAS protein). They inhibit the transcriptional activity of HIF1A and HIF2A by preventing their heterodimerization with HIF1B[50,51].

Under normoxia, HIF1A proteins in the cytoplasm are continuously degraded, through the proteasome pathway[52]. However, when the O_2 concentration decreases, HIF1A proteins are not degraded, but rather they are accumulated and translocated into the nucleus (Figure 1). Regulation of HIF1A levels depends on the presence of an oxygen-dependent degradation domain in the protein. This domain is constituted by Fe^{2+} and two prolyl residues (Pro402 and Pro564). Such residues undergo hydroxylation through prolyl hydroxylases (PHD1, PHD2, and PHD3) in the presence of oxygen and α-ketoglutarate, allowing HIF1A to be recognized by the von Hippel-Lindau tumor suppressor protein, a component of the E3 ubiquitin-ligase complex. That way, it is degraded by the ubiquitin-proteosome pathway[53,54] (Figure 1).

In addition to PHD, another enzyme, called factor inhibiting HIF1 (FIH), can inhibit the transcriptional activity of HIF1A. In this case, FIH hydrolyzes residues within the C-terminal transactivation domain of HIF1A, preventing their binding to coactivators to initiate transcription in the nucleus[55]. Under hypoxic conditions, prolyl hydroxylation is inhibited, and, thus, the degradation of HIF1A is also inhibited. They accumulate and translocate into the nucleus, where they form heterodimers with HIF1B. That way, they can induce gene transcription through binding to pentanucleotide sequences (A/GCGTG) called hypoxic-response elements in the promoters of target genes. For transcription of target genes to occur, coactivators are recruited, mainly p300/CBP[56] (Figure 1).

It has been described that more than 1000 genes can be directly or indirectly regulated by HIF. These genes are involved in adaptation of cells to hypoxic conditions. They affect different physiological processes including metabolism, angiogenesis, inflammatory response, cell differentiation, migration, and apoptosis[57]. In order to present an overview of the various functions of genes regulated by HIF1α and HIF2α, we have analyzed the information contained in Qiagen Ingenuity Pathway Analysis (Qiagen IPA) web-based software application (https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-ipa)[58]. This platform allows querying information gathered from databases and findings described in the literature for a given gene. Information from 493 references related to genes regulated by HIF1A and 215 for HIF2A were integrated in the description of functions of human HIF at the time of writing. From the information of these references, the application shows 191 genes regulated by HIF1α and 111 by HIF2α. Among them, 72 are common to both.

Functional analyses of HIF1A- and HIF2A-regulated genes with IPA show categories and functional annotations in which they are involved. Tables 1 and 2 show these data together with *P* values and the number of genes identified for each of the categories obtained from Qiagen IPA. Regarding the functional annotations, a maximum of the five most significant annotations in each category are shown. The list of genes for HIF1A and HIF2A obtained from such application, as well as genes corresponding to each of the categories presented in Tables 1 and 2, are shown in elsewhere (Supplementary Tables 1-3). Among the functions of genes regulated by HIF1A and HIF2A are those related to glucose metabolism, vessel formation, inflammatory response, cell proliferation, cell migration, and apoptosis. Interestingly, they play relevant roles in tissue regeneration[57].

The importance of HIF1A in tissue regeneration has been further demonstrated using a Murphy Roths large mouse model. These animals are characterized by high basal expression of *HIF1A* gene which has been associated with the ability of these animals to regenerate significant ear lesions without the appearance of fibrotic areas[59]. Indeed, *HIF1A* induction upregulates genes such as vascular-endothelial growth factor (*VEGF*), stromal cell-derived factor-1 alpha protein (*SDF-1A*), transforming

Table 1 Functional categories and annotations in ingenuity pathway analyses of genes regulated by hypoxia-inducible factor 1A

Categories	*P* value	Top five functional annotations	Number of genes
Carbohydrate Metabolism	3.50E-20	Glycolysis of cells	22
Cardiovascular System Development and Function	3.31E-15 to 4.96E-54	Angiogenesis, Vasculogenesis, Growth of blood vessel	84
Cell Cycle	7.42E-16 to 1.82E-38	Binding of DNA, Cell cycle progression, Interphase, Binding of protein binding site, Arrest in interphase	99
Cell Death and Survival	3.79E15 to 7.3E-74	Apoptosis, Necrosis, Cell death of tumor cell lines, Apoptosis of tumor cell lines, Cell viability	150
Cell Morphology	1.66E-16 to 3.21E-24	Morphology of tumor cell lines, Tubulation of cells, Transmembrane potential of mitochondria, Cell spreading, Orientation of cells	77
Cell Signaling	2.83E-19 to 1.1E-26	Cytokine and chemokine mediated signaling pathway, Quantity of Ca^{2+}	48
Cell-To-Cell Signaling and Interaction	3.31E15 to 1.99E-36	Binding of tumor cell lines, Binding of blood cells, Adhesion of blood cells, Binding of leukocytes, Adhesion of immune cells	105
Cellular Assembly and Organization	5.56E-16 to 1.42E-17	Organization of cytoskeleton, Microtubule dynamics, Fibrogenesis	52
Cellular Development	3.47-15 to 2.85E-73	Cell proliferation of tumor cell lines, Cell proliferation of carcinoma cell lines, Cell proliferation of breast cancer cell lines, Proliferation of muscle cells, Assembly of cells	151
Cellular Function and Maintenance	7.07E-32	Cellular homeostasis	67
Cellular Growth and Proliferation	9.07E-27 to 2.12E-30	Proliferation of vascular cells, Colony formation, Proliferation of connective tissue cells, Proliferation of lymphatic system cells, Proliferation of epithelial cells	101
Cellular Movement	3.31E-15 to 3.35E-67	Cell movement, Migration of cells, Invasion of cells, Cell movement of tumor cell lines, Migration of tumor cell lines	132
Connective Tissue Development and Function	1.36E-16 to 1.73E-30	Growth of connective tissue, Quantity of connective tissue	46
DNA Replication, Recombination, and Repair	1.67E-18 to 8.06E-31	Synthesis of DNA, Metabolism of DNA, Degradation of DNA	47
Drug Metabolism, Lipid Metabolism, Small Molecule Biochemistry	2.36E-16	Synthesis of prostaglandin E2	16
Free Radical Scavenging	6.09E-16 to 5.5E-33	Synthesis of reactive oxygen species, Production of reactive oxygen species, Generation of reactive oxygen species, Quantity of reactive oxygen species	43
Gene Expression	1.373-18 to 4.01E-31	Expression of RNA, Transcription, Transcription of RNA, Transactivation, Transactivation of RNA	88
Inflammatory Response	5.48E-20 to 2.72E-26	Inflammation of absolute anatomical region, Inflammation of organ, Inflammatory response, Inflammation of body cavity, Immune response of cells	77
Lipid Metabolism	6.24E-16 to 9.37E-24	Synthesis of eicosanoid, Metabolism of eicosanoid, Fatty acid metabolism, Synthesis of fatty acid, Synthesis of prostaglandin	48
Organismal Survival	9.55E-37 to 2.76E-39	Organismal death, Survival of organism	81
Post-Translational Modification	1.86E-18	Phosphorylation of protein	34
Protein Synthesis	2.10E-15	Metabolism of protein	44
Tissue Development	1.65E-19 to 4.13E-46	Growth of epithelial tissue, Development of epithelial tissue, Growth of nervous tissue	62
Tissue Morphology	3.02E-17 to 3.06E-26	Quantity of cells, Quantity of tumor cell lines	42

growth factor beta 1 (*TGFB1*), platelet-derived growth factor (*PDGF*), and matrix metallopeptidase 9 (*MMP9*), among others. All of them have important functions in healing processes. Therefore, upregulating *HIF1A* can accelerate wound healing. This has been observed in hyperbaric oxygen therapy (HBOT) treatments of diabetic skin ulcers. Interestingly, HBOT treatments increased HIF1A levels[60], probably due to high reactive oxygen species (ROS) concentrations produced by increased O_2 in the tissue, which may inhibit PHD and FIH, thus stabilizing HIF1A. In fact, HIF1A activity is decreased in diabetics, being associated with wound healing difficulty in these patients[61].

Table 2 Functional categories and annotations in Ingenuity Pathway Analyses of genes regulated by hypoxia-inducible factor 1A

Categories	*P* value	Top five functional annotations	Number of genes
Carbohydrate Metabolism	2.45E-12 to 2.81E-17	Quantity of carbohydrate, Uptake of D-glucose, Synthesis of polysaccharide, Glycolysis, Uptake of monosaccharide	44
Cardiovascular System Development and Function	1.00E-11 to 8.41E-31	Angiogenesis, Development of vasculature, Vasculogenesis, Endothelial cell development, Proliferation of endothelial cells	68
Cell Cycle	6.82E-12 to 4.39E-19	Mitogenesis, Binding of DNA, Interphase, Arrest in interphase, Cell cycle progression	53
Cell Death and Survival	1.08E-11 to 5.12E-36	Cell death of tumor cell lines, Apoptosis of tumor cell lines, Cell viability, Cell survival, Apoptosis	88
Cell Morphology	1.11E-11 to 4.76E-19	Tubulation of cells, Morphology of tumor cell lines, Autophagy of cells, Formation of cellular protrusions, Autophagy	67
Cell Signaling	6.16E-12 to 1.73E-12	Quantity of Ca^{2+}, Synthesis of nitric oxide	29
Cell-To-Cell Signaling and Interaction	7.17E-12 to 1.36E-26	Activation of cells, Interaction of tumor cell lines, Binding of tumor cell lines, Activation of blood cells, Binding of professional phagocytic cells	62
Cell-mediated Immune Response	4.19E-14 to 7.70E-15	T cell development, T cell homeostasis	26
Cellular Assembly and Organization	1.74E-12 to 3.51E-20	Microtubule dynamics, Organization of cytoskeleton, Organization of cytoplasm, Development of cytoplasm	52
Cellular Development	1.10E-11 to 2.98E-39	Cell proliferation of tumor cell lines, Colony formation of cells, Proliferation of smooth muscle cells, Cell proliferation of carcinoma cell lines, Cell proliferation of breast cancer cell lines	89
Cellular Function and Maintenance	4.81E-13 to 6.37E-27	Cellular homeostasis, Lymphocyte homeostasis, Function of blood cells	63
Cellular Growth and Proliferation	8.67E-13 to 3.51E-27	Colony formation, Proliferation of connective tissue cells, Proliferation of vascular cells, Proliferation of lymphatic system cells, Proliferation of epithelial cells	72
Cellular Movement	9.45E-12 to 2.99E-30	Invasion of cells, Cellular infiltration, Cell movement of myeloid cells, Migration of cells, Cell movement of tumor cell lines	72
Connective Tissue Development and Function	1.56E-16 to 1.63E-25	Growth of connective tissue, Quantity of connective tissue cells, Quantity of connective tissue, Inflammation of joint, Rheumatic Disease	50
DNA Replication, Recombination, and Repair	4.08E-22	Synthesis of DNA	29
Digestive System Development and Function	1.42E-13	Morphology of digestive system	28
Embryonic Development	1.52E-12 to 1.2E-22	Development of body trunk, Development of abdomen, Growth of embryo, Formation of lymphoid tissue, Formation of lung	60
Free Radical Scavenging	3.11E-13 to 1.77E-17	Metabolism of reactive oxygen species, Synthesis of reactive oxygen species, Production of reactive oxygen species	29
Hair and Skin Development and Function	1.33E-13	Growth of skin	17
Hematological System Development and Function	1.17E-11 to 8.08E-21	Quantity of blood cells, Quantity of leukocytes, Quantity of lymphocytes, Quantity of myeloid cells, Quantity of T lymphocytes	52
Inflammatory Response	9.18E-22 to 4E-24	Inflammation of absolute anatomical region, Inflammatory response, Inflammation of body cavity	59
Lipid Metabolism	8.00E-12 to 3.77E-16	Concentration of lipid, Synthesis of lipid	41
Lymphoid Tissue Structure and Development	3.73E-18	Quantity of lymphatic system cells	35
Molecular Transport	7.49E-15	Transport of molecule	44
Nervous System Development and Function	4.54E-12	Sensory system development	22
Organ Development, Renal and Urological System Development	8.50E-12 to 3.25E-12	Growth of kidney, Growth of renal glomerulus	11

Organ Morphology	1.78E-13	Morphology of gland	22
Organismal Development	1.92E-12 to 7.70E-25	Morphology of body cavity, Formation of vessel, Morphology of head, Development of genitourinary system, Growth of organism	73
Organismal Survival	7.68E-26 to 1.24E-28	Organismal death, Survival of organism	75
Post-Translational Modification	4.05E-12 to 1.23E-15	Phosphorylation of protein, Activation of protein	31
Skeletal and Muscular System Development and Function	1.08E-11 to 1.22E-26	Morphology of muscle, Function of muscle, Growth of smooth muscle	39
Tissue Development	1.73E-13 to 3.23E-30	Development of epithelial tissue, Growth of epithelial tissue, Growth of nervous tissue, Accumulation of cells, Formation of epithelial tissue	58
Tissue Morphology	3.33E-14 to 2.65E-24	Quantity of cells, Quantity of progenitor cells	60

DOI: 10.4252/wjsc.v14.i7.453 **Copyright** ©The Author(s) 2022.
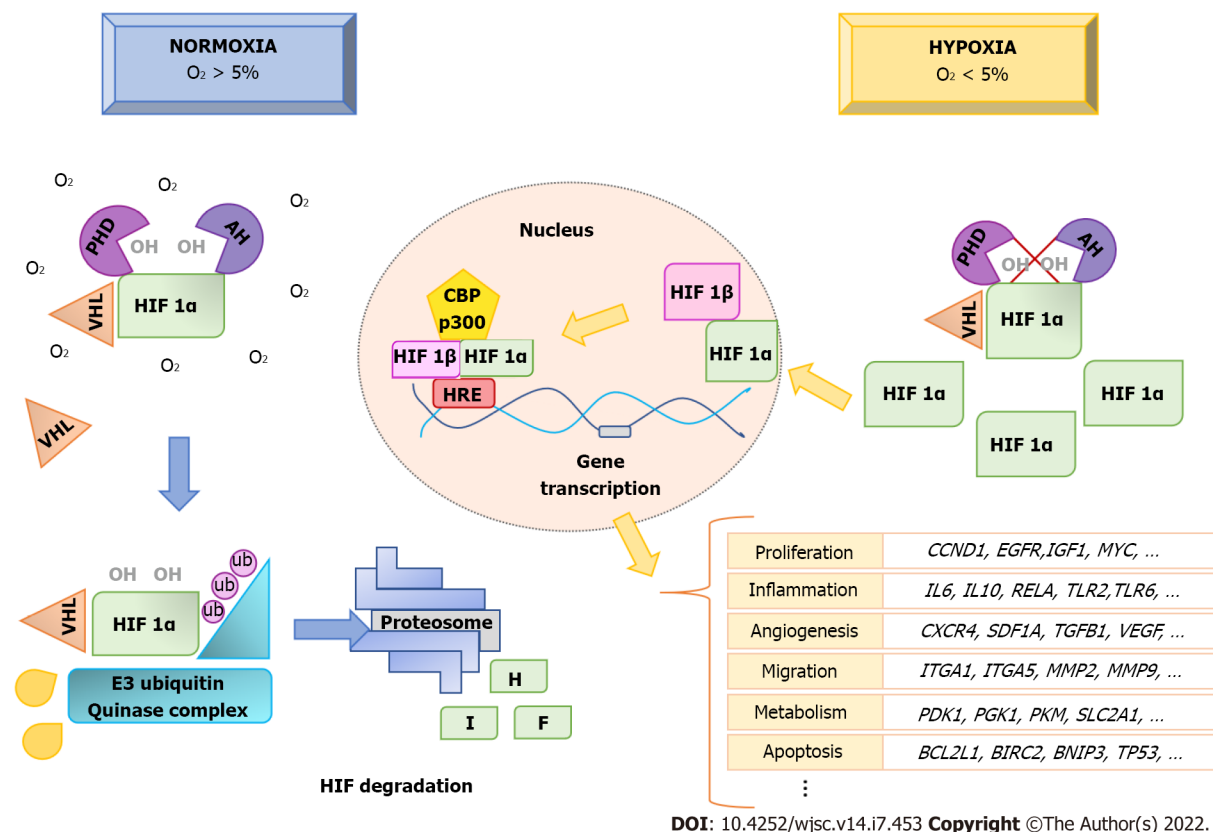
Figure 1 Hypoxia-inducible factor 1A protein regulation in hypoxia. Hypoxia-inducible factor (HIF) 1A is continuously hydroxylated and degraded by the proteosome, when O_2 concentrations are greater than 5%. However, in hypoxia ($O_2 < 5\%$) HIF1A hydroxylation is inhibited and it accumulates in the cytoplasm. It then translocates to the nucleus, where it forms a heterodimer with HIF1B. This, together with the coactivator CBP/P300, binds to hypoxic-response elements at gene-promoter sites, activating transcription of genes involved in biological processes such as angiogenesis, proliferation, migration, inflammatory response, metabolism, and apoptosis, among others. This produces physiological adaptive responses of cells to hypoxia. HIF: Hypoxia-inducible factor; HRE: Hypoxic-response elements; PHD: Prolyl HyDroxylases; VHL: Von Hippel-Lindau.

However, if hypoxia is maintained, wounds may become chronic, and fibrotic processes may appear. This is because, among the genes regulated by the HIF pathway, there are some that encode pro-fibrotic enzymes, producing an excess of extracellular matrix. Some of these genes are related to collagen biosynthesis like collagen type–IV, V, IX and XVIII–Alpha–1 and 2–chains (*COL4A1*, *COL4A2*, *COL5A1*, *COL9A1* and *COL18A1*, accordingly), and the ones encoding enzymes that produce modifications in collagen, such as procollagen PHD and lysyl hydroxylases[62].

Inflammation is the first phase activated by injuries, and hypoxia is related to inflammatory responses. Several protein-encoding genes of nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) cell complex, such as reticuloendotheliosis (REL)-Associated (RELA) proto-oncogene (transcription factor p65, also known as nuclear factor NF-kappa-B p65 subunit p65, involved in NF-κB heterodimer formation, nuclear translocation, and activation) are induced by HIF1A[63]. NF-κB is a

family of transcription factors whose activation regulates different physiological processes. They include inflammatory response, as well as cell differentiation, proliferation, and survival[64]. Among the genes that NF-κB regulates is *HIF1A*, thus producing a reciprocal regulation[65]. The HIF1A proteins also induce expression of genes encoding proteins belonging to the Toll-Like Receptor (TLR) family. Thus, it enhances the activation of NF-κB[66]. This is because TLR have the capacity to recognize pathogen-associated molecules, inducing immune responses through activation of transcription factors, such as NF-κB[67].

Upon injury, the resulting hypoxia promotes macrophage recruitment, through regulation of Sphingosine 1-Phosphate (S1P) levels. It acts as a signal for recruitment, activation, differentiation, and polarization of macrophages[68]. This may be mediated by induction of expression of genes such as sphingosine kinase 1. this gene is involved in the last step of S1P synthesis. It has been described that HIF1A and HIF2A act on M1 and M2 macrophages through different pathways. While HIF1A induces the gene encoding inducible nitric-oxidase synthase, HIF2A acts through arginase-1, maintaining nitric oxide homeostasis during inflammation. In the case of HIF1A, its overexpression induces glycolysis metabolism, resulting in macrophage polarization to M1 (proinflammatory)[69]. However, although HIF2A has also been associated with the M1 phenotype, other studies have shown that it may promote anti-inflammatory and pro-resolving/regenerative M2 macrophages[70]. HIF1A may also produce immunosuppression through induction of Programmed Death-ligand 1 (PD-L1) encoding gene (*CD274*). Binding of PD-L1 to its programmed cell death protein 1 receptor on activated T cells inhibits immunity by counteracting T cell-activating signals[71].

Adaptation to hypoxia also requires metabolic changes. Cells must reduce mitochondrial oxygen consumption. In this sense, glycolysis is activated as the only way to produce adenosine triphosphate (ATP) under such hypoxic conditions. Not surprisingly, HIF1A upregulates genes related to glucose metabolism. Among them is solute carrier family 2 member A1, encoding glucose transporter-1, necessary for glucose uptake by cells[72]. Also, the genes encoding phosphoglycerate kinase 1 and pyruvate kinase M1/2 are transcriptionally upregulated by HIF1A[73,74]. Additionally, in the adaptation to hypoxia, the tricarboxylic acid (TCA) (or Krebs cycle) must be suppressed to prevent accumulation of ROS in mitochondria. For this purpose, HIF1A induces the gene encoding pyruvate dehydrogenase (PDH) kinase 1. This inactivates PDH, which is responsible for converting pyruvate to acetyl-CoA in the TCA[75].

In response to hypoxia caused by tissue damage, cells produce angiogenic factors to induce generation of vessels, restore oxygen levels, and increase nutrient delivery. HIF1A and HIF2A induce expression of genes encoding these factors. Among them, *VEGF*, *SDF-1A*, C-X-C chemokine-Receptor type 4 (*CXCR4*), angiopoietin-2 (*ANG-2*), *PDGF*, and *TGFB*[76] stand out. These factors favor endothelial-cell proliferation, differentiation, and migration for vessel formation. That also involves the mobilization and recruitment of endothelial progenitor cells (EPC) from bone marrow[61]. Mobilization of EPC is mediated by production of SDF-1 in hypoxic tissues. It acts as a chemoattractant of EPC expressing the CXCR4 receptor[77]. On the other hand, regulation of EPC migration to ischemic tissues through CXCR4/SDF1 axis is specific to HIF2A[78].

Tissue regeneration also induces cell proliferation and migration processes. HIF activation can affect cell-cycle progression due to regulation of genes such as cyclin D1 and *cellular myelocytomatosis* (*c-MYC* or *MYC*)[79,80]. Interestingly, while HIF1A downregulates *c-MYC* expression and results in cell-cycle arrest[79], HIF2A upregulates *c-MYC* expression, promoting cell-cycle progression and proliferation [81]. Regarding cell migration, HIF regulates genes encoding *integrin–alpha* and *beta–1, 3 and 5* (*ITGA1*, *ITGA5*, *ITGAV*, *ITGB3*, and *ITGB5*, accordingly) and *MMP2*, *MMP7*, and *MMP9*, which are important in such processes[82]. The induction of cell migration by hypoxia is essential under physiological conditions for tissue regeneration after injury. This favors recruitment and homing of inflammatory and precursor cells, eliminating pathogens and cellular debris, further regenerating damaged tissues[68].

Hypoxia causes important changes in cellular microenvironments that might condition cell viability. Therefore, another set of important genes regulated by hypoxia are related to cell survival and death. Thus, HIF1A regulates genes activators of apoptosis such as the ones encoding *tumor protein p53* (*TP53*; antioncogene) and *B-Cell Lymphoma 2* (*BCL2*)/*adenovirus E1B 19 kDa protein-interacting protein 3*[83], as well as anti-apoptotic genes, such as *baculoviral inhibitor of apoptosis protein (IAP) repeat containing 2* and *BCL2*[83,84]. The balance of expression of these genes, and thus cell survival, will depend on the adaptation of cells to hypoxic conditions. Thus, cell survival may predominate under mild hypoxia, but apoptosis is preferentially activated under severe hypoxia[85].

HYPOXIA AND MSC

MSC reside in areas of 3%-9% of oxygen tension, allowing this hypoxic niche it's capacities for self-renewal, proliferation, migration, and ultimately, their differentiation[86,87]. Based on this, MSC in culture have been grown at low levels of oxygen to condition or acclimate them before their therapeutic use[88]. These cells exposed to hypoxic conditions activate protein kinase B (also known as Akt, name derived from Ak mouse strain with thymoma transforming tumors) or AKT signaling pathway

mediated by HIF-1 activation to improve their survival and proliferation[89]. However, different modes, severity, and duration of hypoxic exposure could provoke different responses on MSC. Indeed, cells can become stressed and even undergo apoptosis under extreme (< 1.5%) oxygen levels[87]. Furthermore, if hypoxic exposures are maintained, internal energy reserves of glucose are rapidly consumed. That is due to glycolysis characteristic of MSC causing poor survival after implantation[90].

Ischemic conditions could be solved by providing glucose supplementation to hypoxic MSC. That allows them to retain their proliferative capacity and differentiation potency[91]. Therefore, survival of MSC could be improved by preconditioning them at 1%-4% O_2 for 24 to 48 h prior to implantation[88]. Hypoxia could also reduce cell viability and proliferation of MSC. Nevertheless, reoxygenation processes might promote recovery of cells, enhancing expression of pro-survival genes, as well as various trophic factors[92], further promoting multipotency of MSC[93,94]. Therefore, maintenance of MSC cultures in hypoxia may influence processes such as proliferation[87,94,95], migration[87], differentiation[93,95], metabolism[87], and apoptosis[88,96], which may affect their regenerative capacity. Interestingly, cyclic hypoxic exposure, defined as periodic exposure to hypoxia interrupted by normoxic exposure or lower levels of hypoxia[97], could have positive effects on proliferation and migration abilities of MSC[98].

THERAPEUTIC POTENTIAL OF EV DERIVED FROM MSC PRECONDITIONED IN HYPOXIA

Microenvironments in which MSC are cultivated are extremely important for their proliferation, differentiation, and therapeutic potential. Factors, such as time in culture, oxygen levels, medium composition, or cell-material interactions, should be considered[99]. As indicated in previous sections, many factors induced by hypoxia are involved in processes related to tissue regeneration, such as inflammation, angiogenesis, cell proliferation, and migration[100]. Thus, priming MSC in hypoxia favors generation of EV enriched in hypoxia-induced factors. Their functions include alterations of microenvironments for tissue adaptations to low O_2 concentrations[101-103]. Production and isolation of these EV for use in regenerative medicine is of great interest from a clinical point of view. Therefore, numerous studies have evaluated their potential therapeutic applications. In this scenario, time exposure and degree of hypoxia may represent relevant factors influencing contents and therapeutic properties of EV (Table 3).

Hypoxia is an important inducer of angiogenesis, which plays a key role in tissue regeneration. Therefore, diverse studies have analyzed whether MSC-derived EV exposed to low levels of O_2 are enriched in angiogenic factors, and likewise, whether this has an impact on their ability to induce vessel formation. One of these studies showed that MSC cultivated for 72 h under hypoxic conditions (1% O_2) produced exosomes with proangiogenic effects, through overexpression of genes encoding urokinase receptor [also known as urokinase *plasminogen-activator surface receptor (uPAR)*], *angiogenin (ANG)*, *VEGF , insulin-like growth Factor (IGF)*, *angiopoietin receptor tyrosine kinase with immunoglobulin-like and epidermal growth factor (EGF)-like domains 2 (Tie-2)* [also known as *tyrosine endothelial kinase (TEK)*], and *interleukin 6 (IL-6)*[104]. Additionally, umbilical cord MSC-derived EV have the ability to enhance endothelial-cell angiogenesis *in vitro* and in a rat hindlimb ischemia model, being able to restore blood flow[103]. Preconditioning adipose-derived MSC in moderate hypoxia (5% O_2) also produced EV with capacity to increase formation of tubular structures in human umbilical-vein endothelial cells (HUVEC) with respect to EV obtained in normoxia. On the other hand, effects of EV were greater than those produced by media obtained after isolation of microvesicles. This indicates that EV, rather than soluble factors in the media, are responsible for angiogenic induction[32].

In vivo studies have also shown the potential of EV derived from MSC grown under hypoxia on angiogenesis. For example, in a mouse model of fat grafting, co-transplantation of exosomes in subcutaneous fat grafting enhanced angiogenesis, neovascularization, and graft survival[105]. A significant rise in protein synthesis of EGF, fibroblast growth-factors, VEGF/VEGF receptors (VEGF-R), Ang-1, and angiopoietin receptor tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie-1) were shown in grafted animals, 30 d after transplantation[106]. Inclusion in hydrogels that allow local release of EV with high angiogenic capacity has also been used for treatment of spinal-cord injuries [107]. One of the proteins that has been found to be over synthesized in MSC-derived EV under hypoxia, compared to those obtained in normoxia, is jagged-1 (JAG1). This is one of the notch ligands. The notch pathway modulates processes such as angiogenesis, embryonic development, and hematopoietic stem-cell (HSC) biology[108,109]. HSC from umbilical-cord blood were treated *in vitro* with EV from MSC preconditioned in 1% O_2 for 48 h. As expected, their expansion capacity, self-renewal, and clonogenic potential was increased through JAG1/notch pathway regulation[110].

Treatment of endothelial cells with hypoxia-conditioned MSC-derived EV modulates angiogenesis-related signaling pathways. For instance, it has recently been described that EV obtained from MSC maintained in 5% O_2 for 6 d induced angiogenesis in HUVEC. That was accomplished through increased synthesis of high-mobility group box 1. It activates c-Jun N-terminal Kinases pathway (name derived from viral homolog v-jun, discovered in avian sarcoma virus 17 and named ju-nana, the Japanese word for 17), and consequently upregulated *HIF1A*/ *VEGF* expression[111]. The angiogenic

Table 3 Use of extracellular vesicles, derived from hypoxic mesenchymal stem cells, in regenerative medicine

Source of MSC	Culture	Hypoxic preconditions, O_2%, percentage	Time of exposition	Major findings	Ref.
Human umbilical-cord	α-Mem deprived of FBS	1	72 h	Proangiogenic effects with an increase in UPAR, angiogenin, VEGF, IGF, Tie-2/TEK and IL-6 expression	[104]
	α-Mem deprived of FBS	1	72 h	Promoted angiogenesis *in vitro* and *in vivo*	[103]
	DMEM/high glucose media with 10% Exo depleted FBS and 1% penicillin/streptomycin	1	48 h	Enhanced of miRNA-126 exerting a pro-angiogenic effect in endothelial cells thereby activating Spred 1/Ras/Erk pathway	[117]
	α-Mem 10% EV free FBS	1	Not defined	EV encapsulated in a hyaluronic acid adhesive hydrogel have angiogenic properties and nerve regeneration effects after traumatic spinal cord injury	[107]
Olfactory mucosa	DMEM supplemented with 10% EV-depleted FBS	3	48 h	Promoted angiogenesis *via* miR-612 transfer	[112]
Adipose tissue	α-Mem 10% EV free serum	5	48 h	Promoted vessel formation *in vitro*. Enhanced angiogenesis, neovascularization and graft survival *in vivo*. Activation of VEGF/VEGF-R	[105,106]
	EV depleted standard medium	5	72 h	Promoted angiogenesis	[32]
	RPMI medium	1	72 h	Promoted angiogenesis, inhibition of apoptosis, immunomodulation, intracellular ATP recovery and reduction of ROS	[122]
	Microvascular endothelial cell growth medium 2 media deprived of FBS with supplement of 1× serum	1	24 h	Improved diabetic wound healing. Enhanced fibroblasts proliferation and migration activating PI3K/Akt pathway	[132]
	DMEM/F12 with 10% EV-free FBS	0–20 (5 cycles)	Hypoxia 60 min–reoxygenation 30 min	miRNA-224-5p in EV decreases TXNIP expression in cardiomyocytes and protects them from hypoxia mediated injury	[128]
Bone marrow	DMEM with low glucose containing inactivated 15% FBS	—	12 h	Increased of miRNA-21. Synaptic dysfunction restoration, inactivation of STAT3 and NF-kB, reduced plaque deposition and amyloid-β. Regulation of inflammatory responses in APP/PS1 mouse model	[114]
	DMEM with 10% FBS and 1% penicillin-streptomycin	5	6 d	High HMGB1 expression. Activation of JNK pathway and induction of HIF-1α/VEGF expression promoting angiogenesis	[111]
	Exosome-depleted fetal bovine serum	1	48 h	Increased exosomal levels of miRNA-216a-5p. Inhibition of TLR4/NF-κB and activation of PI3KAKT signaling pathway shifting microglial M1/M2 polarization	[115]
	α-Mem 10% exosomes-depleted FBS	2	48 h	Promoted angiogenesis	[118]
	Mesenchymal Stem Cells Medium (Sciencell) 5% exosomes-depleted FBS	1	48 h	Alleviate intervertebral disc degeneration by delivering miR-17-5p	[119]
	DMEM/F12 10% exosomes-depleted FBS	3	48 h	promote cartilage regeneration *via* the miR-205–5p/PTEN/AKT	[120]

				pathway	
	DMEM/F12 10% exosomes-depleted FBS	5	48 h	EV improved chondrocyte proliferation and migration and suppressed chondrocyte apoptosis. miRNA-18-3P/JAK/STAT or miRNA-181c-5p/MAPK signaling pathway may be involved	[121]
	DMEM low glucose 10% platelet lysate	1	48 h	EV increase angiogenesis, reduced neuronal degeneration, brain atrophy and improved neurological recovery	[116]
Murine bone	α-Mem 10% Exo-removed FBS	0.5	24 h	Significant enrichment of miRNA-210. Promoted survival and recovery of cardiac functions. Also, reduced apoptosis and fibrosis and increased the mobilization of cardiac progenitor cells	[124]
	DMEM/F12 supplemented with 10% fetal bovine serum	1	72 h	Overexpression of miR-210 regulated PI3K/AKT and p53 signaling by targeting AIFM3 reducing apoptosis and tissue death after a myocardial infarction	[125]
	α-Mem 10% Exo-removed FBS	1	72 h	Overexpression of miR-125b-5p. Ability to modify the direction of exosomes to ischemic tissue	[126]

AIFM3: Apoptosis-inducing factor, mitochondria-associated 3; AKT: Protein kinase B (PKB), named derived from kinase encoded by oncogene in transforming retrovirus from thymoma cell line AKT-8 of stock A, strain k, AKR mouse; APP: Amyloid precursor protein; ATP: Adenosine triphosphate; DMEM: Dulbecco's modified Eagle medium; Erk: Extracellular signal-regulated kinase; Exo: Exosomes; FBS: Fetal bovine serum; GM-CSF: Granulocyte macrophage colony-stimulating factor; HMGB1: High mobility group box 1 protein; IGF: Insulin-like growth factor; IL-6: Interleukin 6; JNK: Jun N-terminal kinase; MAPK: Mitogen-activated protein kinase; NF-□B: Nuclear factor kappa B; P53: Tumor protein 53 (antioncogene); PI3K: Phosphatidylinositol 3-kinase; PS1: PreSenilin 1; RKCM: Growth medium stem cell; ROS: Reactive oxygen species; RPMI: Gibco Roswell Park Memorial Institute; Spred 1: Sprouty-related EVH1 domain-containing protein 1; STAT3: Signal transducer and activator of transcription 3; TEK: Tyrosine endothelial kinase; Tie-2: Tyrosine kinase receptor 2; TLR4: Toll-like receptor 4; TXNIP: Thioredoxin-interacting protein.; UPAR: Urokinase-type plasminogen activator receptor; VEGF: Vascular endothelial growth factor.

effects of MSC-derived EV exposed to hypoxia are mediated, in part, by their cargos, specifically by certain miRNA. One of these miRNAs is miR-612, which inhibits translation of TP53 mRNA, favoring the activity of HIF-1A-VEGF signaling, and consequently angiogenesis[112].

According to the properties of MSC-derived EV under hypoxia, their applications may be useful in multiple disease treatments (Figure 2). Among them is Alzheimer's disease, which is characterized by neuronal and synaptic loss caused by deposition of beta-amyloid peptides[113] due to erroneous protein folding. Experiments have been carried out with an Alzheimer's transgenic mouse model overexpressing mutated forms of human amyloid-precursor protein (APP) and presenilin 1 (PS1). Interestingly, they improved learning and memory functions after treatment with exosomes from MSC preconditioned for 12 h under hypoxia. These improvements could be due to reduced β-amyloid accumulation through increased levels of miR-21 in the brain, synthesis of synaptic proteins, and a decrease of inflammatory factors[114].

Repair of traumatic injuries of spinal cords have also been studied using exosomes released during 48 h under hypoxia (1% O$_2$). An enrichment of miR-216a-5p in exosomes was observed involving TLR4/NF-κB/phosphoinositide 3-kinase (PI3K)/AKT signaling cascades. These miR-216a-5p-enriched exosomes promoted functional behavioral recovery using both *in vitro* and *in vivo* models carried out by shifting microglial polarization from classically-activated macrophage (M1) to alternatively-activated macrophage (M2) phenotype, effectively switching from pro-inflammatory to non-inflammatory states [115]. Also, application of EV derived from bone-marrow MSC preconditioned in hypoxia (1% O$_2$) reduced neuronal degeneration, brain atrophy, and improved neurological recovery. These effects were due to the EV effects on angiogenesis in a mouse model of cerebral ischemia[116].

In relation to the skeletal system, EV may be used in bone-fracture healing. Thus, exosomes generated by MSC obtained from human umbilical cord were exposed to 1% O$_2$ during 48 h. They promoted bone fracture healing in an animal model. These exosomes are enriched in miR-126 by the action of HIF1A exerting proangiogenic effects by means of sprouty-related, N-terminal enabled/vasodilator-stimulated phosphoprotein homology-1 domain-containing protein 1/Ras (name derived from Rat sarcoma-virus protein)/mitogen-activated protein kinase (originally called extracellular signal-regulated kinase or Erk) pathway activation[117].
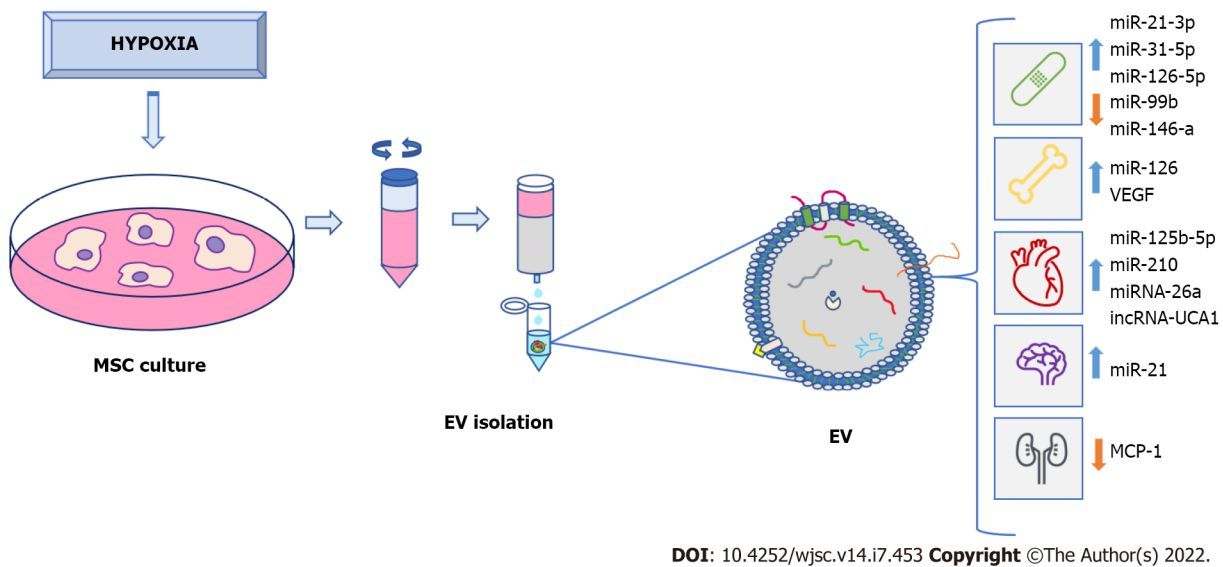
Figure 2 Clinical potential of extracellular vesicles from preconditioned mesenchymal stem-cells under hypoxia. Mesenchymal stem-cell (MSC) exposed to hypoxia secrete extracellular vesicles (EV) that can be isolated and used for clinical purposes, such as treatment of wound healing and bone fractures, as well as cardiovascular, neurodegenerative, and renal diseases, among others. Isolation of EV is made from MSC culture medium, which can be carried out in different ways. In this case, the use of a size-exclusion column is shown. EV secreted under hypoxia were enriched in various proteins, nucleic acids (like microRNA), as well as grow factors that are implicated in modulation and improvement of different biological processes related to tissue regeneration in different pathologies. EV: Extracellular vesicles; MSC: Mesenchymal stem-cell.

Also, treatment with EV, released from MSC preconditioned in 2% of oxygen, prevented bone loss, increasing blood-vessel formation in a rat model of steroid-induced osteonecrosis of femoral head[118]. Additionally, other studies have showed that MSC-derived EV, grown in hypoxia, protect from intervertebral-disc degeneration through their content in mir-17-5p, which modulates proliferation of nucleus-pulposus cell (NPC) matrix, *via* the TLR4/PI3K/AKT pathway[119]. Furthermore, preconditioning in hypoxia also increased the capacity of MSC-derived EV in cartilage regeneration by positively acting on chondrocytes. Thus, *in vivo* assays have shown that an injectable silk-fibroin hydrogel, containing articular chondrocytes and MSC-derived EV in hypoxia promoted cartilage regeneration [120]. Several miRNA were involved in this process, including miR-18a-3p, miR-181c-5p, miR-205-5p, miR-337-5p, and miR-376a-5p[120,121].

Treatment with EV derived from MSC has also been proposed for kidney injury. Thus, EV from adipose tissue-derived MSC cultured 72 h under hypoxia (1% O_2) or normoxia conditions were compared in treatment of kidney injury induced by ischemia in a rat model. Both conditions reduced tissue damage, but renal regeneration was higher under hypoxia conditions, triggering antiapoptotic, angiogenetic, immunomodulatory, and anti-oxidative stress responses. This could be due to differences in proteomic profiles of EV types[122].

On the other hand, EV derived from MSC cultured in hypoxia have been applied, using models of myocardial infarction, in several studies. Generally, protective effects of cardiac tissues from ischemic injury were observed. They were due, at least in part, to the ability of these EV to promote blood-vessel formation[123]. Additionally, exosomes from conditioned bone-marrow MSC cultured in hypoxia (24 h, 0.5% O_2) or normoxia were used. They were injected intramyocardially into infarcted hearts of C57 Black 6 inbred mice strain. Treatment with hypoxia-derived exosomes produced interesting results: (1) Decrease in fibrotic tissue and apoptotic cardiomyocytes; and (2) Increase in cardiac-progenitor cells. These exosomes, compared to normoxia ones, had a significant increase in expression of miR-210, which had positive effects on endothelial cells and cardiomyocytes[124].

Such miRNA were also abundant in EV secreted by rat bone-marrow MSC, cultured in 1% O_2 for 72 h. Their antiapoptotic effects in cardiomyocytes have also been demonstrated in a rat model of myocardium infarction[125]. Other EV derived from MSC cultured under hypoxia were also enriched in miRNA, showing antiapoptotic activity in cardiomyocytes. They include miR-125b-5p, which works through repression of *p53* and BCL2 Antagonist/Killer 1[126]. It has also been shown that EV obtained from MSC cultured in hypoxia were enriched in miR-26a in relation to EV obtained in normoxia. Such miRNA is involved in upregulating glycogen-synthase kinase 3 beta (*GSK3B*) expression, which enhanced the beta-catenin pathway, reducing ischemia-reperfusion injury in a rat model[127].

Other miRNA enriched in EV, derived from adipose and bone-marrow MSC preconditioned in hypoxia were miR-224-5p and miR-24. The former decreased synthesis of thioredoxin-interacting protein, which facilitates degradation of HIF1A. EV enriched in miR-224-5p favored adaptation of cardiomyocytes to hypoxia, therefore protecting them against myocardial infarction[128]. On the other

hand, miR-24 decreased in infarcted myocardium of rats. Thus, application of EV containing this miRNA protected cardiomyocytes from apoptosis, reducing infarct size, and improving cardiac function [129].

In addition to miRNA, other RNA types have also been identified, showing cardioprotective effects in EV generated by MSC, under hypoxia conditions. This is the case of long non-coding RNA of urothelial carcinoma-associated 1, which is related to the anti-apoptotic miR-873-5p/X-linked inhibitor of apoptosis protein/phosphorylated AMP-activated protein kinase pathway[130].

Exosomes derived from MSC grown under hypoxia may be also useful for treatments of chronic skin-ulcers. They are associated with pathologies such as diabetes. Their healing is difficult and isa serious problem for patients and public health systems[131]. Recently, a study has evaluated the potential application of EV obtained from adipose-tissue stem cells maintained at 1% O_2 for 24 h. *In vitro* assays showed that they promoted fibroblast proliferation and migration. That was accomplished by activating PI3K/AKT pathway in a more effective way than when EV obtained under normoxia were used. Differential expression analyses of miRNA contents between both types of EV showed upregulated miR-21-3p, miR-31-5p, and miR-126-5p and downregulated miR-99b and miR-146a. They may be involved in signaling pathways related to fibroblast proliferation and migration, modulating immune responses. Indeed, treatment with hypoxia-derived EV improved healing in a diabetic nude mice model of wound healing, which was carried out *via* downregulation of *IL-6*, upregulation of *VEGF* and modulation of extracellular matrix[132]. Additionally, EV derived from umbilical cord MSC exposed to 1% O_2 for 3 to 6 h were used in a full-thickness skin-injury mouse model, improving wound healing with respect to EV obtained in normoxia. In this case, it was demonstrated that EV in hypoxia had anti-apoptotic effects on endothelial cells due to miR-125b, which suppressed expression of TP53-inducible nuclear-protein 1 [133].

CONCLUSION

In recent years, the therapeutic potential of using MSC-derived EV has become apparent. This is because the regenerative effects of MSC are partly due to their paracrine activity. Besides, the contents of EV can be modulated through preconditioning of MSC under different culture conditions. Among they, exposure to hypoxia stands out. HIF activation affects hundreds of genes involved in processes such as inflammation, migration, proliferation, differentiation, metabolism, and cell apoptosis. That is related to the contents of secreted EV, and thus their therapeutic potential, which is better than the one of EV obtained under normoxic conditions. Therefore, hypoxia preconditioning of MSC is a very attractive strategy for isolation of therapeutic EV. They have a high potential for use in regenerative medicine and can be applied to different pathologies. However, studies published to date show a great variability. That includes sources of MSC, culture media, O_2 concentrations, and exposure times to hypoxia, as well as methods of EV isolation. Such factors may influence the degree of induction of *HIF1A* and *HIF2A*, and therefore MSC responses and EV cargos. Thus, it would be necessary to perform studies to optimize and standardize conditions for obtaining EV in the future according to their therapeutic applications. Also, *in vivo* studies carried out so far have been performed mainly in animal models. Only two active MSC-derived EV clinical trials in recruitment phase in which hypoxia is being evaluated are shown in ClinicalTrials (https://clinicaltrials.gov): "Treatment of Severe COVID-19 Patients Using Secretome of Hypoxia-Mesenchymal Stem Cells in Indonesia" (ID: NCT04753476) and "Regeneration of Posterior Cruciate Ligament Injury Using Hypoxic Conditioned Allogenic Adipose Mesenchymal Stem Cell and Condition Medium" (ID: NCT04889963). Therefore, in order to ascertain the greater potential effectiveness of EV obtained from MSC preconditioned in hypoxia, it would be necessary to carry out a greater number of properly designed clinical trials.

Using EV in regenerative medicine is very promising, as shown above. Yet, possible adverse effects associated with the use of the ones derived from MSC in human clinical practice must be taken into account. One of them is that the contents of EV may enhance tumor-cell activity[134]. In any case, that should be significantly lower –if it exists– than using whole stem-cells. Therefore, these risks should be properly evaluated in animal models and potential clinical trials. In this regard, there are several challenges for the use of MSC-derived EV in regenerative medicine that must be properly addressed beforehand. These include: (1) Identification of the most suitable MSC sources for each pathology; (2) Optimization and consensus of culture methods and conditions to obtain EV with greater regenerative capacity; (3) Scaling up of production for clinical use; (4) Control of variability and stability of produced EV; (5) Increase in clinical trials to make them statistically significant; and (6) A better understanding of pharmacokinetics and biodistribution of applied EV[135].

FOOTNOTES

ORCID number: Victoria Pulido-Escribano 0000-0002-1107-1547; Bárbara Torrecillas-Baena 0000-0001-6934-3958; Marta Camacho-Cardenosa 0000-0002-0921-2670; Gabriel Dorado 0000-0002-0648-5278; María Ángeles Gálvez-Moreno 0000-0002-1680-1932; Antonio Casado-Díaz 0000-0002-8520-8278.

REFERENCES

1 **Heo JS**, Choi Y, Kim HS, Kim HO. Comparison of molecular profiles of human mesenchymal stem cells derived from bone marrow, umbilical cord blood, placenta and adipose tissue. *Int J Mol Med* 2016; **37**: 115-125 [PMID: 26719857 DOI: 10.3892/ijmm.2015.2413]

2 **Méndez-Ferrer S**, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, Scadden DT, Ma'ayan A, Enikolopov GN, Frenette PS. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 2010; **466**: 829-834 [PMID: 20703299 DOI: 10.1038/nature09262]

3 **Xu X**, Zhu F, Zhang M, Zeng D, Luo D, Liu G, Cui W, Wang S, Guo W, Xing W, Liang H, Li L, Fu X, Jiang J, Huang H. Stromal cell-derived factor-1 enhances wound healing through recruiting bone marrow-derived mesenchymal stem cells to the wound area and promoting neovascularization. *Cells Tissues Organs* 2013; **197**: 103-113 [PMID: 23207453 DOI: 10.1159/000342921]

4 **Brown C**, McKee C, Bakshi S, Walker K, Hakman E, Halassy S, Svinarich D, Dodds R, Govind CK, Chaudhry GR. Mesenchymal stem cells: Cell therapy and regeneration potential. *J Tissue Eng Regen Med* 2019; **13**: 1738-1755 [PMID: 31216380 DOI: 10.1002/term.2914]

5 **Dzobo K**. Recent Trends in Multipotent Human Mesenchymal Stem/Stromal Cells: Learning from History and Advancing Clinical Applications. *OMICS* 2021; **25**: 342-357 [PMID: 34115524 DOI: 10.1089/omi.2021.0049]

6 **Sohni A**, Verfaillie CM. Mesenchymal stem cells migration homing and tracking. *Stem Cells Int* 2013; **2013**: 130763 [PMID: 24194766 DOI: 10.1155/2013/130763]

7 **Bernardo ME**, Cometa AM, Pagliara D, Vinti L, Rossi F, Cristantielli R, Palumbo G, Locatelli F. Ex vivo expansion of mesenchymal stromal cells. *Best Pract Res Clin Haematol* 2011; **24**: 73-81 [PMID: 21396595 DOI: 10.1016/j.beha.2010.11.002]

8 **Tamama K**, Kerpedjieva SS. Acceleration of Wound Healing by Multiple Growth Factors and Cytokines Secreted from Multipotential Stromal Cells/Mesenchymal Stem Cells. *Adv Wound Care (New Rochelle)* 2012; **1**: 177-182 [PMID: 24527301 DOI: 10.1089/wound.2011.0296]

9 **González-González A**, García-Sánchez D, Dotta M, Rodríguez-Rey JC, Pérez-Campo FM. Mesenchymal stem cells secretome: The cornerstone of cell-free regenerative medicine. *World J Stem Cells* 2020; **12**: 1529-1552 [PMID: 33505599 DOI: 10.4252/wjsc.v12.i12.1529]

10 **Casado-Díaz A**, Quesada-Gómez JM, Dorado G. Extracellular Vesicles Derived From Mesenchymal Stem Cells (MSC) in Regenerative Medicine: Applications in Skin Wound Healing. *Front Bioeng Biotechnol* 2020; **8**: 146 [PMID: 32195233 DOI: 10.3389/fbioe.2020.00146]

11 **Ruthenborg RJ**, Ban JJ, Wazir A, Takeda N, Kim JW. Regulation of wound healing and fibrosis by hypoxia and hypoxia-inducible factor-1. *Mol Cells* 2014; **37**: 637-643 [PMID: 24957212 DOI: 10.14348/molcells.2014.0150]

12 **Friedenstein AJ**, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 1970; **3**: 393-403 [PMID: 5523063 DOI: 10.1111/j.1365-2184.1970.tb00347.x]

13 **Rani S**, Ryan AE, Griffin MD, Ritter T. Mesenchymal Stem Cell-derived Extracellular Vesicles: Toward Cell-free Therapeutic Applications. *Mol Ther* 2015; **23**: 812-823 [PMID: 25868399 DOI: 10.1038/mt.2015.44]

14 **Dominici M**, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy

position statement. *Cytotherapy* 2006; **8**: 315-317 [PMID: 16923606 DOI: 10.1080/14653240600855905]

15   **Huang S**, Leung V, Peng S, Li L, Lu FJ, Wang T, Lu W, Cheung KM, Zhou G. Developmental definition of MSCs: new insights into pending questions. *Cell Reprogram* 2011; **13**: 465-472 [PMID: 21919705 DOI: 10.1089/cell.2011.0045]

16   **Chen L**, Qu J, Cheng T, Chen X, Xiang C. Menstrual blood-derived stem cells: toward therapeutic mechanisms, novel strategies, and future perspectives in the treatment of diseases. *Stem Cell Res Ther* 2019; **10**: 406 [PMID: 31864423 DOI: 10.1186/s13287-019-1503-7]

17   **Abbaszadeh H**, Ghorbani F, Derakhshani M, Movassaghpour AA, Yousefi M, Talebi M, Shamsasenjan K. Regenerative potential of Wharton's jelly-derived mesenchymal stem cells: A new horizon of stem cell therapy. *J Cell Physiol* 2020; **235**: 9230-9240 [PMID: 32557631 DOI: 10.1002/jcp.29810]

18   **Liu P**, Deng Z, Han S, Liu T, Wen N, Lu W, Geng X, Huang S, Jin Y. Tissue-engineered skin containing mesenchymal stem cells improves burn wounds. *Artif Organs* 2008; **32**: 925-931 [PMID: 19133020 DOI: 10.1111/j.1525-1594.2008.00654.x]

19   **Maranda EL**, Rodriguez-Menocal L, Badiavas EV. Role of Mesenchymal Stem Cells in Dermal Repair in Burns and Diabetic Wounds. *Curr Stem Cell Res Ther* 2017; **12**: 61-70 [PMID: 27412677 DOI: 10.2174/1574888x11666160714115926]

20   **Shao J**, Zhang W, Yang T. Using mesenchymal stem cells as a therapy for bone regeneration and repairing. *Biol Res* 2015; **48**: 62 [PMID: 26530042 DOI: 10.1186/s40659-015-0053-4]

21   **Harris VK**, Vyshkina T, Sadiq SA. Clinical safety of intrathecal administration of mesenchymal stromal cell-derived neural progenitors in multiple sclerosis. *Cytotherapy* 2016; **18**: 1476-1482 [PMID: 27727015 DOI: 10.1016/j.jcyt.2016.08.007]

22   **Petrou P**, Kassis I, Levin N, Paul F, Backner Y, Benoliel T, Oertel FC, Scheel M, Hallimi M, Yaghmour N, Hur TB, Ginzberg A, Levy Y, Abramsky O, Karussis D. Beneficial effects of autologous mesenchymal stem cell transplantation in active progressive multiple sclerosis. *Brain* 2020; **143**: 3574-3588 [PMID: 33253391 DOI: 10.1093/brain/awaa333]

23   **Shi M**, Zhang Z, Xu R, Lin H, Fu J, Zou Z, Zhang A, Shi J, Chen L, Lv S, He W, Geng H, Jin L, Liu Z, Wang FS. Human mesenchymal stem cell transfusion is safe and improves liver function in acute-on-chronic liver failure patients. *Stem Cells Transl Med* 2012; **1**: 725-731 [PMID: 23197664 DOI: 10.5966/sctm.2012-0034]

24   **Gnecchi M**, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. *Circ Res* 2008; **103**: 1204-1219 [PMID: 19028920 DOI: 10.1161/CIRCRESAHA.108.176826]

25   **Wagner J**, Kean T, Young R, Dennis JE, Caplan AI. Optimizing mesenchymal stem cell-based therapeutics. *Curr Opin Biotechnol* 2009; **20**: 531-536 [PMID: 19783424 DOI: 10.1016/j.copbio.2009.08.009]

26   **Zhou Y**, Yamamoto Y, Xiao Z, Ochiya T. The Immunomodulatory Functions of Mesenchymal Stromal/Stem Cells Mediated *via* Paracrine Activity. *J Clin Med* 2019; **8** [PMID: 31336889 DOI: 10.3390/jcm8071025]

27   **Tan SSH**, Tjio CKE, Wong JRY, Wong KL, Chew JRJ, Hui JHP, Toh WS. Mesenchymal Stem Cell Exosomes for Cartilage Regeneration: A Systematic Review of Preclinical *In Vivo* Studies. *Tissue Eng Part B Rev* 2021; **27**: 1-13 [PMID: 32159464 DOI: 10.1089/ten.TEB.2019.0326]

28   **Khan K**, Caron C, Mahmoud I, Derish I, Schwertani A, Cecere R. Extracellular Vesicles as a Cell-free Therapy for Cardiac Repair: a Systematic Review and Meta-analysis of Randomized Controlled Preclinical Trials in Animal Myocardial Infarction Models. *Stem Cell Rev Rep* 2022; **18**: 1143-1167 [PMID: 35107768 DOI: 10.1007/s12015-021-10289-6]

29   **Prasai A**, Jay JW, Jupiter D, Wolf SE, El Ayadi A. Role of Exosomes in Dermal Wound Healing: A Systematic Review. *J Invest Dermatol* 2022; **142**: 662-678.e8 [PMID: 34461128 DOI: 10.1016/j.jid.2021.07.167]

30   **Khalaj K**, Figueira RL, Antounians L, Lauriti G, Zani A. Systematic review of extracellular vesicle-based treatments for lung injury: are EVs a potential therapy for COVID-19? *J Extracell Vesicles* 2020; **9**: 1795365 [PMID: 32944185 DOI: 10.1080/20013078.2020.1795365]

31   **Kalluri R**, LeBleu VS. The biology**,** function**,** and biomedical applications of exosomes. *Science* 2020; **367** [PMID: 32029601 DOI: 10.1126/science.aau6977]

32   **Almeria C**, Weiss R, Roy M, Tripisciano C, Kasper C, Weber V, Egger D. Hypoxia Conditioned Mesenchymal Stem Cell-Derived Extracellular Vesicles Induce Increased Vascular Tube Formation *in vitro*. *Front Bioeng Biotechnol* 2019; **7**: 292 [PMID: 31709251 DOI: 10.3389/fbioe.2019.00292]

33   **Doyle LM**, Wang MZ. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. *Cells* 2019; **8** [PMID: 31311206 DOI: 10.3390/cells8070727]

34   **Théry C**, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A, Arab T, Archer F, Atkin-Smith GK, Ayre DC, Bach JM, Bachurski D, Baharvand H, Balaj L, Baldacchino S, Bauer NN, Baxter AA, Bebawy M, Beckham C, Bedina Zavec A, Benmoussa A, Berardi AC, Bergese P, Bielska E, Blenkiron C, Bobis-Wozowicz S, Boilard E, Boireau W, Bongiovanni A, Borràs FE, Bosch S, Boulanger CM, Breakefield X, Breglio AM, Brennan MÁ, Brigstock DR, Brisson A, Broekman ML, Bromberg JF, Bryl-Górecka P, Buch S, Buck AH, Burger D, Busatto S, Buschmann D, Bussolati B, Buzás EI, Byrd JB, Camussi G, Carter DR, Caruso S, Chamley LW, Chang YT, Chen C, Chen S, Cheng L, Chin AR, Clayton A, Clerici SP, Cocks A, Cocucci E, Coffey RJ, Cordeiro-da-Silva A, Couch Y, Coumans FA, Coyle B, Crescitelli R, Criado MF, D'Souza-Schorey C, Das S, Datta Chaudhuri A, de Candia P, De Santana EF, De Wever O, Del Portillo HA, Demaret T, Deville S, Devitt A, Dhondt B, Di Vizio D, Dieterich LC, Dolo V, Dominguez Rubio AP, Dominici M, Dourado MR, Driedonks TA, Duarte FV, Duncan HM, Eichenberger RM, Ekström K, El Andaloussi S, Elie-Caille C, Erdbrügger U, Falcón-Pérez JM, Fatima F, Fish JE, Flores-Bellver M, Försönits A, Frelet-Barrand A, Fricke F, Fuhrmann G, Gabrielsson S, Gámez-Valero A, Gardiner C, Gärtner K, Gaudin R, Gho YS, Giebel B, Gilbert C, Gimona M, Giusti I, Goberdhan DC, Görgens A, Gorski SM, Greening DW, Gross JC, Gualerzi A, Gupta GN, Gustafson D, Handberg A, Haraszti RA, Harrison P, Hegyesi H, Hendrix A, Hill AF, Hochberg FH, Hoffmann KF, Holder B, Holthofer H, Hosseinkhani B, Hu G, Huang Y, Huber V, Hunt S, Ibrahim AG, Ikezu T, Inal JM, Isin M, Ivanova A, Jackson HK, Jacobsen S, Jay SM, Jayachandran M, Jenster G, Jiang L, Johnson SM, Jones JC, Jong A, Jovanovic-Talisman T, Jung S, Kalluri R, Kano SI, Kaur S, Kawamura Y, Keller ET, Khamari D, Khomyakova E, Khvorova A, Kierulf P, Kim KP, Kislinger T, Klingeborn M, Klinke DJ 2nd, Kornek M, Kosanović MM, Kovács ÁF, Krämer-Albers EM, Krasemann S,

Krause M, Kurochkin IV, Kusuma GD, Kuypers S, Laitinen S, Langevin SM, Languino LR, Lannigan J, Lässer C, Laurent LC, Lavieu G, Lázaro-Ibáñez E, Le Lay S, Lee MS, Lee YXF, Lemos DS, Lenassi M, Leszczynska A, Li IT, Liao K, Libregts SF, Ligeti E, Lim R, Lim SK, Linē A, Linnemannstöns K, Llorente A, Lombard CA, Lorenowicz MJ, Lörincz ÁM, Lötvall J, Lovett J, Lowry MC, Loyer X, Lu Q, Lukomska B, Lunavat TR, Maas SL, Malhi H, Marcilla A, Mariani J, Mariscal J, Martens-Uzunova ES, Martin-Jaular L, Martinez MC, Martins VR, Mathieu M, Mathivanan S, Maugeri M, McGinnis LK, McVey MJ, Meckes DG Jr, Meehan KL, Mertens I, Minciacchi VR, Möller A, Møller Jørgensen M, Morales-Kastresana A, Morhayim J, Mullier F, Muraca M, Musante L, Mussack V, Muth DC, Myburgh KH, Najrana T, Nawaz M, Nazarenko I, Nejsum P, Neri C, Neri T, Nieuwland R, Nimrichter L, Nolan JP, Nolte-'t Hoen EN, Noren Hooten N, O'Driscoll L, O'Grady T, O'Loghlen A, Ochiya T, Olivier M, Ortiz A, Ortiz LA, Osteikoetxea X, Østergaard O, Ostrowski M, Park J, Pegtel DM, Peinado H, Perut F, Pfaffl MW, Phinney DG, Pieters BC, Pink RC, Pisetsky DS, Pogge von Strandmann E, Polakovicova I, Poon IK, Powell BH, Prada I, Pulliam L, Quesenberry P, Radeghieri A, Raffai RL, Raimondo S, Rak J, Ramirez MI, Raposo G, Rayyan MS, Regev-Rudzki N, Ricklefs FL, Robbins PD, Roberts DD, Rodrigues SC, Rohde E, Rome S, Rouschop KM, Rughetti A, Russell AE, Saá P, Sahoo S, Salas-Huenuleo E, Sánchez C, Saugstad JA, Saul MJ, Schiffelers RM, Schneider R, Schøyen TH, Scott A, Shahaj E, Sharma S, Shatnyeva O, Shekari F, Shelke GV, Shetty AK, Shiba K, Siljander PR, Silva AM, Skowronek A, Snyder OL 2nd, Soares RP, Sódar BW, Soekmadji C, Sotillo J, Stahl PD, Stoorvogel W, Stott SL, Strasser EF, Swift S, Tahara H, Tewari M, Timms K, Tiwari S, Tixeira R, Tkach M, Toh WS, Tomasini R, Torrecilhas AC, Tosar JP, Toxavidis V, Urbanelli L, Vader P, van Balkom BW, van der Grein SG, Van Deun J, van Herwijnen MJ, Van Keuren-Jensen K, van Niel G, van Royen ME, van Wijnen AJ, Vasconcelos MH, Vechetti IJ Jr, Veit TD, Vella LJ, Velot É, Verweij FJ, Vestad B, Viñas JL, Visnovitz T, Vukman KV, Wahlgren J, Watson DC, Wauben MH, Weaver A, Webber JP, Weber V, Wehman AM, Weiss DJ, Welsh JA, Wendt S, Wheelock AM, Wiener Z, Witte L, Wolfram J, Xagorari A, Xander P, Xu J, Yan X, Yáñez-Mó M, Yin H, Yuana Y, Zappulli V, Zarubova J, Žėkas V, Zhang JY, Zhao Z, Zheng L, Zheutlin AR, Zickler AM, Zimmermann P, Zivkovic AM, Zocco D, Zuba-Surma EK. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 2018; **7**: 1535750 [PMID: 30637094 DOI: 10.1080/20013078.2018.1535750]

35   **Huang-Doran I**, Zhang CY, Vidal-Puig A. Extracellular Vesicles: Novel Mediators of Cell Communication In Metabolic Disease. *Trends Endocrinol Metab* 2017; **28**: 3-18 [PMID: 27810172 DOI: 10.1016/j.tem.2016.10.003]

36   **Ding J**, Wang X, Chen B, Zhang J, Xu J. Exosomes Derived from Human Bone Marrow Mesenchymal Stem Cells Stimulated by Deferoxamine Accelerate Cutaneous Wound Healing by Promoting Angiogenesis. *Biomed Res Int* 2019; **2019**: 9742765 [PMID: 31192260 DOI: 10.1155/2019/9742765]

37   **Pitt JM**, Kroemer G, Zitvogel L. Extracellular vesicles: masters of intercellular communication and potential clinical interventions. *J Clin Invest* 2016; **126**: 1139-1143 [PMID: 27035805 DOI: 10.1172/JCI87316]

38   **Gurunathan S**, Kang MH, Jeyaraj M, Qasim M, Kim JH. Review of the Isolation, Characterization, Biological Function, and Multifarious Therapeutic Approaches of Exosomes. *Cells* 2019; **8** [PMID: 30987213 DOI: 10.3390/cells8040307]

39   **Rani S**, Ritter T. The Exosome - A Naturally Secreted Nanoparticle and its Application to Wound Healing. *Adv Mater* 2016; **28**: 5542-5552 [PMID: 26678528 DOI: 10.1002/adma.201504009]

40   **Chuo ST**, Chien JC, Lai CP. Imaging extracellular vesicles: current and emerging methods. *J Biomed Sci* 2018; **25**: 91 [PMID: 30580764 DOI: 10.1186/s12929-018-0494-5]

41   **Vizoso FJ**, Eiro N, Cid S, Schneider J, Perez-Fernandez R. Mesenchymal Stem Cell Secretome: Toward Cell-Free Therapeutic Strategies in Regenerative Medicine. *Int J Mol Sci* 2017; **18** [PMID: 28841158 DOI: 10.3390/ijms18091852]

42   **Lee SM**, Jun DW, Kang HT, Oh JH, Saeed WK, Ahn SB. Optimal Hypoxic Preconditioning of Human Embryonic Stem Cell-Derived Mesenchymal Stem Cells (hES-MSCs) and Their Characteristics. *Int J Stem Cells* 2021; **14**: 221-228 [PMID: 33632987 DOI: 10.15283/ijsc20096]

43   **Wang GL**, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc Natl Acad Sci U S A* 1995; **92**: 5510-5514 [PMID: 7539918 DOI: 10.1073/pnas.92.12.5510]

44   **Yellowley CE**, Genetos DC. Hypoxia Signaling in the Skeleton: Implications for Bone Health. *Curr Osteoporos Rep* 2019; **17**: 26-35 [PMID: 30725321 DOI: 10.1007/s11914-019-00500-6]

45   **Weidemann A**, Johnson RS. Biology of HIF-1alpha. *Cell Death Differ* 2008; **15**: 621-627 [PMID: 18259201 DOI: 10.1038/cdd.2008.12]

46   **Wang V**, Davis DA, Haque M, Huang LE, Yarchoan R. Differential gene up-regulation by hypoxia-inducible factor-1alpha and hypoxia-inducible factor-2alpha in HEK293T cells. *Cancer Res* 2005; **65**: 3299-3306 [PMID: 15833863 DOI: 10.1158/0008-5472.CAN-04-4130]

47   **Tian H**, McKnight SL, Russell DW. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev* 1997; **11**: 72-82 [PMID: 9000051 DOI: 10.1101/gad.11.1.72]

48   **Lin Q**, Cong X, Yun Z. Differential hypoxic regulation of hypoxia-inducible factors 1alpha and 2alpha. *Mol Cancer Res* 2011; **9**: 757-765 [PMID: 21571835 DOI: 10.1158/1541-7786.MCR-11-0053]

49   **Kumar H**, Choi DK. Hypoxia Inducible Factor Pathway and Physiological Adaptation: A Cell Survival Pathway? *Mediators Inflamm* 2015; **2015**: 584758 [PMID: 26491231 DOI: 10.1155/2015/584758]

50   **Yamashita T**, Ohneda O, Nagano M, Iemitsu M, Makino Y, Tanaka H, Miyauchi T, Goto K, Ohneda K, Fujii-Kuriyama Y, Poellinger L, Yamamoto M. Abnormal heart development and lung remodeling in mice lacking the hypoxia-inducible factor-related basic helix-loop-helix PAS protein NEPAS. *Mol Cell Biol* 2008; **28**: 1285-1297 [PMID: 18070924 DOI: 10.1128/MCB.01332-07]

51   **Hara S**, Hamada J, Kobayashi C, Kondo Y, Imura N. Expression and characterization of hypoxia-inducible factor (HIF)-3alpha in human kidney: suppression of HIF-mediated gene expression by HIF-3alpha. *Biochem Biophys Res Commun* 2001; **287**: 808-813 [PMID: 11573933 DOI: 10.1006/bbrc.2001.5659]

52   **Ema M**, Hirota K, Mimura J, Abe H, Yodoi J, Sogawa K, Poellinger L, Fujii-Kuriyama Y. Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. *EMBO J* 1999; **18**: 1905-1914 [PMID: 10202154 DOI: 10.1093/emboj/18.7.1905]

53   **Jaakkola P**, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, von Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science* 2001; **292**: 468-472 [PMID: 11292861 DOI: 10.1126/science.1059796]

54   **Ivan M**, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG Jr. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science* 2001; **292**: 464-468 [PMID: 11292862 DOI: 10.1126/science.1059817]

55   **Lando D**, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev* 2002; **16**: 1466-1471 [PMID: 12080085 DOI: 10.1101/gad.991402]

56   **Kaluz S**, Kaluzová M, Stanbridge EJ. Regulation of gene expression by hypoxia: integration of the HIF-transduced hypoxic signal at the hypoxia-responsive element. *Clin Chim Acta* 2008; **395**: 6-13 [PMID: 18505681 DOI: 10.1016/j.cca.2008.05.002]

57   **DeFrates KG**, Franco D, Heber-Katz E, Messersmith PB. Unlocking mammalian regeneration through hypoxia inducible factor one alpha signaling. *Biomaterials* 2021; **269**: 120646 [PMID: 33493769 DOI: 10.1016/j.biomaterials.2020.120646]

58   **Krämer A**, Green J, Pollard J Jr, Tugendreich S. Causal analysis approaches in Ingenuity Pathway Analysis. *Bioinformatics* 2014; **30**: 523-530 [PMID: 24336805 DOI: 10.1093/bioinformatics/btt703]

59   **Naviaux RK**, Le TP, Bedelbaeva K, Leferovich J, Gourevitch D, Sachadyn P, Zhang XM, Clark L, Heber-Katz E. Retained features of embryonic metabolism in the adult MRL mouse. *Mol Genet Metab* 2009; **96**: 133-144 [PMID: 19131261 DOI: 10.1016/j.ymgme.2008.11.164]

60   **Sunkari VG**, Lind F, Botusan IR, Kashif A, Liu ZJ, Ylä-Herttuala S, Brismar K, Velazquez O, Catrina SB. Hyperbaric oxygen therapy activates hypoxia-inducible factor 1 (HIF-1), which contributes to improved wound healing in diabetic mice. *Wound Repair Regen* 2015; **23**: 98-103 [PMID: 25532619 DOI: 10.1111/wrr.12253]

61   **Catrina SB**, Zheng X. Disturbed hypoxic responses as a pathogenic mechanism of diabetic foot ulcers. *Diabetes Metab Res Rev* 2016; **32** Suppl 1: 179-185 [PMID: 26453314 DOI: 10.1002/dmrr.2742]

62   **Strowitzki MJ**, Ritter AS, Kimmer G, Schneider M. Hypoxia-adaptive pathways: A pharmacological target in fibrotic disease? *Pharmacol Res* 2019; **147**: 104364 [PMID: 31376431 DOI: 10.1016/j.phrs.2019.104364]

63   **Luo R**, Zhang W, Zhao C, Zhang Y, Wu H, Jin J, Grenz A, Eltzschig HK, Tao L, Kellems RE, Xia Y. Elevated Endothelial Hypoxia-Inducible Factor-1α Contributes to Glomerular Injury and Promotes Hypertensive Chronic Kidney Disease. *Hypertension* 2015; **66**: 75-84 [PMID: 25987665 DOI: 10.1161/HYPERTENSIONAHA.115.05578]

64   **Mitchell S**, Vargas J, Hoffmann A. Signaling *via* the NFκB system. *Wiley Interdiscip Rev Syst Biol Med* 2016; **8**: 227-241 [PMID: 26990581 DOI: 10.1002/wsbm.1331]

65   **Eltzschig HK**, Carmeliet P. Hypoxia and inflammation. *N Engl J Med* 2011; **364**: 656-665 [PMID: 21323543 DOI: 10.1056/NEJMra0910283]

66   **Kuhlicke J**, Frick JS, Morote-Garcia JC, Rosenberger P, Eltzschig HK. Hypoxia inducible factor (HIF)-1 coordinates induction of Toll-like receptors TLR2 and TLR6 during hypoxia. *PLoS One* 2007; **2**: e1364 [PMID: 18159247 DOI: 10.1371/journal.pone.0001364]

67   **Lim KH**, Staudt LM. Toll-like receptor signaling. *Cold Spring Harb Perspect Biol* 2013; **5**: a011247 [PMID: 23284045 DOI: 10.1101/cshperspect.a011247]

68   **Hutami IR**, Izawa T, Khurel-Ochir T, Sakamaki T, Iwasa A, Tanaka E. Macrophage Motility in Wound Healing Is Regulated by HIF-1α *via* S1P Signaling. *Int J Mol Sci* 2021; **22** [PMID: 34445695 DOI: 10.3390/ijms22168992]

69   **Wang T**, Liu H, Lian G, Zhang SY, Wang X, Jiang C. HIF1α-Induced Glycolysis Metabolism Is Essential to the Activation of Inflammatory Macrophages. *Mediators Inflamm* 2017; **2017**: 9029327 [PMID: 29386753 DOI: 10.1155/2017/9029327]

70   **Takeda N**, O'Dea EL, Doedens A, Kim JW, Weidemann A, Stockmann C, Asagiri M, Simon MC, Hoffmann A, Johnson RS. Differential activation and antagonistic function of HIF-{alpha} isoforms in macrophages are essential for NO homeostasis. *Genes Dev* 2010; **24**: 491-501 [PMID: 20194441 DOI: 10.1101/gad.1881410]

71   **Sun C**, Mezzadra R, Schumacher TN. Regulation and Function of the PD-L1 Checkpoint. *Immunity* 2018; **48**: 434-452 [PMID: 29562194 DOI: 10.1016/j.immuni.2018.03.014]

72   **Xu O**, Li X, Qu Y, Liu S, An J, Wang M, Sun Q, Zhang W, Lu X, Pi L, Zhang M, Shen Y. Regulation of glucose transporter protein-1 and vascular endothelial growth factor by hypoxia inducible factor 1α under hypoxic conditions in Hep-2 human cells. *Mol Med Rep* 2012; **6**: 1418-1422 [PMID: 22971798 DOI: 10.3892/mmr.2012.1075]

73   **Semenza GL**, Roth PH, Fang HM, Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem* 1994; **269**: 23757-23763 [PMID: 8089148]

74   **Xu C**, Gu L, Kuerbanjiang M, Wen S, Xu Q, Xue H. Thrombospondin 2/Toll-Like Receptor 4 Axis Contributes to HIF-1α -Derived Glycolysis in Colorectal Cancer. *Front Oncol* 2020; **10**: 557730 [PMID: 33244454 DOI: 10.3389/fonc.2020.557730]

75   **Kim JW**, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab* 2006; **3**: 177-185 [PMID: 16517405 DOI: 10.1016/j.cmet.2006.02.002]

76   **Zimna A**, Kurpisz M. Hypoxia-Inducible Factor-1 in Physiological and Pathophysiological Angiogenesis: Applications and Therapies. *Biomed Res Int* 2015; **2015**: 549412 [PMID: 26146622 DOI: 10.1155/2015/549412]

77   **Hoenig MR**, Bianchi C, Sellke FW. Hypoxia inducible factor-1 alpha, endothelial progenitor cells, monocytes, cardiovascular risk, wound healing, cobalt and hydralazine: a unifying hypothesis. *Curr Drug Targets* 2008; **9**: 422-435 [PMID: 18473772 DOI: 10.2174/138945008784221215]

78   **Tu TC**, Nagano M, Yamashita T, Hamada H, Ohneda K, Kimura K, Ohneda O. A Chemokine Receptor, CXCR4, Which Is Regulated by Hypoxia-Inducible Factor 2α, Is Crucial for Functional Endothelial Progenitor Cells Migration to Ischemic Tissue and Wound Repair. *Stem Cells Dev* 2016; **25**: 266-276 [PMID: 26620723 DOI: 10.1089/scd.2015.0290]

79   **Koshiji M**, Kageyama Y, Pete EA, Horikawa I, Barrett JC, Huang LE. HIF-1alpha induces cell cycle arrest by

functionally counteracting Myc. *EMBO J* 2004; **23**: 1949-1956 [PMID: 15071503 DOI: 10.1038/sj.emboj.7600196]

80   **Baba M**, Hirai S, Yamada-Okabe H, Hamada K, Tabuchi H, Kobayashi K, Kondo K, Yoshida M, Yamashita A, Kishida T, Nakaigawa N, Nagashima Y, Kubota Y, Yao M, Ohno S. Loss of von Hippel-Lindau protein causes cell density dependent deregulation of CyclinD1 expression through hypoxia-inducible factor. *Oncogene* 2003; **22**: 2728-2738 [PMID: 12743597 DOI: 10.1038/sj.onc.1206373]

81   **Gordan JD**, Bertout JA, Hu CJ, Diehl JA, Simon MC. HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. *Cancer Cell* 2007; **11**: 335-347 [PMID: 17418410 DOI: 10.1016/j.ccr.2007.02.006]

82   **Friedl P**. Prespecification and plasticity: shifting mechanisms of cell migration. *Curr Opin Cell Biol* 2004; **16**: 14-23 [PMID: 15037300 DOI: 10.1016/j.ceb.2003.11.001]

83   **Greijer AE**, van der Wall E. The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. *J Clin Pathol* 2004; **57**: 1009-1014 [PMID: 15452150 DOI: 10.1136/jcp.2003.015032]

84   **Kilic M**, Kasperczyk H, Fulda S, Debatin KM. Role of hypoxia inducible factor-1 alpha in modulation of apoptosis resistance. *Oncogene* 2007; **26**: 2027-2038 [PMID: 17043658 DOI: 10.1038/sj.onc.1210008]

85   **Jing X**, Yang F, Shao C, Wei K, Xie M, Shen H, Shu Y. Role of hypoxia in cancer therapy by regulating the tumor microenvironment. *Mol Cancer* 2019; **18**: 157 [PMID: 31711497 DOI: 10.1186/s12943-019-1089-9]

86   **Basciano L**, Nemos C, Foliguet B, de Isla N, de Carvalho M, Tran N, Dalloul A. Long term culture of mesenchymal stem cells in hypoxia promotes a genetic program maintaining their undifferentiated and multipotent status. *BMC Cell Biol* 2011; **12**: 12 [PMID: 21450070 DOI: 10.1186/1471-2121-12-12]

87   **Ejtehadifar M**, Shamsasenjan K, Movassaghpour A, Akbarzadehlaleh P, Dehdilani N, Abbasi P, Molaeipour Z, Saleh M. The Effect of Hypoxia on Mesenchymal Stem Cell Biology. *Adv Pharm Bull* 2015; **5**: 141-149 [PMID: 26236651 DOI: 10.15171/apb.2015.021]

88   **Sylakowski K**, Bradshaw A, Wells A. Mesenchymal Stem Cell/Multipotent Stromal Cell Augmentation of Wound Healing: Lessons from the Physiology of Matrix and Hypoxia Support. *Am J Pathol* 2020; **190**: 1370-1381 [PMID: 32294456 DOI: 10.1016/j.ajpath.2020.03.017]

89   **Lee JH**, Yoon YM, Lee SH. Hypoxic Preconditioning Promotes the Bioactivities of Mesenchymal Stem Cells *via* the HIF-1α-GRP78-Akt Axis. *Int J Mol Sci* 2017; **18** [PMID: 28635661 DOI: 10.3390/ijms18061320]

90   **Moya A**, Paquet J, Deschepper M, Larochette N, Oudina K, Denoeud C, Bensidhoum M, Logeart-Avramoglou D, Petite H. Human Mesenchymal Stem Cell Failure to Adapt to Glucose Shortage and Rapidly Use Intracellular Energy Reserves Through Glycolysis Explains Poor Cell Survival After Implantation. *Stem Cells* 2018; **36**: 363-376 [PMID: 29266629 DOI: 10.1002/stem.2763]

91   **Hawkins KE**, Sharp TV, McKay TR. The role of hypoxia in stem cell potency and differentiation. *Regen Med* 2013; **8**: 771-782 [PMID: 24147532 DOI: 10.2217/rme.13.71]

92   **Kim YS**, Noh MY, Cho KA, Kim H, Kwon MS, Kim KS, Kim J, Koh SH, Kim SH. Hypoxia/Reoxygenation-Preconditioned Human Bone Marrow-Derived Mesenchymal Stromal Cells Rescue Ischemic Rat Cortical Neurons by Enhancing Trophic Factor Release. *Mol Neurobiol* 2015; **52**: 792-803 [PMID: 25288154 DOI: 10.1007/s12035-014-8912-5]

93   **Boyette LB**, Creasey OA, Guzik L, Lozito T, Tuan RS. Human bone marrow-derived mesenchymal stem cells display enhanced clonogenicity but impaired differentiation with hypoxic preconditioning. *Stem Cells Transl Med* 2014; **3**: 241-254 [PMID: 24436440 DOI: 10.5966/sctm.2013-0079]

94   **Kheirandish M**, Gavgani SP, Samiee S. The effect of hypoxia preconditioning on the neural and stemness genes expression profiling in human umbilical cord blood mesenchymal stem cells. *Transfus Apher Sci* 2017; **56**: 392-399 [PMID: 28428031 DOI: 10.1016/j.transci.2017.03.015]

95   **Samal JRK**, Rangasami VK, Samanta S, Varghese OP, Oommen OP. Discrepancies on the Role of Oxygen Gradient and Culture Condition on Mesenchymal Stem Cell Fate. *Adv Healthc Mater* 2021; **10**: e2002058 [PMID: 33533187 DOI: 10.1002/adhm.202002058]

96   **Antebi B**, Rodriguez LA 2nd, Walker KP 3rd, Asher AM, Kamucheka RM, Alvarado L, Mohammadipoor A, Cancio LC. Short-term physiological hypoxia potentiates the therapeutic function of mesenchymal stem cells. *Stem Cell Res Ther* 2018; **9**: 265 [PMID: 30305185 DOI: 10.1186/s13287-018-1007-x]

97   **Powell FL**, Garcia N. Physiological effects of intermittent hypoxia. *High Alt Med Biol* 2000; **1**: 125-136 [PMID: 11256564 DOI: 10.1089/15270290050074279]

98   **Hu C**, Li L. Preconditioning influences mesenchymal stem cell properties in vitro and in vivo. *J Cell Mol Med* 2018; **22**: 1428-1442 [PMID: 29392844 DOI: 10.1111/jcmm.13492]

99   **Patel DB**, Gray KM, Santharam Y, Lamichhane TN, Stroka KM, Jay SM. Impact of cell culture parameters on production and vascularization bioactivity of mesenchymal stem cell-derived extracellular vesicles. *Bioeng Transl Med* 2017; **2**: 170-179 [PMID: 28932818 DOI: 10.1002/btm2.10065]

100   **Rosová I**, Dao M, Capoccia B, Link D, Nolta JA. Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. *Stem Cells* 2008; **26**: 2173-2182 [PMID: 18511601 DOI: 10.1634/stemcells.2007-1104]

101   **Salomon C**, Ryan J, Sobrevia L, Kobayashi M, Ashman K, Mitchell M, Rice GE. Exosomal signaling during hypoxia mediates microvascular endothelial cell migration and vasculogenesis. *PLoS One* 2013; **8**: e68451 [PMID: 23861904 DOI: 10.1371/journal.pone.0068451]

102   **Yu J**, Liu XL, Cheng QG, Lu SS, Xu XQ, Zu QQ, Liu S. G-CSF and hypoxic conditioning improve the proliferation, neural differentiation and migration of canine bone marrow mesenchymal stem cells. *Exp Ther Med* 2016; **12**: 1822-1828 [PMID: 27588100 DOI: 10.3892/etm.2016.3535]

103   **Zhang HC**, Liu XB, Huang S, Bi XY, Wang HX, Xie LX, Wang YQ, Cao XF, Lv J, Xiao FJ, Yang Y, Guo ZK. Microvesicles derived from human umbilical cord mesenchymal stem cells stimulated by hypoxia promote angiogenesis both *in vitro* and in vivo. *Stem Cells Dev* 2012; **21**: 3289-3297 [PMID: 22839741 DOI: 10.1089/scd.2012.0095]

104   **Chen J**, Liu Z, Hong MM, Zhang H, Chen C, Xiao M, Wang J, Yao F, Ba M, Liu J, Guo ZK, Zhong J. Proangiogenic compositions of microvesicles derived from human umbilical cord mesenchymal stem cells. *PLoS One* 2014; **9**: e115316

[PMID: 25514634 DOI: 10.1371/journal.pone.0115316]

105 **Han YD**, Bai Y, Yan XL, Ren J, Zeng Q, Li XD, Pei XT, Han Y. Co-transplantation of exosomes derived from hypoxia-preconditioned adipose mesenchymal stem cells promotes neovascularization and graft survival in fat grafting. *Biochem Biophys Res Commun* 2018; **497**: 305-312 [PMID: 29428734 DOI: 10.1016/j.bbrc.2018.02.076]

106 **Han Y**, Ren J, Bai Y, Pei X, Han Y. Exosomes from hypoxia-treated human adipose-derived mesenchymal stem cells enhance angiogenesis through VEGF/VEGF-R. *Int J Biochem Cell Biol* 2019; **109**: 59-68 [PMID: 30710751 DOI: 10.1016/j.biocel.2019.01.017]

107 **Mu J**, Li L, Wu J, Huang T, Zhang Y, Cao J, Ma T, Chen J, Zhang C, Zhang X, Lu T, Kong X, Sun J, Gao J. Hypoxia-stimulated mesenchymal stem cell-derived exosomes loaded by adhesive hydrogel for effective angiogenic treatment of spinal cord injury. *Biomater Sci* 2022; **10**: 1803-1811 [PMID: 35234220 DOI: 10.1039/d1bm01722e]

108 **Lomelí H**, Castillo-Castellanos F. Notch signaling and the emergence of hematopoietic stem cells. *Dev Dyn* 2020; **249**: 1302-1317 [PMID: 32996661 DOI: 10.1002/dvdy.230]

109 **Hori K**, Sen A, Artavanis-Tsakonas S. Notch signaling at a glance. *J Cell Sci* 2013; **126**: 2135-2140 [PMID: 23729744 DOI: 10.1242/jcs.127308]

110 **Niazi V**, Ghafouri-Fard S, Verdi J, Jeibouei S, Karami F, Pourhadi M, Ahani M, Atarodi K, Soleimani M, Zali H, Zomorrod MS. Hypoxia preconditioned mesenchymal stem cell-derived exosomes induce *ex vivo* expansion of umbilical cord blood hematopoietic stem cells CD133+ by stimulation of Notch signaling pathway. *Biotechnol Prog* 2022; **38**: e3222 [PMID: 34734683 DOI: 10.1002/btpr.3222]

111 **Gao W**, He R, Ren J, Zhang W, Wang K, Zhu L, Liang T. Exosomal HMGB1 derived from hypoxia-conditioned bone marrow mesenchymal stem cells increases angiogenesis *via* the JNK/HIF-1α pathway. *FEBS Open Bio* 2021; **11**: 1364-1373 [PMID: 33711197 DOI: 10.1002/2211-5463.13142]

112 **Ge L**, Xun C, Li W, Jin S, Liu Z, Zhuo Y, Duan D, Hu Z, Chen P, Lu M. Extracellular vesicles derived from hypoxia-preconditioned olfactory mucosa mesenchymal stem cells enhance angiogenesis *via* miR-612. *J Nanobiotechnology* 2021; **19**: 380 [PMID: 34802444 DOI: 10.1186/s12951-021-01126-6]

113 **Crous-Bou M**, Minguillón C, Gramunt N, Molinuevo JL. Alzheimer's disease prevention: from risk factors to early intervention. *Alzheimers Res Ther* 2017; **9**: 71 [PMID: 28899416 DOI: 10.1186/s13195-017-0297-z]

114 **Cui GH**, Wu J, Mou FF, Xie WH, Wang FB, Wang QL, Fang J, Xu YW, Dong YR, Liu JR, Guo HD. Exosomes derived from hypoxia-preconditioned mesenchymal stromal cells ameliorate cognitive decline by rescuing synaptic dysfunction and regulating inflammatory responses in APP/PS1 mice. *FASEB J* 2018; **32**: 654-668 [PMID: 28970251 DOI: 10.1096/fj.201700600R]

115 **Liu W**, Rong Y, Wang J, Zhou Z, Ge X, Ji C, Jiang D, Gong F, Li L, Chen J, Zhao S, Kong F, Gu C, Fan J, Cai W. Exosome-shuttled miR-216a-5p from hypoxic preconditioned mesenchymal stem cells repair traumatic spinal cord injury by shifting microglial M1/M2 polarization. *J Neuroinflammation* 2020; **17**: 47 [PMID: 32019561 DOI: 10.1186/s12974-020-1726-7]

116 **Gregorius J**, Wang C, Stambouli O, Hussner T, Qi Y, Tertel T, Börger V, Mohamud Yusuf A, Hagemann N, Yin D, Dittrich R, Mouloud Y, Mairinger FD, Magraoui FE, Popa-Wagner A, Kleinschnitz C, Doeppner TR, Gunzer M, Meyer HE, Giebel B, Hermann DM. Small extracellular vesicles obtained from hypoxic mesenchymal stromal cells have unique characteristics that promote cerebral angiogenesis, brain remodeling and neurological recovery after focal cerebral ischemia in mice. *Basic Res Cardiol* 2021; **116**: 40 [PMID: 34105014 DOI: 10.1007/s00395-021-00881-9]

117 **Liu W**, Li L, Rong Y, Qian D, Chen J, Zhou Z, Luo Y, Jiang D, Cheng L, Zhao S, Kong F, Wang J, Xu T, Gong F, Huang Y, Gu C, Zhao X, Bai J, Wang F, Zhao W, Zhang L, Li X, Yin G, Fan J, Cai W. Hypoxic mesenchymal stem cell-derived exosomes promote bone fracture healing by the transfer of miR-126. *Acta Biomater* 2020; **103**: 196-212 [PMID: 31857259 DOI: 10.1016/j.actbio.2019.12.020]

118 **Yuan N**, Ge Z, Ji W, Li J. Exosomes Secreted from Hypoxia-Preconditioned Mesenchymal Stem Cells Prevent Steroid-Induced Osteonecrosis of the Femoral Head by Promoting Angiogenesis in Rats. *Biomed Res Int* 2021; **2021**: 6655225 [PMID: 33928159 DOI: 10.1155/2021/6655225]

119 **Zhou ZM**, Bao JP, Peng X, Gao JW, Vlf C, Zhang C, Sun R, Kun-Wang, Wu XT. Small extracellular vesicles from hypoxic mesenchymal stem cells alleviate intervertebral disc degeneration by delivering miR-17-5p. *Acta Biomater* 2022; **140**: 641-658 [PMID: 34879291 DOI: 10.1016/j.actbio.2021.11.044]

120 **Shen K**, Duan A, Cheng J, Yuan T, Zhou J, Song H, Chen Z, Wan B, Liu J, Zhang X, Zhang Y, Xie R, Liu F, Fan W, Zuo Q. Exosomes derived from hypoxia preconditioned mesenchymal stem cells laden in a silk hydrogel promote cartilage regeneration *via* the miR-205-5p/PTEN/AKT pathway. *Acta Biomater* 2022; **143**: 173-188 [PMID: 35202856 DOI: 10.1016/j.actbio.2022.02.026]

121 **Zhang B**, Tian X, Qu Z, Hao J, Zhang W. Hypoxia-Preconditioned Extracellular Vesicles from Mesenchymal Stem Cells Improve Cartilage Repair in Osteoarthritis. *Membranes (Basel)* 2022; **12** [PMID: 35207146 DOI: 10.3390/membranes12020225]

122 **Collino F**, Lopes JA, Corrêa S, Abdelhay E, Takiya CM, Wendt CHC, de Miranda KR, Vieyra A, Lindoso RS. Adipose-Derived Mesenchymal Stromal Cells Under Hypoxia: Changes in Extracellular Vesicles Secretion and Improvement of Renal Recovery after Ischemic Injury. *Cell Physiol Biochem* 2019; **52**: 1463-1483 [PMID: 31099507 DOI: 10.33594/000000102]

123 **Bian S**, Zhang L, Duan L, Wang X, Min Y, Yu H. Extracellular vesicles derived from human bone marrow mesenchymal stem cells promote angiogenesis in a rat myocardial infarction model. *J Mol Med (Berl)* 2014; **92**: 387-397 [PMID: 24337504 DOI: 10.1007/s00109-013-1110-5]

124 **Zhu J**, Lu K, Zhang N, Zhao Y, Ma Q, Shen J, Lin Y, Xiang P, Tang Y, Hu X, Chen J, Zhu W, Webster KA, Wang J, Yu H. Myocardial reparative functions of exosomes from mesenchymal stem cells are enhanced by hypoxia treatment of the cells *via* transferring microRNA-210 in an nSMase2-dependent way. *Artif Cells Nanomed Biotechnol* 2018; **46**: 1659-1670 [PMID: 29141446 DOI: 10.1080/21691401.2017.1388249]

125 **Cheng H**, Chang S, Xu R, Chen L, Song X, Wu J, Qian J, Zou Y, Ma J. Hypoxia-challenged MSC-derived exosomes deliver miR-210 to attenuate post-infarction cardiac apoptosis. *Stem Cell Res Ther* 2020; **11**: 224 [PMID: 32513270 DOI:

10.1186/s13287-020-01737-0]

126   **Zhu LP**, Tian T, Wang JY, He JN, Chen T, Pan M, Xu L, Zhang HX, Qiu XT, Li CC, Wang KK, Shen H, Zhang GG, Bai YP. Hypoxia-elicited mesenchymal stem cell-derived exosomes facilitates cardiac repair through miR-125b-mediated prevention of cell death in myocardial infarction. *Theranostics* 2018; **8**: 6163-6177 [PMID: 30613290 DOI: 10.7150/thno.28021]

127   **Park H**, Park H, Mun D, Kang J, Kim H, Kim M, Cui S, Lee SH, Joung B. Extracellular Vesicles Derived from Hypoxic Human Mesenchymal Stem Cells Attenuate GSK3β Expression *via* miRNA-26a in an Ischemia-Reperfusion Injury Model. *Yonsei Med J* 2018; **59**: 736-745 [PMID: 29978610 DOI: 10.3349/ymj.2018.59.6.736]

128   **Mao CY**, Zhang TT, Li DJ, Zhou E, Fan YQ, He Q, Wang CQ, Zhang JF. Extracellular vesicles from hypoxia-preconditioned mesenchymal stem cells alleviates myocardial injury by targeting thioredoxin-interacting protein-mediated hypoxia-inducible factor-1α pathway. *World J Stem Cells* 2022; **14**: 183-199 [PMID: 35432732 DOI: 10.4252/wjsc.v14.i2.183]

129   **Zhang CS**, Shao K, Liu CW, Li CJ, Yu BT. Hypoxic preconditioning BMSCs-exosomes inhibit cardiomyocyte apoptosis after acute myocardial infarction by upregulating microRNA-24. *Eur Rev Med Pharmacol Sci* 2019; **23**: 6691-6699 [PMID: 31378912 DOI: 10.26355/eurrev_201908_18560]

130   **Sun L**, Zhu W, Zhao P, Wang Q, Fan B, Zhu Y, Lu Y, Chen Q, Zhang J, Zhang F. Long noncoding RNA UCA1 from hypoxia-conditioned hMSC-derived exosomes: a novel molecular target for cardioprotection through miR-873-5p/XIAP axis. *Cell Death Dis* 2020; **11**: 696 [PMID: 32826854 DOI: 10.1038/s41419-020-02783-5]

131   **Raghav A**, Khan ZA, Labala RK, Ahmad J, Noor S, Mishra BK. Financial burden of diabetic foot ulcers to world: a progressive topic to discuss always. *Ther Adv Endocrinol Metab* 2018; **9**: 29-31 [PMID: 29344337 DOI: 10.1177/2042018817744513]

132   **Wang J**, Wu H, Peng Y, Zhao Y, Qin Y, Zhang Y, Xiao Z. Hypoxia adipose stem cell-derived exosomes promote high-quality healing of diabetic wound involves activation of PI3K/Akt pathways. *J Nanobiotechnology* 2021; **19**: 202 [PMID: 34233694 DOI: 10.1186/s12951-021-00942-0]

133   **Zhang XF**, Wang T, Wang ZX, Huang KP, Zhang YW, Wang GL, Zhang HJ, Chen ZH, Wang CY, Zhang JX, Wang H. Hypoxic ucMSC-secreted exosomal miR-125b promotes endothelial cell survival and migration during wound healing by targeting TP53INP1. *Mol Ther Nucleic Acids* 2021; **26**: 347-359 [PMID: 34513314 DOI: 10.1016/j.omtn.2021.07.014]

134   **Zhang X**, Sai B, Wang F, Wang L, Wang Y, Zheng L, Li G, Tang J, Xiang J. Hypoxic BMSC-derived exosomal miRNAs promote metastasis of lung cancer cells *via* STAT3-induced EMT. *Mol Cancer* 2019; **18**: 40 [PMID: 30866952 DOI: 10.1186/s12943-019-0959-5]

135   **Fuloria S**, Subramaniyan V, Dahiya R, Dahiya S, Sudhakar K, Kumari U, Sathasivam K, Meenakshi DU, Wu YS, Sekar M, Malviya R, Singh A, Fuloria NK. Mesenchymal Stem Cell-Derived Extracellular Vesicles: Regenerative Potential and Challenges. *Biology (Basel)* 2021; **10** [PMID: 33668707 DOI: 10.3390/biology10030172]

Application of exosome-derived noncoding RNAs in bone regeneration: Opportunities and challenges

Yuan-Zhong Ren, Shan-Shan Ding, Ya-Ping Jiang, Hui Wen, Tao Li

Yuan-Zhong Ren, Hui Wen, Department of Emergency Trauma Surgery, Luoyang Central Hospital Affiliated to Zhengzhou University, Luoyang 471000, Henan Province, China

Shan-Shan Ding, Department of Geriatrics, Luoyang Central Hospital Affiliated to Zhengzhou University, Luoyang 471000, Henan Province, China

Ya-Ping Jiang, Department of Oral Implantology, The Affiliated Hospital of Qingdao University, Qingdao 266000, Shandong Province, China

Tao Li, Department of Joint Surgery, The Affiliated Hospital of Qingdao University, Qingdao 266003, Shandong Province, China

Corresponding author: Tao Li, MD, Postdoc, Surgeon, Department of Joint Surgery, The Affiliated Hospital of Qingdao University, No. 59 Haier Road, Qingdao 266003, Shandong Province, China. qdult@qdu.edu.cn

Abstract

With advances in the fields of regenerative medicine, cell-free therapy has received increased attention. Exosomes have a variety of endogenous properties that provide stability for molecular transport across biological barriers to cells, as a form of cell-to-cell communication that regulates function and phenotype. In addition, exosomes are an important component of paracrine signaling in stem-cell-based therapy and can be used as a stand-alone therapy or as a drug delivery system. The remarkable potential of exosomes has paved the pathway for cell-free treatment in bone regeneration. Exosomes are enriched in distinct noncoding RNAs (ncRNAs), including microRNAs, long ncRNAs and circular RNAs. Different ncRNAs have multiple functions. Altered expression of ncRNA in exosomes is associated with the regenerative potential and development of various diseases, such as femoral head osteonecrosis, myocardial infarction, and cancer. Although there is increasing evidence that exosome-derived ncRNAs (exo-ncRNAs) have the potential for bone regeneration, the detailed mechanisms are not fully understood. Here, we review the biogenesis of exo-ncRNA and the effects of ncRNAs on angiogenesis and osteoblast- and osteoclast-related pathways in different diseases. However, there are still many unsolved problems and challenges in the clinical application of ncRNA; for instance, production, storage, targeted delivery and therapeutic potency assessment. Advancements in exo-ncRNA methods and design will promote the development of therapeutics, revolutionizing the present landscape.

Key Words: Exosomes; Non-coding RNA; Bone; Osteogenesis; Angiogenesis; Osteoclasts

Core Tip: The key to bone regeneration is the mutual balance between osteoblasts, osteoclasts and angiogenesis. As a critical factor in bone regeneration, exosome-derived noncoding RNA (exo-ncRNA) has been extensively studied. However, the detailed mechanism of exo-ncRNA in bone regeneration is still unclear, and further research is necessary. This article summarizes the research on exo-ncRNA in bone regeneration.

INTRODUCTION

Exosomes

Although extracellular vesicles (EVs) were first mentioned in the late 1960s[1], it was not until the last decade that they were named[2]. According to characteristics and cell sources, EVs can be divided into three types: Apoptotic bodies, microvesicles, and exosomes[3]. Exosomes are small EVs[4] that were first found in reticulocytes[5]. Johnston named these structures as exosomes in 1987[6]. The formation and secretion of exosomes is a complex biological process. Exosomes express biological effects through the paracrine pathway and transport bioactive substances to regulate intercellular communication[7]. Therefore, stable and efficient separation and extraction methods are the prerequisites for their clinical application. Although diverse exosome isolation techniques have been developed based on their biophysical and biochemical properties, there is still a lack of standardized and large-scale clinical isolation and purification methods.

Biogenesis and isolation of exosomes

Exosomes are spherical endocytic vesicles with a diameter of 40–150 nm. They are formed in intracellular multivesicular bodies (MVBs) and removed from various cell types[8,9]. Although the biogenesis mechanism of exosomes has not been fully elucidated[10], recent studies have implicated that exosomes originate from the endocytotic–exogenous pathway[11]. The formation process of exosomes mainly includes the following three phases: (1) The constitution of endocytic vesicles by invagination of the plasma membrane; (2) MVBs with intracavitary vesicles are produced in the Golgi complex; and (3) Mature MVBs are fused with the plasma membrane and then released into the extracellular space as exosomes (Figure 1)[2,12-14].

Exosomes can be identified from the extracellular matrix (ECM) in almost all types of eukaryotic cells [15]. Based on the different physiochemical properties of exosomes, various separation and purification techniques have been developed[16,17]. Ultracentrifugation is the most widely used and most basic isolation method[18,19]. However, exosomes obtained by ultracentrifugation are time-consuming and low-yield and contain other vesicles, proteins, or aggregates of proteins and RNAs. Martínez-Greene *et al*[20] enhanced the production and purity of exosome preparations by combining polymer-based precipitation and size exclusion chromatography. Recently, the application of microfluidics in exosome isolation has received more attention. Wang *et al*[21] used a three-dimensional nanostructured microfluidic chip to capture exosomes. Ultrafiltration with size exclusion indicated higher yields with satisfactory purity[22]. Unfortunately, so far, no extraction/separation method is perfect.

Identification of exosomes

As a subclass of EVs, exosomes can be obtained from various cell types and extracellular media[23]. Although there is no consensus on the specific markers of EV subtypes[24], exosomes are mostly 30–200 nm in diameter and round or oval in shape[25]. Exosomes are composed of diverse molecules such as RNA, proteins and carbohydrates[26]. The proteins are composed of the transmembrane family and endosomal proteins. Various proteins in exosomes can be used as potential biomarkers, such as annexin, MVB-producing proteins such as ALIX, and tumor susceptibility gene 101 protein[27]. The tetraspanins CD9, CD81 and CD63 are well-established markers of exosomes[28].
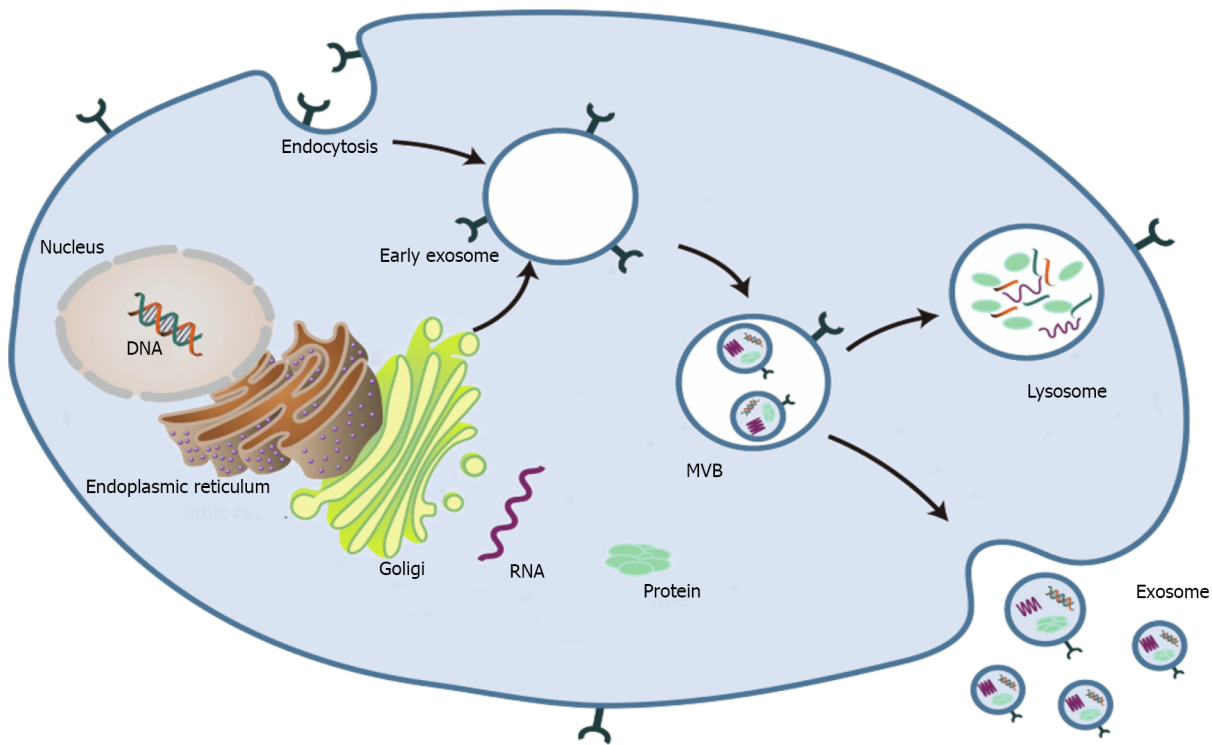
Figure 1 Schematic profile of the biogenesis of exosomes[2]. MVB: Multivesicular body. Citation: Liu Y, Wang Y, Lv Q, Li X. Exosomes: From garbage bins to translational medicine. *Int J Pharm* 2020; 583: 119333. Copyright© The Authors 2020. Published by Elsevier B.V.

Exosomes and noncoding RNA

Exosomes have been demonstrated to contain proteins and nucleic acids, and exosomes can regulate the functional activity of proteins and nucleic acids *via* transcriptional and translational regulation[29]. Currently, the genome-wide analysis has demonstrated that a significant portion (> 66%) is actively transcribed into noncoding RNAs (ncRNAs) that have functional roles in regulating the expression of protein-coding genes[30]. NcRNAs include microRNAs (miRNAs), long ncRNAs (lncRNAs) and circular RNAs (circRNAs)[31]. Length is an essential criterion for defining ncRNA. ncRNA with more than 200nt is called lncRNA and miRNA is about 20 nt and is the best-known group of small ncRNA [32]. Mature miRNA sequences are located in introns or exons of ncRNA, many of which are produced by introns (mirtron) of Pri-miRNAs (pre-mRNAs)[33]. CircRNAs range from 100 nt to over 4 kb in length and have remarkable stability due to their lack of exposed ends that are susceptible to nuclear degradation and contain single or multiple exons[34-36]. Numerous publications have indicated that exosomes are closely related to bone regeneration[37-40]. In particular, exosome-derived ncRNAs (exo-ncRNAs) have obtained extensive attention as an essential component of exosomes[41].

Bone regeneration

Bone is a dynamic tissue that remodels and regenerates itself throughout life activities[42]. Bone regeneration is a complex procedure that demands coordinating multiple cell types and biogenesis, such as osteoblasts, osteoclasts, endothelial cells, chondrocytes and mesenchymal stem cells (MSCs) (Figure 2)[43-46].

Mechanistically, intramembranous or endochondral ossification is a pathway to bone regeneration [47]. Intramembranous ossification is primarily the differentiation of stem cells into osteoblasts, which in turn deposit a mineralized ECM. The sources of stem cells mainly comprise bone marrow, fat, peripheral blood, and the umbilical cord[48]. In endochondral ossification, osteoblast progenitor cells, osteoclasts, and vascular endothelial cells enter the hypertrophic cartilage. The next step is to differentiate osteoblast progenitor cells into osteoblasts to form trabecular bone, hematopoietic cells, and endothelial cells to form bone marrow so that hypertrophic cartilage is absorbed[49]. Finally, the balance and coordination among various cells complete bone regeneration. During these processes, several signaling pathways are involved in osteogenesis, including bone morphogenetic proteins (BMPs), Notch, Hedgehog, and Wnt/β-catenin[50]. The BMP group is one of three subfamilies of the transforming growth factor (TGF) family[51]. Smad1/5 is regulated by BMP receptor complex[52]. Multiple miRNAs regulate osteogenesis by balancing bone morphogenetic protein receptor 2 (BMPR2)/Activin receptor type 2b competition for BMPR-triggered phosphorylation of Smads[53].
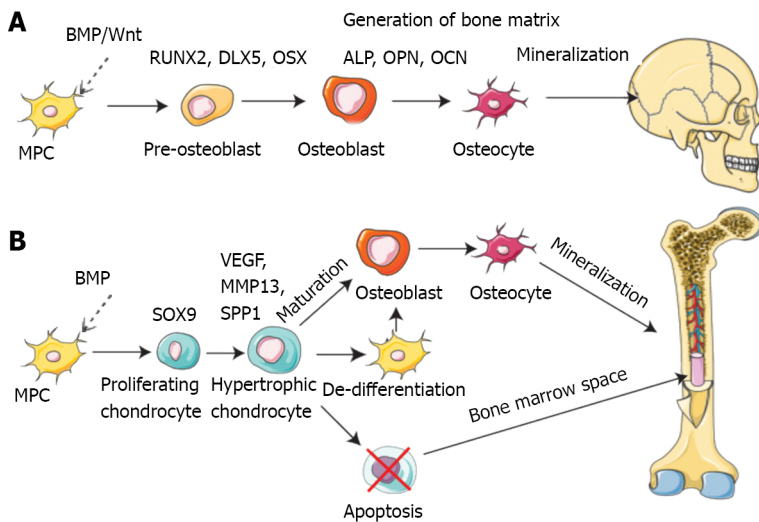
Figure 2 Pathways of bone formation during development[46]. A: Direct (intramembranous); B: Indirect (endochondral). BMP: Bone morphogenetic protein; MPC: Muscle precursor cell; VEGF: Vascular endothelial growth factor; RUNX2: Runt-related transcription factor 2; DLX5: Distal-less homeobox gene 5; ALP: Alkaline phosphatase; OPN: Osteopontin; OCN: Osteocalcin; MMP13: Matrix metallopeptidase-13. Citation: Schott NG, Friend NE, Stegemann JP. Coupling osteogenesis and vasculogenesis in engineered orthopedic tissues. *Tissue Eng Part B Rev* 2021; 27: 199-214. Copyright© The Authors 2021. Published by Mary Ann Liebert, Inc.

ROLES OF EXO-NCRNAS IN BONE REGENERATION

Roles of exo-ncRNAs in osteogenic differentiation

Osteocytes are fully mature and differentiated osteoblasts usually derived from mesenchymal cells, including bone-marrow- and adipose-derived MSCs[54]. Growing evidence indicates that ncRNAs influence MSC differentiation[55]. For example, miR-214 impedes the osteogenic differentiation of MSCs by reducing the expression of BMP2[56]. Furthermore, ncRNAs promote osteogenic differentiation in addition to their inhibitory effect. Dai *et al*[57] remarked that miR-217 improves the expression of Runt-related transcription factor (RUNX2) to promote proliferation and osteogenic differentiation of bone MSCs (BMSCs) significantly by targeting Dickkopf-1[57]. Nevertheless, little is understood about the regulatory functions of exo-ncRNAs in these procedures, and we summarize their role in osteogenic differentiation (Table 1).

Regulatory mechanisms of exosome-derived miRNAs in osteogenic differentiation

MiRNAs are 19–24 nucleotide ncRNA molecules[58] that can an regulate mRNA transcription and inhibit protein translation[59]. Exosome-derived miRNAs (exo-miRNAs) are important components of exosomes and largely determine the impact of exosomes on target cells[60]. Recent studies implicate that BMPR2 is the target gene of exo-miR-100-5p. Further research has confirmed that miR-100-5p inhibits osteogenesis of human BMSCs *via* targeting BMPR2 and inhibiting the BMPR2/smad1/5 pathway[61]. Exo-miRNA-128-3p promotes osteogenic differentiation *via* targeting Smad5[62]. Jiang *et al* [63] observed that exo-miRNA-21 inhibits osteogenesis through regulating MSC-derived exosomes pulled from osteoporosis patients *via* targeting Smad7. Additionally, exo-miR-424-5p attenuates osteogenesis *via* regulating the WIF1-mediated Wnt/β-catenin axis[64]. The CXCL12/CXCR4 axis regulates osteogenic differentiation by regulating the BMP2/Smad/Osterix axis[65,66]. Exo-miR-23a released from fibroblasts inhibits osteogenic differentiation *via* silencing CXCL12[25]. As a highly conserved signaling pathway, Wnt signaling positively affects osteogenic differentiation[67]. Previous studies have confirmed that many sirtuin family members are closely related to the Wnt signaling pathway[68,69]. Yang *et al*[70] showed that exo-miR-130a-3p promotes the osteogenic differentiation of Adipose-derived stem cells *via* inhibiting sirtuin 7[70]. Wnt activates Yes-associated protein (YAP)/transcriptional co-activator with PDZ-binding motif (TAZ), and the Hippo pathway regulates YAP[71,72].

As an essential gene in the Hippo signaling pathway, Mps One binder 1 (MOB1) regulates the expression of downstream genes, including YAP/TAZ[73]. Exo-miR-186 promotes osteogenesis by targeting MOB1 in postmenopausal osteoporosis[74]. A study has indicated that FZD4 explicitly activates the Wnt signaling pathway[75]. In contrast, exo-miR-129–5p from the jaw of diabetic rats targets FZD4 to inhibit the β-catenin signaling pathway[76]. As an E3 ubiquitin ligase, FBXW7 can inhibit osteogenic differentiation by regulating the degradation of substrates[77]. Exo-miR-101 increases osteogenic differentiation *via* inhibiting FBXW7 to control the HIF1α/FOXP3 axis[78]. As a tumor suppressor gene, PDCD4 wields antitumor activity *via* facilitating apoptosis[79]. Zhang *et al*[80]

Table 1 The role of exosome-derived ncRNA in bone regeneration

Origin of exosomes	NcRNA	Pathway	Up/down	Mechanism	Animal model	Ref.
BMSC	lncRNA H19	HOXa H10	Up	Promoted osteogenesis	Mice	[105]
HBMSC	lncRNA MALAT1	SATB2	Up	Promoted osteogenesis	Mice	[108]
MM	lncRNARUNX2-AS1	RUNX2	Up	Inhibit osteoblast differentiation	Mice	[102]
Prostate cancer cells	lncRNANEAT1	RUNX2	Up	Promoted osteogenesis	Mice	[109]
BMSC	miR-101	FBXW7	Up	Promoted osteogenesis	N/A	[78]
BMSC	miR-122-5p	SPRY2	Down	Promoted osteogenesis	Rabbit	[86]
HiPS-MSC	miR-135b	PDCD4	Down	Promoted osteogenesis	Rat	[80]
HBMSC	miR-935	STAT1	Down	Promoted osteogenesis	Rat	[87]
BMSC	miR-21	SMAD7	Down	Inhibit osteoblast differentiation	N/A	[63]
BMSC	miR-424-5p	WIF1	Down	Promoted osteogenesis	N/A	[64]
Osteoclast	miR-23a-5p	RUNX2	Down	Inhibit osteoblast differentiation	N/A	[89]
Fibroblasts	miR-23a	CXCL12	Down	Inhibit osteoblast differentiation	N/A	[25]
BMSC	miR-186	Mob1	Down	Promoted osteogenesis	Rat	[74]
hASCs	miR-375	IGFBP3	Down	Promoted osteogenesis	Rat	[83]
ADSC	miR-130a-3p	SIRT7/Wnt	Down	Promoted osteogenesis	N/A	[70]
Bone Tissues	miR-100-5p	BMPR2/smad1/5/9	Down	Inhibit osteoblast differentiation	Rat	[61]
ADSCs	miR-141-5p	KCNQ1OT1	Up	Promoted osteogenesis	N/A	[90]
Serum	circ_0006859	ROCK1	Up	Inhibit osteoblast differentiation	N/A	[118]
DPSCs	circLPAR1	SATB2	Up	Promoted osteogenesis	N/A	[115]
EPC	lncRNAMALAT1	ITGB1	Up	Promoted osteoclastogenesis	Mice	[153]
Osteoblast	circ_0008542	RANK	Up	Promoted osteoclastogenesis	Mice	[156]
ASCs	miR-378	Sufu	Up	Promoted angiogenic	Rat	[140]
BMMSC	miR-126	PI3K/Akt	Down	Promoted angiogenic	Mice	[132]
BMSC	miR-21-5p	AKT and MAPK	Up	Promoted angiogenic	Rat	[136]
HRMECs	lncRNA SNHG7	XBP1	Up	Inhibit angiogenic	N/A	[142]
BMSC	miR-126	SPRED1	Down	Promoted angiogenic	Rats	[128]
BMSC	miR-224-3p	RB1CC1	Up	Promoted angiogenic	Rats	[125]
BMSC	miR-21-5p	SPRY2	Down	Promoted angiogenic	Rats	[138]
SHED	miR-26a	TGF-β/SMAD2/3	Up	Promoted angiogenic	Mice	[131]

BMSC: Bone mesenchymal stem cell; HRMEC: Human retinal microvascular endothelial cells; BMMSC: Bone marrow mesenchymal stem cell; ASC: Atypical squamous cell; EPC: Endothelial progenitor cell; ADSC: Adipose-derived stem cell; HASC: Human adipose-derived stem cell; N/A: No animal; MM: Multiple myeloma.

demonstrated that exo-miR-135b relieves the harshness of Osteonecrosis of femoral head (ONFH) *via* diminishing the level of PDCD4-induced apoptosis of osteoblasts. Insulin-like-growth-factor-binding proteins (IGFBPs) are regulators of the functions of IGF[81]. A previous study reported that IGF-2 enhanced BMP9-induced osteogenic differentiation[82]. Recently, it has been reported that exo-miR-375 restricts the expression of IGFBP3 from plying osteogenic effects[83]. Previous studies have shown that SPRY2 inhibits the Ras/MAPK signaling pathway[84,85]. Liao *et al*[86] demonstrated that miR-122-5p

promotes osteoblast differentiation by suppressing the SPRY2 declaration and creating receptor tyrosine kinase (RTK) activity through RTK/Ras/MAPK signaling. As noted by Zhang *et al*[87], exo-miR-935 deters signal transducer and activator of transcription 1 expression and stimulates osteoblast expansion and differentiation potential. Also, upregulated exo-miR-935 alleviates osteoporosis presentation[87]. RUNX2 is concerned with the regulation of bone metabolism *via* multiple pathways[88]. Yang *et al*[89] found that osteoclast-derived exosomes including exo-miR-23a-5p inhibit osteogenic differentiation *via* abating RUNX2. There is an interaction between exo-lncRNAs and exo-miRNAs. According to one study, exo-lncRNA-KCNQ1OT1 inhibits apoptosis of primary osteoblasts by sponging miR-141-5p[90].

Regulatory mechanisms of exo-lncRNAs and circRNA in osteogenic differentiation

LncRNAs are abundant in the genome, and > 27000 have already been recognized in the human genome [91]. LncRNA can serve not only as a critical molecule in regulating bone and cartilage degeneration, promoting bone metastasis, and repairing spinal cord injury, but also as a new class of potential biomarkers and therapeutic targets for the treatment of cancer[92,93].

More importantly, lncRNAs can action as miRNAs sponges *via* binding miRNAs[94]. Furthermore, lncRNAs and miRNAs exert their biological functions by forming a large and complex regulatory network interacting with each other, leading to regulation of gene expression. LncRNA/miRNA interactions allow proper function of the musculoskeletal system, control of bone homeostasis and regeneration, and osteogenic differentiation of stem cells[95,96].

It was reported that lncRNA MEG3 could inhibit the adipogenic and osteogenic differentiation of human adipose-derived stem cells by regulating the expression of miR-140-5p[97]. Previous studies indicated that lncRNA Rmst was induced by BMP9 *via* the Smad signaling pathway. Further studies found that the lncRNA Rmst-miRNA-Notch regulatory axis could be a key mediator of BMP9-induced osteogenic differentiation of MSCs[98]. Moreover, lncPCAT1 promotes the osteogenic differentiation of PDLSCs by sponging miR-106a-5p and upregulating the expression of the miR-106a-5p-targeted gene BMP2[99]. Jia *et al*[100] have found that LINC00707 is involved in the osteogenic differentiation of BMSCs. Mechanistically, LINC00707 can sponge miR-370-3p and upregulate Wnt2B to promote the osteogenic differentiation of HBMSCs[100]. All these data demonstrate that lncRNAs can promote osteogenesis through multiple pathways. Numerous publications have indicated that the *RUNX2* gene plays a vital role in the osteogenic differentiation process[101]. Li *et al*[102] identified that myeloma-cell-derived exo-lncRNA RUNX2-AS1 could be loaded into exosomes and delivered to MSCs, thereby inhibiting the osteogenesis of MSCs[102]. Mechanistically, RUNX2-AS1 can form RNA duplexes with RUNX2 pre-mRNA, and this duplex transcriptionally suppresses RUNX2 expression *via* decreasing splicing efficiency[101,103]. Previous studies have confirmed that Hoxa10 can participate in regulating osteogenic differentiation[104]. More recently, Wang *et al*[105] demonstrated that exo-H19 can regulate the expression of Hoxa10 through competitive binding to miR-467 and promote osteogenic differentiation[105]. Moreover, exo-lncRNA-H19 stimulates osteogenesis *via* mediating Angpt1/Tie2-NO signaling in mice[106]. Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1) may act as a prognostic biomarker for lung cancer metastasis[107]. exo-lncRNA MALAT1 improves osteoblast action *via* moderating the miR-34c/SATB2 axis, which may enhance the osteogenic activity *via* functioning as a miR-34c sponge to upregulate SATB2 expression[108]. In addition, exo-lncRNA NEAT1 boosts osteogenic differentiation of human BMSCs. Mechanistically, NEAT1 upregulates RUNX2 expression through competitively binding to miR-205-5p[109].

Exosome-derived circRNAs play essential roles in osteogenic differentiation. circRNAs are endogenous covalently linked RNA molecules that do not have 5'–3'polarity or poly A tails[110]. CircRNAs have diverse roles, such as modulating translation and functioning as miRNA sponges[111]. CircRNAs are enriched in exosomes, implying the potential of circRNAs as biomarkers for complicated diseases[112]. For example, hsa-miR-31 is considered a miRNA inhibitor of osteogenic differentiation [113,114]. Xie *et al*[115] demonstrated that exo-circLPAR1 has an osteogenic effect *via* competitively binding to hsa-miR-31[115]. Also, previous studies reported Hsa_circ_0006859 expression in different diseases but did not discuss the related cell signaling[116,117]. Zhi *et al*[118] reported that exo-Hsa_circ_0006859 inhibits osteogenesis by sponging miR-431-5p to upregulate Rho-associated kinase 1 (ROCK1)[118].

Roles of exo-ncRNAs in angiogenic differentiation

Blood vessels serve as channels for transporting nutrients and oxygen[119]. There is a firm connection between the growth of blood vessels in bones and osteogenesis[120]. Bone tissue is a highly vascularized tissue, and the development of the vascular system requires a synergistic interaction between osteoblasts and angioblasts[121]. Endothelial progenitor cells (EPCs) are acknowledged to stimulate bone restoration *via* facilitating neovascularization and osteogenesis[122,123]. In line with this, endothelial cells are also implicated in the vascularization of the bone tissue[54].

Regulatory mechanisms of exo-miRNAs and lncRNAs in angiogenic differentiation

Exosomal miRNAs regulate the progression of angiogenic differentiation through diverse mechanisms. ONFH is naturally known to develop at a cellular level, inferring the value of adopting cytotherapy

[124]. Liao *et al*[86] demonstrated that BMSC-derived exosomes modified through miR-122-5p promote angiogenesis and healing.

However, there is a lack of specifically related mechanisms. According to one study, lower levels miR-224-3p promote angiogenesis of ONFH by upregulating FIP200[125]. In contrast, according to another study, exo-miR-100-5p inhibits angiogenesis of human umbilical vein endothelial cells (HUVECs) *via* targeting BMPR2[61]. Vascular endothelial growth factor (VEGF) is a critical factor in blood vessel growth and is also involved in bone development and regeneration[126]. For example, miR-126 promotes angiogenesis *via* inhibiting negative regulators of the VEGF pathway[127]. Huang *et al*[128] demonstrated that exo-miR-126 promoted angiogenesis of HUVECs by suppressing SPRED1 and PIK3R2[128]. As a member of the miRNA-26 family, miR-26a has a vital role in bone regeneration by promoting angiogenesis–osteogenesis coupling[129,130]. Wu *et al*[131] reported that exo-miR-26a stimulates angiogenesis by upregulation of the TGF-β/Smad2/3 pathway[131]. Exo-miR-126 downregulated PIK3R2 to trigger the PI3K/Akt signaling pathway in HUVECs. Further analyses in mice confirmed that exo-miR-126 improved angiogenesis in the wound site[132]. In addition, MSC exo-miRNAs promotes diabetic foot repair. exo-miRNA-210-3p can stimulate angiogenesis *via* promoting VEGF gene expression and triggering proangiogenic essential proteins[133]. However, exo-miRNA-100 inhibits angiogenesis by regulating the mTOR/HIF-1α/VEGF pathway[134]. Exosome-derived miR-let-7c promotes angiogenesis in multiple myeloma (MM)[135]. Huang *et al*[136] demonstrated that exo-miRNA-21-5p stimulates angiogenesis *via* VEGFR and AKT and MAPK pathway upregulation[136]. However, exo-miR150 inhibits HUVEC tube formation by downregulation of VEGF[137]. miR-21-5p is highly expressed in mag-BMSC-Exos and acts as a key mediator of mag-BMSC-Exo-induced regulation; mechanistically, exo-miR-21-5p boosts angiogenesis in HUVECs *via* regulating SPRY[138]. ASCs-Exos can stimulate angiogenesis and neovascularization in ischemic disease[139]. Mechanistically, miR-378-ASCs-exos not only promote osteogenic differentiation, but also increase cell migration and angiogenic capacity. miR-378-ASCs-Exos upregulate the Shh signaling pathway by targeting Sufu to enhance osteogenesis and angiogenesis[140].

Behera *et al*[106] demonstrated that exo-lnc-H19 acts as a sponge to absorb miR-106 and control the expression of Angpt1. In line with this, exosomes promotion of angiogenesis *via* Angpt1 triggers lnc-H19/Tie2-NO signaling in endothelial cells[106]. XBP1, as a primary transcription factor, has been shown to regulate protein homeostasis in cells under endoplasmic reticulum stress[141]. Cao *et al*[142] reported that exo-lncRNA SNHG7 inhibited tube formation of human retinal microvascular endothelial cells *via* regulating the miR-34a-5p/XBP1 signal pathway[142].

Roles of exo-ncRNAs in osteoclast differentiation

Osteoclasts are generated by monocyte–macrophage precursors of the hematopoietic lineage in the bone marrow[143]. The role of osteoclasts is to remove the organic and inorganic parts of bone, and is crucial to healthy bone function[144]. miR-124 reduces the proliferation of osteoclast precursors and negatively regulates osteoclastogenesis[145]. miR-214 and miR-21 promote osteoclastogenesis *via* targeting the PTEN/PI3K/AKT pathway[146]. Although the regulation of osteoclasts by ncRNA is currently comprehended, there have been few studies on the role of exo-ncRNA in osteoclast differentiation.

Regulatory mechanisms of exo-ncRNAs in osteoclast differentiation

Signaling between osteoblasts and osteoclasts is vital for osteoclast maturation[147]. miRNAs play paramount functions in the post□transcriptional control of gene expression[148]. miRNAs induce the translational repression or degradation of their target genes *via* binding to the complementary sequences in the 3′-UTRs of their marker mRNAs[149]. Xu *et al*[150] investigated the effects of osteoclast-secreted exo-lncRNAs on osteogenesis in the process of particle-induced osteolysis. The results showed that miR-214 levels in exosomes were significantly up-regulated in osteoclast-specific miR-214 transgenic mice. Further studies have found that in ovariectomized mice, preventing exosome formation by downregulating Rab27a increased osteoblast activity. Taken together, osteoclast-derived exosomes transferred miR-214 into osteoblasts to suppress their activity[151]. Coculture systems of osteoblasts and osteoclasts to simulate bone regeneration have been reviewed by Borciani *et al*[152]. Cui *et al*[153] reported that EPC-derived exosomes stimulated osteoclastogenesis *via* the lncRNA-MALAT1/miR124 pathway. Exo-lncRNA-MALAT1 can negatively control miR-124 activity. Furthermore, there was a negative correlation between miR-124 mRNA and ITGB1. They also indicated that EPC-derived exosomes increased neovascularization in a mouse femoral fracture model. CircRNAs were discovered as ncRNAs with covalently closed structures, and they regulate disease occurrence and development[154]. m6A methylation is an ordinary state of RNA methylation, and it participates in and regulates many vital functions of RNA[155]. circ_0008542 in osteoblast exosomes enables osteoclast-induced bone resorption *via* m6A methylation[156].

NcRNA includes not only several types that have been introduced above but also other types. For example, tRNA-derived small RNAs (tsRNAs) are a recently discovered form of ncRNA[157,158]. TsRNAs participate in translation inhibition and exert control in various physiological phenomena [159]. Fang *et al*[160] reported that tsRNA-10277-loaded BMSC exosomes improved osteogenic differentiation capacity of dexamethasone-induced BMSCs[160]. In addition, the ECM plays a significant role in bone repair and regeneration[161]. Hyaluronic acid (HA) exists naturally as a critical component of the

ECM. Zhai *et al*[162] focused on the recent applications of HA in bone regeneration. Recently, the emergence of decellularized ECM scaffolds have been studied in bone regeneration[163]. Decellularized ECM scaffolds promote osteogenic differentiation of stem cells and maintain cytokines that regulate bone regeneration[164,165].

CONCLUSION

Opportunities
Over the past few decades, due to the development of genetic engineering, the surface of exosomes has been packed with inhibitors of ncRNA and marker molecules by modifying the isolation and purification of exosomes. Researchers have delivered targeted ncRNAs to designated tissues or organs through exosome carriers.

Different research methods can have multiple effects. The most common delivery of miRNAs to target cells is *via* exosomes or liposomes[166]. Tahmasebi *et al*[167] proposed novel tissue-engineering methods premised upon miRNA-incorporated polycaprolactone nanofibers in treating bone lesions and defects. It is well established that ECs and MSCs are critical performers in orthopedic tissue regeneration and vascularization. Coculture studies have demonstrated that ECs and MSCs have synergistic effects on tissue regeneration[46,168].

Hypoxic preconditioning of MSCs can enhance their biological functions[169]. In fact, hypoxic MSCs are close to the *in vivo* environment[170]. Liu *et al*[171] showed that hypoxia enhanced the production of exo-miR-126, which further promoted fracture healing[171]. Liu *et al*[172] explored biomaterial-mediated chemical signaling through a model lithium-binding bioactive glass-ceramic (Li-BGC). Mechanistically, Li-BGC-exo transfers proangiogenic miR-130a and in turn, promotes the angiogenesis of ECs *via* activating the AKT pathway[172]. Liu *et al*[173] previously reported that knee loading protects against osteonecrosis of the femoral head *via* enhancing vessel remodeling[173]. Knee loading stimulates type H vessel formation and promotes angiogenesis *via* downregulating exo-miR-214–3p [174].

In diagnostics, numerous studies have found differential expression of exo-ncRNAs in various diseases[175,176]. It implies that exo-ncRNAs have advantages as biomarkers over non-exo-ncRNAs. Meanwhile, exo-ncRNAs are concerned with bone regeneration processes like cell proliferation, migration, and angiogenesis. Exo-ncRNAs promote intercellular and intertissue crosstalk in a paracrine and autocrine manner, leading to multiple applications in diseases like femoral head necrosis, bone defects, and osteoporosis.

In therapeutics, upregulation or downregulation of exo-ncRNAs may have different clinical consequences. For instance, Lv *et al*[177] utilized electroporation for packaging miR-21-5p mimic into exosomes. The study indicated that the miR-21-5p promotes angiogenesis and vessel maturation[177]. Additionally, overexpressed exo-miR-122-5p weakens ONFH aggravation[87]. There may also be therapeutic effects by decreasing the number of harmful ncRNAs in exosomes. In addition, the use of exo-ncRNAs has multiple potential benefits. Exosomes holding a characteristic cargo can function as a drug delivery system. The use of exosomes as endogenous vehicles can evade the immune response. Despite the great potential of exo-ncRNAs as biomarkers and therapeutics for bone regeneration, there are still many obstacles before their clinical application.

Challenges
The investigations and clinical transformation of exo-ncRNAs in bone regeneration have exposed several challenges. First, we need to explore more efficient exosome purification methods to exclude exogenous exosomes and RNA interference. Exosomes, microvesicles, and smaller vesicles are different but still disorganized[178,179]. In addition, according to the existing technology, there is still a lack of efficient and fast methods for extracting and isolating exosomes. Second, further study is needed on the pharmacokinetics and toxicity of potential exo-ncRNAs. Expansion of exosome production by increasing intracellular calcium concentration and serum starvation or transfer of the oncogene c-myc may alter exosome content (including ncRNA) and increase tumorigenic potential[180,181]. Third, an extensive study is needed to comprehend fully the mechanism by which exo-ncRNAs exert their physiological roles. Fourth, further investigations are demanded in the future to characterize whether miRNAs, circRNAs, lncRNAs, and other ncRNAs may form competing endogenous RNA networks. Hence, if we better understand the bioactive molecules' exact mechanism, we can improve bone tissue regeneration. Fifth, the expression profile of miRNAs is altered with age. For example, miR-183-5p increases with aging, suppresses osteogenic differentiation in BMSCs, and reduces Hmox1 levels[182]. Sixth, most of the current research focuses on cellular experiments and a small number of animal models. It is urgent to use large-scale animal and clinical models to conduct investigations to determine whether exo-ncRNAs can play a role in regulating homeostasis. Overall, the transition of exo-ncRNAs from basic laboratory studies to clinical application remains challenging. Nonetheless, these studies provide renewed approaches for potential clinical diagnosis and therapeutic direction for exosome-mediated human diseases.

FOOTNOTES

Country/Territory of origin: China

ORCID number: Yuan-Zhong Ren 0000-0003-0020-4006; Shan-Shan Ding 0000-0003-4870-283X; Ya-Ping Jiang 0000-0002-6614-0079; Hui Wen 0000-0003-0306-1557; Tao Li 0000-0002-0698-2960.

REFERENCES

1 **Wolf P**. The nature and significance of platelet products in human plasma. *Br J Haematol* 1967; **13**: 269-288 [PMID: 6025241 DOI: 10.1111/j.1365-2141.1967.tb08741.x]

2 **Liu Y**, Wang Y, Lv Q, Li X. Exosomes: From garbage bins to translational medicine. *Int J Pharm* 2020; **583**: 119333 [PMID: 32348800 DOI: 10.1016/j.ijpharm.2020.119333]

3 **McBride JD**, Rodriguez-Menocal L, Badiavas EV. Extracellular Vesicles as Biomarkers and Therapeutics in Dermatology: A Focus on Exosomes. *J Invest Dermatol* 2017; **137**: 1622-1629 [PMID: 28648952 DOI: 10.1016/j.jid.2017.04.021]

4 **Chen JH**, Wu ATH, Bamodu OA, Yadav VK, Chao TY, Tzeng YM, Mukhopadhyay D, Hsiao M, Lee JC. Ovatodiolide Suppresses Oral Cancer Malignancy by Down-Regulating Exosomal Mir-21/STAT3/β-Catenin Cargo and Preventing Oncogenic Transformation of Normal Gingival Fibroblasts. *Cancers (Basel)* 2019; **12** [PMID: 31878245 DOI: 10.3390/cancers12010056]

5 **Harding C**, Heuser J, Stahl P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J Cell Biol* 1983; **97**: 329-339 [PMID: 6309857 DOI: 10.1083/jcb.97.2.329]

6 **Johnstone RM**, Bianchini A, Teng K. Reticulocyte maturation and exosome release: transferrin receptor containing exosomes shows multiple plasma membrane functions. *Blood* 1989; **74**: 1844-1851 [PMID: 2790208]

7 **Han C**, Sun X, Liu L, Jiang H, Shen Y, Xu X, Li J, Zhang G, Huang J, Lin Z, Xiong N, Wang T. Exosomes and Their Therapeutic Potentials of Stem Cells. *Stem Cells Int* 2016; **2016**: 7653489 [PMID: 26770213 DOI: 10.1155/2016/7653489]

8 **Mostafazadeh M**, Samadi N, Kahroba H, Baradaran B, Haiaty S, Nouri M. Potential roles and prognostic significance of exosomes in cancer drug resistance. *Cell Biosci* 2021; **11**: 1 [PMID: 33407894 DOI: 10.1186/s13578-020-00515-y]

9 **Zhang Y**, Liu Y, Liu H, Tang WH. Exosomes: biogenesis, biologic function and clinical potential. *Cell Biosci* 2019; **9**: 19 [PMID: 30815248 DOI: 10.1186/s13578-019-0282-2]

10 **Wang X**, Thomsen P. Mesenchymal stem cell-derived small extracellular vesicles and bone regeneration. *Basic Clin Pharmacol Toxicol* 2021; **128**: 18-36 [PMID: 32780530 DOI: 10.1111/bcpt.13478]

11 **Ma ZJ**, Yang JJ, Lu YB, Liu ZY, Wang XX. Mesenchymal stem cell-derived exosomes: Toward cell-free therapeutic strategies in regenerative medicine. *World J Stem Cells* 2020; **12**: 814-840 [PMID: 32952861 DOI: 10.4252/wjsc.v12.i8.814]

12 **Feng ZY**, Zhang QY, Tan J, Xie HQ. Techniques for increasing the yield of stem cell-derived exosomes: what factors may be involved? *Sci China Life Sci* 2021 [PMID: 34637101 DOI: 10.1007/s11427-021-1997-2]

13 **Gurunathan S**, Kang MH, Jeyaraj M, Qasim M, Kim JH. Review of the Isolation, Characterization, Biological Function, and Multifarious Therapeutic Approaches of Exosomes. *Cells* 2019; **8** [PMID: 30987213 DOI: 10.3390/cells8040307]

14 **Kalluri R**, LeBleu VS. The biology, function, and biomedical applications of exosomes. *Science* 2020; **367** [PMID: 32029601 DOI: 10.1126/science.aau6977]

15 **Zhao Y**, Li X, Zhang W, Yu L, Wang Y, Deng Z, Liu M, Mo S, Wang R, Zhao J, Liu S, Hao Y, Wang X, Ji T, Zhang L, Wang C. Trends in the biological functions and medical applications of extracellular vesicles and analogues. *Acta Pharm Sin B* 2021; **11**: 2114-2135 [PMID: 34522580 DOI: 10.1016/j.apsb.2021.03.012]

16 **Yang D**, Zhang W, Zhang H, Zhang F, Chen L, Ma L, Larcher LM, Chen S, Liu N, Zhao Q, Tran PHL, Chen C, Veedu RN, Wang T. Progress, opportunity, and perspective on exosome isolation - efforts for efficient exosome-based

theranostics. *Theranostics* 2020; **10**: 3684-3707 [PMID: 32206116 DOI: 10.7150/thno.41580]

17   **Alzhrani GN**, Alanazi ST, Alsharif SY, Albalawi AM, Alsharif AA, Abdel-Maksoud MS, Elsherbiny N. Exosomes: Isolation, characterization, and biomedical applications. *Cell Biol Int* 2021; **45**: 1807-1831 [PMID: 33913604 DOI: 10.1002/cbin.11620]

18   **Livshits MA**, Khomyakova E, Evtushenko EG, Lazarev VN, Kulemin NA, Semina SE, Generozov EV, Govorun VM. Isolation of exosomes by differential centrifugation: Theoretical analysis of a commonly used protocol. *Sci Rep* 2015; **5**: 17319 [PMID: 26616523 DOI: 10.1038/srep17319]

19   **Gross JC**, Chaudhary V, Bartscherer K, Boutros M. Active Wnt proteins are secreted on exosomes. *Nat Cell Biol* 2012; **14**: 1036-1045 [PMID: 22983114 DOI: 10.1038/ncb2574]

20   **Martínez-Greene JA**, Hernández-Ortega K, Quiroz-Baez R, Resendis-Antonio O, Pichardo-Casas I, Sinclair DA, Budnik B, Hidalgo-Miranda A, Uribe-Querol E, Ramos-Godínez MDP, Martínez-Martínez E. Quantitative proteomic analysis of extracellular vesicle subgroups isolated by an optimized method combining polymer-based precipitation and size exclusion chromatography. *J Extracell Vesicles* 2021; **10**: e12087 [PMID: 33936570 DOI: 10.1002/jev2.12087]

21   **Wang J**, Li W, Zhang L, Ban L, Chen P, Du W, Feng X, Liu BF. Chemically Edited Exosomes with Dual Ligand Purified by Microfluidic Device for Active Targeted Drug Delivery to Tumor Cells. *ACS Appl Mater Interfaces* 2017; **9**: 27441-27452 [PMID: 28762264 DOI: 10.1021/acsami.7b06464]

22   **Gao M**, Cai J, Zitkovsky HS, Chen B, Guo L. Comparison of Yield, Purity, and Functional Properties of Large-Volume Exosome Isolation Using Ultrafiltration and Polymer-Based Precipitation. *Plast Reconstr Surg* 2022; **149**: 638-649 [PMID: 35196679 DOI: 10.1097/PRS.0000000000008830]

23   **van der Pol E**, Böing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol Rev* 2012; **64**: 676-705 [PMID: 22722893 DOI: 10.1124/pr.112.005983]

24   **Théry C**, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A, Arab T, Archer F, Atkin-Smith GK, Ayre DC, Bach JM, Bachurski D, Baharvand H, Balaj L, Baldacchino S, Bauer NN, Baxter AA, Bebawy M, Beckham C, Bedina Zavec A, Benmoussa A, Berardi AC, Bergese P, Bielska E, Blenkiron C, Bobis-Wozowicz S, Boilard E, Boireau W, Bongiovanni A, Borràs FE, Bosch S, Boulanger CM, Breakefield X, Breglio AM, Brennan MÁ, Brigstock DR, Brisson A, Broekman ML, Bromberg JF, Bryl-Górecka P, Buch S, Buck AH, Burger D, Busatto S, Buschmann D, Bussolati B, Buzás EI, Byrd JB, Camussi G, Carter DR, Caruso S, Chamley LW, Chang YT, Chen C, Chen S, Cheng L, Chin AR, Clayton A, Clerici SP, Cocks A, Cocucci E, Coffey RJ, Cordeiro-da-Silva A, Couch Y, Coumans FA, Coyle B, Crescitelli R, Criado MF, D'Souza-Schorey C, Das S, Datta Chaudhuri A, de Candia P, De Santana EF, De Wever O, Del Portillo HA, Demaret T, Deville S, Devitt A, Dhondt B, Di Vizio D, Dieterich LC, Dolo V, Dominguez Rubio AP, Dominici M, Dourado MR, Driedonks TA, Duarte FV, Duncan HM, Eichenberger RM, Ekström K, El Andaloussi S, Elie-Caille C, Erdbrügger U, Falcón-Pérez JM, Fatima F, Fish JE, Flores-Bellver M, Försönits A, Frelet-Barrand A, Fricke F, Fuhrmann G, Gabrielsson S, Gámez-Valero A, Gardiner C, Gärtner K, Gaudin R, Gho YS, Giebel B, Gilbert C, Gimona M, Giusti I, Goberdhan DC, Görgens A, Gorski SM, Greening DW, Gross JC, Gualerzi A, Gupta GN, Gustafson D, Handberg A, Haraszti RA, Harrison P, Hegyesi H, Hendrix A, Hill AF, Hochberg FH, Hoffmann KF, Holder B, Holthofer H, Hosseinkhani B, Hu G, Huang Y, Huber V, Hunt S, Ibrahim AG, Ikezu T, Inal JM, Isin M, Ivanova A, Jackson HK, Jacobsen S, Jay SM, Jayachandran M, Jenster G, Jiang L, Johnson SM, Jones JC, Jong A, Jovanovic-Talisman T, Jung S, Kalluri R, Kano SI, Kaur S, Kawamura Y, Keller ET, Khamari D, Khomyakova E, Khvorova A, Kierulf P, Kim KP, Kislinger T, Klingeborn M, Klinke DJ 2nd, Kornek M, Kosanović MM, Kovács ÁF, Krämer-Albers EM, Krasemann S, Krause M, Kurochkin IV, Kusuma GD, Kuypers S, Laitinen S, Langevin SM, Languino LR, Lannigan J, Lässer C, Laurent LC, Lavieu G, Lázaro-Ibáñez E, Le Lay S, Lee MS, Lee YXF, Lemos DS, Lenassi M, Leszczynska A, Li IT, Liao K, Libregts SF, Ligeti E, Lim R, Lim SK, Linē A, Linnemannstöns K, Llorente A, Lombard CA, Lorenowicz MJ, Lörincz ÁM, Lötvall J, Lovett J, Lowry MC, Loyer X, Lu Q, Lukomska B, Lunavat TR, Maas SL, Malhi H, Marcilla A, Mariani J, Mariscal J, Martens-Uzunova ES, Martin-Jaular L, Martinez MC, Martins VR, Mathieu M, Mathivanan S, Maugeri M, McGinnis LK, McVey MJ, Meckes DG Jr, Meehan KL, Mertens I, Minciacchi VR, Möller A, Møller Jørgensen M, Morales-Kastresana A, Morhayim J, Mullier F, Muraca M, Musante L, Mussack V, Muth DC, Myburgh KH, Najrana T, Nawaz M, Nazarenko I, Nejsum P, Neri C, Neri T, Nieuwland R, Nimrichter L, Nolan JP, Nolte-'t Hoen EN, Noren Hooten N, O'Driscoll L, O'Grady T, O'Loghlen A, Ochiya T, Olivier M, Ortiz A, Ortiz LA, Osteikoetxea X, Østergaard O, Ostrowski M, Park J, Pegtel DM, Peinado H, Perut F, Pfaffl MW, Phinney DG, Pieters BC, Pink RC, Pisetsky DS, Pogge von Strandmann E, Polakovicova I, Poon IK, Powell BH, Prada I, Pulliam L, Quesenberry P, Radeghieri A, Raffai RL, Raimondo S, Rak J, Ramirez MI, Raposo G, Rayyan MS, Regev-Rudzki N, Ricklefs FL, Robbins PD, Roberts DD, Rodrigues SC, Rohde E, Rome S, Rouschop KM, Rughetti A, Russell AE, Saá P, Sahoo S, Salas-Huenuleo E, Sánchez C, Saugstad JA, Saul MJ, Schiffelers RM, Schneider R, Schøyen TH, Scott A, Shahaj E, Sharma S, Shatnyeva O, Shekari F, Shelke GV, Shetty AK, Shiba K, Siljander PR, Silva AM, Skowronek A, Snyder OL 2nd, Soares RP, Sódar BW, Soekmadji C, Sotillo J, Stahl PD, Stoorvogel W, Stott SL, Strasser EF, Swift S, Tahara H, Tewari M, Timms K, Tiwari S, Tixeira R, Tkach M, Toh WS, Tomasini R, Torrecilhas AC, Tosar JP, Toxavidis V, Urbanelli L, Vader P, van Balkom BW, van der Grein SG, Van Deun J, van Herwijnen MJ, Van Keuren-Jensen K, van Niel G, van Royen ME, van Wijnen AJ, Vasconcelos MH, Vechetti IJ Jr, Veit TD, Vella LJ, Velot É, Verweij FJ, Vestad B, Viñas JL, Visnovitz T, Vukman KV, Wahlgren J, Watson DC, Wauben MH, Weaver A, Webber JP, Weber V, Wehman AM, Weiss DJ, Welsh JA, Wendt S, Wheelock AM, Wiener Z, Witte L, Wolfram J, Xagorari A, Xander P, Xu J, Yan X, Yáñez-Mó M, Yin H, Yuana Y, Zappulli V, Zarubova J, Žėkas V, Zhang JY, Zhao Z, Zheng L, Zheutlin AR, Zickler AM, Zimmermann P, Zivkovic AM, Zocco D, Zuba-Surma EK. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 2018; **7**: 1535750 [PMID: 30637094 DOI: 10.1080/20013078.2018.1535750]

25   **Zhuang XM**, Zhou B. Exosome secreted by human gingival fibroblasts in radiation therapy inhibits osteogenic differentiation of bone mesenchymal stem cells by transferring miR-23a. *Biomed Pharmacother* 2020; **131**: 110672 [PMID: 32889404 DOI: 10.1016/j.biopha.2020.110672]

26   **Cheng H**, Yang Q, Wang R, Luo R, Zhu S, Li M, Li W, Chen C, Zou Y, Huang Z, Xie T, Wang S, Zhang H, Tian Q. Emerging Advances of Detection Strategies for Tumor-Derived Exosomes. *Int J Mol Sci* 2022; **23** [PMID: 35055057 DOI:

10.3390/ijms23020868]

27   **Liu J**, Ren L, Li S, Li W, Zheng X, Yang Y, Fu W, Yi J, Wang J, Du G. The biology, function, and applications of exosomes in cancer. *Acta Pharm Sin B* 2021; **11**: 2783-2797 [PMID: 34589397 DOI: 10.1016/j.apsb.2021.01.001]

28   **Larios J**, Mercier V, Roux A, Gruenberg J. ALIX- and ESCRT-III-dependent sorting of tetraspanins to exosomes. *J Cell Biol* 2020; **219** [PMID: 32049272 DOI: 10.1083/jcb.201904113]

29   **Corrado C**, Barreca MM, Zichittella C, Alessandro R, Conigliaro A. Molecular Mediators of RNA Loading into Extracellular Vesicles. *Cells* 2021; **10** [PMID: 34943863 DOI: 10.3390/cells10123355]

30   **Yang C**, Zhang Y, Yang B. MIAT, a potent CVD-promoting lncRNA. *Cell Mol Life Sci* 2021; **79**: 43 [PMID: 34921634 DOI: 10.1007/s00018-021-04046-8]

31   **Hulshoff MS**, Del Monte-Nieto G, Kovacic J, Krenning G. Non-coding RNA in endothelial-to-mesenchymal transition. *Cardiovasc Res* 2019; **115**: 1716-1731 [PMID: 31504268 DOI: 10.1093/cvr/cvz211]

32   **Beermann J**, Piccoli MT, Viereck J, Thum T. Non-coding RNAs in Development and Disease: Background, Mechanisms, and Therapeutic Approaches. *Physiol Rev* 2016; **96**: 1297-1325 [PMID: 27535639 DOI: 10.1152/physrev.00041.2015]

33   **Saliminejad K**, Khorram Khorshid HR, Soleymani Fard S, Ghaffari SH. An overview of microRNAs: Biology, functions, therapeutics, and analysis methods. *J Cell Physiol* 2019; **234**: 5451-5465 [PMID: 30471116 DOI: 10.1002/jcp.27486]

34   **Jeck WR**, Sorrentino JA, Wang K, Slevin MK, Burd CE, Liu J, Marzluff WF, Sharpless NE. Circular RNAs are abundant, conserved, and associated with ALU repeats. *RNA* 2013; **19**: 141-157 [PMID: 23249747 DOI: 10.1261/rna.035667.112]

35   **Zhang Y**, Zhang XO, Chen T, Xiang JF, Yin QF, Xing YH, Zhu S, Yang L, Chen LL. Circular intronic long noncoding RNAs. *Mol Cell* 2013; **51**: 792-806 [PMID: 24035497 DOI: 10.1016/j.molcel.2013.08.017]

36   **Quinn JJ**, Chang HY. Unique features of long non-coding RNA biogenesis and function. *Nat Rev Genet* 2016; **17**: 47-62 [PMID: 26666209 DOI: 10.1038/nrg.2015.10]

37   **Shan SK**, Lin X, Li F, Xu F, Zhong JY, Guo B, Wang Y, Zheng MH, Wu F, Yuan LQ. Exosomes and Bone Disease. *Curr Pharm Des* 2019; **25**: 4536-4549 [PMID: 31775592 DOI: 10.2174/1381612825666191127114054]

38   **Zhai M**, Zhu Y, Yang M, Mao C. Human Mesenchymal Stem Cell Derived Exosomes Enhance Cell-Free Bone Regeneration by Altering Their miRNAs Profiles. *Adv Sci (Weinh)* 2020; **7**: 2001334 [PMID: 33042751 DOI: 10.1002/advs.202001334]

39   **Luo ZW**, Li FX, Liu YW, Rao SS, Yin H, Huang J, Chen CY, Hu Y, Zhang Y, Tan YJ, Yuan LQ, Chen TH, Liu HM, Cao J, Liu ZZ, Wang ZX, Xie H. Aptamer-functionalized exosomes from bone marrow stromal cells target bone to promote bone regeneration. *Nanoscale* 2019; **11**: 20884-20892 [PMID: 31660556 DOI: 10.1039/c9nr02791b]

40   **Al-Sowayan B**, Alammari F, Alshareeda A. Preparing the Bone Tissue Regeneration Ground by Exosomes: From Diagnosis to Therapy. *Molecules* 2020; **25** [PMID: 32937850 DOI: 10.3390/molecules25184205]

41   **Li C**, Ni YQ, Xu H, Xiang QY, Zhao Y, Zhan JK, He JY, Li S, Liu YS. Roles and mechanisms of exosomal non-coding RNAs in human health and diseases. *Signal Transduct Target Ther* 2021; **6**: 383 [PMID: 34753929 DOI: 10.1038/s41392-021-00779-x]

42   **Shang F**, Yu Y, Liu S, Ming L, Zhang Y, Zhou Z, Zhao J, Jin Y. Advancing application of mesenchymal stem cell-based bone tissue regeneration. *Bioact Mater* 2021; **6**: 666-683 [PMID: 33005830 DOI: 10.1016/j.bioactmat.2020.08.014]

43   **Pant T**, Juric M, Bosnjak ZJ, Dhanasekaran A. Recent Insight on the Non-coding RNAs in Mesenchymal Stem Cell-Derived Exosomes: Regulatory and Therapeutic Role in Regenerative Medicine and Tissue Engineering. *Front Cardiovasc Med* 2021; **8**: 737512 [PMID: 34660740 DOI: 10.3389/fcvm.2021.737512]

44   **Malekpour K**, Hazrati A, Zahar M, Markov A, Zekiy AO, Navashenaq JG, Roshangar L, Ahmadi M. The Potential Use of Mesenchymal Stem Cells and Their Derived Exosomes for Orthopedic Diseases Treatment. *Stem Cell Rev Rep* 2022; **18**: 933-951 [PMID: 34169411 DOI: 10.1007/s12015-021-10185-z]

45   **Tan SHS**, Wong JRY, Sim SJY, Tjio CKE, Wong KL, Chew JRJ, Hui JHP, Toh WS. Mesenchymal stem cell exosomes in bone regenerative strategies-a systematic review of preclinical studies. *Mater Today Bio* 2020; **7**: 100067 [PMID: 32695985 DOI: 10.1016/j.mtbio.2020.100067]

46   **Schott NG**, Friend NE, Stegemann JP. Coupling Osteogenesis and Vasculogenesis in Engineered Orthopedic Tissues. *Tissue Eng Part B Rev* 2021; **27**: 199-214 [PMID: 32854589 DOI: 10.1089/ten.TEB.2020.0132]

47   **Pajarinen J**, Lin T, Gibon E, Kohno Y, Maruyama M, Nathan K, Lu L, Yao Z, Goodman SB. Mesenchymal stem cell-macrophage crosstalk and bone healing. *Biomaterials* 2019; **196**: 80-89 [PMID: 29329642 DOI: 10.1016/j.biomaterials.2017.12.025]

48   **Brown C**, McKee C, Bakshi S, Walker K, Hakman E, Halassy S, Svinarich D, Dodds R, Govind CK, Chaudhry GR. Mesenchymal stem cells: Cell therapy and regeneration potential. *J Tissue Eng Regen Med* 2019; **13**: 1738-1755 [PMID: 31216380 DOI: 10.1002/term.2914]

49   **Maes C**, Kobayashi T, Selig MK, Torrekens S, Roth SI, Mackem S, Carmeliet G, Kronenberg HM. Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. *Dev Cell* 2010; **19**: 329-344 [PMID: 20708594 DOI: 10.1016/j.devcel.2010.07.010]

50   **Thomas S**, Jaganathan BG. Signaling network regulating osteogenesis in mesenchymal stem cells. *J Cell Commun Signal* 2022; **16**: 47-61 [PMID: 34236594 DOI: 10.1007/s12079-021-00635-1]

51   **Miyazono K**, Kamiya Y, Morikawa M. Bone morphogenetic protein receptors and signal transduction. *J Biochem* 2010; **147**: 35-51 [PMID: 19762341 DOI: 10.1093/jb/mvp148]

52   **Massagué J**. TGFβ signalling in context. *Nat Rev Mol Cell Biol* 2012; **13**: 616-630 [PMID: 22992590 DOI: 10.1038/nrm3434]

53   **Liu A**, Lin D, Zhao H, Chen L, Cai B, Lin K, Shen SG. Optimized BMSC-derived osteoinductive exosomes immobilized in hierarchical scaffold *via* lyophilization for bone repair through Bmpr2/Acvr2b competitive receptor-activated Smad pathway. *Biomaterials* 2021; **272**: 120718 [PMID: 33838528 DOI: 10.1016/j.biomaterials.2021.120718]

54   **Lee J**, Byun H, Madhurakkat Perikamana SK, Lee S, Shin H. Current Advances in Immunomodulatory Biomaterials for Bone Regeneration. *Adv Healthc Mater* 2019; **8**: e1801106 [PMID: 30328293 DOI: 10.1002/adhm.201801106]

55   **Mazziotta C**, Lanzillotti C, Iaquinta MR, Taraballi F, Torreggiani E, Rotondo JC, Otòn-Gonzalez L, Mazzoni E, Frontini

F, Bononi I, De Mattei M, Tognon M, Martini F. MicroRNAs Modulate Signaling Pathways in Osteogenic Differentiation of Mesenchymal Stem Cells. *Int J Mol Sci* 2021; **22** [PMID: 33673409 DOI: 10.3390/ijms22052362]

56   **Wang CG**, Liao Z, Xiao H, Liu H, Hu YH, Liao QD, Zhong D. LncRNA KCNQ1OT1 promoted BMP2 expression to regulate osteogenic differentiation by sponging miRNA-214. *Exp Mol Pathol* 2019; **107**: 77-84 [PMID: 30703347 DOI: 10.1016/j.yexmp.2019.01.012]

57   **Dai Z**, Jin Y, Zheng J, Liu K, Zhao J, Zhang S, Wu F, Sun Z. MiR-217 promotes cell proliferation and osteogenic differentiation of BMSCs by targeting DKK1 in steroid-associated osteonecrosis. *Biomed Pharmacother* 2019; **109**: 1112-1119 [PMID: 30551361 DOI: 10.1016/j.biopha.2018.10.166]

58   **Khan IN**, Ullah N, Hussein D, Saini KS. Current and emerging biomarkers in tumors of the central nervous system: Possible diagnostic, prognostic and therapeutic applications. *Semin Cancer Biol* 2018; **52**: 85-102 [PMID: 28774835 DOI: 10.1016/j.semcancer.2017.07.004]

59   **Tingö L**, Ahlberg E, Johansson L, Pedersen SA, Chawla K, Sætrom P, Cione E, Simpson MR. Non-Coding RNAs in Human Breast Milk: A Systematic Review. *Front Immunol* 2021; **12**: 725323 [PMID: 34539664 DOI: 10.3389/fimmu.2021.725323]

60   **Alcayaga-Miranda F**, Varas-Godoy M, Khoury M. Harnessing the Angiogenic Potential of Stem Cell-Derived Exosomes for Vascular Regeneration. *Stem Cells Int* 2016; **2016**: 3409169 [PMID: 27127516 DOI: 10.1155/2016/3409169]

61   **Yang W**, Zhu W, Yang Y, Guo M, Qian H, Jiang W, Chen Y, Lian C, Xu Z, Bai H, Chen T, Zhang J. Exosomal miR-100-5p inhibits osteogenesis of hBMSCs and angiogenesis of HUVECs by suppressing the BMPR2/Smad1/5/9 signalling pathway. *Stem Cell Res Ther* 2021; **12**: 390 [PMID: 34256859 DOI: 10.1186/s13287-021-02438-y]

62   **Xu T**, Luo Y, Wang J, Zhang N, Gu C, Li L, Qian D, Cai W, Fan J, Yin G. Exosomal miRNA-128-3p from mesenchymal stem cells of aged rats regulates osteogenesis and bone fracture healing by targeting Smad5. *J Nanobiotechnology* 2020; **18**: 47 [PMID: 32178675 DOI: 10.1186/s12951-020-00601-w]

63   **Jiang LB**, Tian L, Zhang CG. Bone marrow stem cells-derived exosomes extracted from osteoporosis patients inhibit osteogenesis *via* microRNA-21/SMAD7. *Eur Rev Med Pharmacol Sci* 2018; **22**: 6221-6229 [PMID: 30338786 DOI: 10.26355/eurrev_201810_16028]

64   **Wei Y**, Ma H, Zhou H, Yin H, Yang J, Song Y, Yang B. miR-424-5p shuttled by bone marrow stem cells-derived exosomes attenuates osteogenesis *via* regulating WIF1-mediated Wnt/β-catenin axis. *Aging (Albany NY)* 2021; **13**: 17190-17201 [PMID: 34229300 DOI: 10.18632/aging.203169]

65   **Li Z**, Wang W, Xu H, Ning Y, Fang W, Liao W, Zou J, Yang Y, Shao N. Effects of altered CXCL12/CXCR4 axis on BMP2/Smad/Runx2/Osterix axis and osteogenic gene expressions during osteogenic differentiation of MSCs. *Am J Transl Res* 2017; **9**: 1680-1693 [PMID: 28469774]

66   **Zhuang XM**, Zhou B, Yuan KF. Role of p53 mediated miR-23a/CXCL12 pathway in osteogenic differentiation of bone mesenchymal stem cells on nanostructured titanium surfaces. *Biomed Pharmacother* 2019; **112**: 108649 [PMID: 30784930 DOI: 10.1016/j.biopha.2019.108649]

67   **Sethi JK**, Vidal-Puig A. Wnt signalling and the control of cellular metabolism. *Biochem J* 2010; **427**: 1-17 [PMID: 20226003 DOI: 10.1042/BJ20091866]

68   **Wang H**, Diao D, Shi Z, Zhu X, Gao Y, Gao S, Liu X, Wu Y, Rudolph KL, Liu G, Li T, Ju Z. SIRT6 Controls Hematopoietic Stem Cell Homeostasis through Epigenetic Regulation of Wnt Signaling. *Cell Stem Cell* 2016; **18**: 495-507 [PMID: 27058938 DOI: 10.1016/j.stem.2016.03.005]

69   **Chen EEM**, Zhang W, Ye CCY, Gao X, Jiang LLJ, Zhao TTF, Pan ZZJ, Xue DDT. Knockdown of SIRT7 enhances the osteogenic differentiation of human bone marrow mesenchymal stem cells partly *via* activation of the Wnt/β-catenin signaling pathway. *Cell Death Dis* 2017; **8**: e3042 [PMID: 28880264 DOI: 10.1038/cddis.2017.429]

70   **Yang S**, Guo S, Tong S, Sun X. Exosomal miR-130a-3p regulates osteogenic differentiation of Human Adipose-Derived stem cells through mediating SIRT7/Wnt/β-catenin axis. *Cell Prolif* 2020; **53**: e12890 [PMID: 32808361 DOI: 10.1111/cpr.12890]

71   **Park HW**, Kim YC, Yu B, Moroishi T, Mo JS, Plouffe SW, Meng Z, Lin KC, Yu FX, Alexander CM, Wang CY, Guan KL. Alternative Wnt Signaling Activates YAP/TAZ. *Cell* 2015; **162**: 780-794 [PMID: 26276632 DOI: 10.1016/j.cell.2015.07.013]

72   **Pan JX**, Xiong L, Zhao K, Zeng P, Wang B, Tang FL, Sun D, Guo HH, Yang X, Cui S, Xia WF, Mei L, Xiong WC. YAP promotes osteogenesis and suppresses adipogenic differentiation by regulating β-catenin signaling. *Bone Res* 2018; **6**: 18 [PMID: 29872550 DOI: 10.1038/s41413-018-0018-7]

73   **Couzens AL**, Xiong S, Knight JDR, Mao DY, Guettler S, Picaud S, Kurinov I, Filippakopoulos P, Sicheri F, Gingras AC. MOB1 Mediated Phospho-recognition in the Core Mammalian Hippo Pathway. *Mol Cell Proteomics* 2017; **16**: 1098-1110 [PMID: 28373298 DOI: 10.1074/mcp.M116.065490]

74   **Li L**, Zhou X, Zhang JT, Liu AF, Zhang C, Han JC, Zhang XQ, Wu S, Zhang XY, Lv FQ. Exosomal miR-186 derived from BMSCs promote osteogenesis through hippo signaling pathway in postmenopausal osteoporosis. *J Orthop Surg Res* 2021; **16**: 23 [PMID: 33413543 DOI: 10.1186/s13018-020-02160-0]

75   **Li ZT**, Zhang X, Wang DW, Xu J, Kou KJ, Wang ZW, Yong G, Liang DS, Sun XY. Overexpressed lncRNA GATA6-AS1 Inhibits LNM and EMT *via* FZD4 through the Wnt/β-Catenin Signaling Pathway in GC. *Mol Ther Nucleic Acids* 2020; **19**: 827-840 [PMID: 31981860 DOI: 10.1016/j.omtn.2019.09.034]

76   **Wang J**, Xia Y, Li J, Wang W. miR-129-5p in exosomes inhibits diabetes-associated osteogenesis in the jaw *via* targeting FZD4. *Biochem Biophys Res Commun* 2021; **566**: 87-93 [PMID: 34119828 DOI: 10.1016/j.bbrc.2021.05.072]

77   **Yumimoto K**, Matsumoto M, Onoyama I, Imaizumi K, Nakayama KI. F-box and WD repeat domain-containing-7 (Fbxw7) protein targets endoplasmic reticulum-anchored osteogenic and chondrogenic transcriptional factors for degradation. *J Biol Chem* 2013; **288**: 28488-28502 [PMID: 23955342 DOI: 10.1074/jbc.M113.465179]

78   **Li Y**, Wang J, Ma Y, Du W, Feng K, Wang S. miR-101-loaded exosomes secreted by bone marrow mesenchymal stem cells requires the FBXW7/HIF1α/FOXP3 axis, facilitating osteogenic differentiation. *J Cell Physiol* 2021; **236**: 4258-4272 [PMID: 33438204 DOI: 10.1002/jcp.30027]

79   **Lu K**, Chen Q, Li M, He L, Riaz F, Zhang T, Li D. Programmed cell death factor 4 (PDCD4), a novel therapy target for

metabolic diseases besides cancer. *Free Radic Biol Med* 2020; **159**: 150-163 [PMID: 32745771 DOI: 10.1016/j.freeradbiomed.2020.06.016]

80   **Zhang X**, You JM, Dong XJ, Wu Y. Administration of mircoRNA-135b-reinforced exosomes derived from MSCs ameliorates glucocorticoid-induced osteonecrosis of femoral head (ONFH) in rats. *J Cell Mol Med* 2020; **24**: 13973-13983 [PMID: 33089961 DOI: 10.1111/jcmm.16006]

81   **Tencerova M**, Okla M, Kassem M. Insulin Signaling in Bone Marrow Adipocytes. *Curr Osteoporos Rep* 2019; **17**: 446-454 [PMID: 31749085 DOI: 10.1007/s11914-019-00552-8]

82   **Chen L**, Jiang W, Huang J, He BC, Zuo GW, Zhang W, Luo Q, Shi Q, Zhang BQ, Wagner ER, Luo J, Tang M, Wietholt C, Luo X, Bi Y, Su Y, Liu B, Kim SH, He CJ, Hu Y, Shen J, Rastegar F, Huang E, Gao Y, Gao JL, Zhou JZ, Reid RR, Luu HH, Haydon RC, He TC, Deng ZL. Insulin-like growth factor 2 (IGF-2) potentiates BMP-9-induced osteogenic differentiation and bone formation. *J Bone Miner Res* 2010; **25**: 2447-2459 [PMID: 20499340 DOI: 10.1002/jbmr.133]

83   **Chen S**, Tang Y, Liu Y, Zhang P, Lv L, Zhang X, Jia L, Zhou Y. Exosomes derived from miR-375-overexpressing human adipose mesenchymal stem cells promote bone regeneration. *Cell Prolif* 2019; **52**: e12669 [PMID: 31380594 DOI: 10.1111/cpr.12669]

84   **Velasco A**, Pallares J, Santacana M, Gatius S, Fernandez M, Domingo M, Valls J, Yeramian A, Encinas M, Dolcet X, Matias-Guiu X. Promoter hypermethylation and expression of sprouty 2 in endometrial carcinoma. *Hum Pathol* 2011; **42**: 185-193 [PMID: 21111454 DOI: 10.1016/j.humpath.2010.08.001]

85   **Yim DG**, Ghosh S, Guy GR, Virshup DM. Casein kinase 1 regulates Sprouty2 in FGF-ERK signaling. *Oncogene* 2015; **34**: 474-484 [PMID: 24469046 DOI: 10.1038/onc.2013.564]

86   **Liao W**, Ning Y, Xu HJ, Zou WZ, Hu J, Liu XZ, Yang Y, Li ZH. BMSC-derived exosomes carrying microRNA-122-5p promote proliferation of osteoblasts in osteonecrosis of the femoral head. *Clin Sci (Lond)* 2019; **133**: 1955-1975 [PMID: 31387936 DOI: 10.1042/CS20181064]

87   **Zhang Y**, Cao X, Li P, Fan Y, Zhang L, Ma X, Sun R, Liu Y, Li W. microRNA-935-modified bone marrow mesenchymal stem cells-derived exosomes enhance osteoblast proliferation and differentiation in osteoporotic rats. *Life Sci* 2021; **272**: 119204 [PMID: 33581127 DOI: 10.1016/j.lfs.2021.119204]

88   **Komori T**. Roles of Runx2 in Skeletal Development. *Adv Exp Med Biol* 2017; **962**: 83-93 [PMID: 28299652 DOI: 10.1007/978-981-10-3233-2_6]

89   **Yang JX**, Xie P, Li YS, Wen T, Yang XC. Osteoclast-derived miR-23a-5p-containing exosomes inhibit osteogenic differentiation by regulating Runx2. *Cell Signal* 2020; **70**: 109504 [PMID: 31857240 DOI: 10.1016/j.cellsig.2019.109504]

90   **Wang SZ**, Jia J, Chen CH. lncRNA-KCNQ1OT1: A Potential Target in Exosomes Derived from Adipose-Derived Stem Cells for the Treatment of Osteoporosis. *Stem Cells Int* 2021; **2021**: 7690006 [PMID: 34712334 DOI: 10.1155/2021/7690006]

91   **Zahid KR**, Raza U, Chen J, Raj UJ, Gou D. Pathobiology of pulmonary artery hypertension: role of long non-coding RNAs. *Cardiovasc Res* 2020; **116**: 1937-1947 [PMID: 32109276 DOI: 10.1093/cvr/cvaa050]

92   **Bhan A**, Soleimani M, Mandal SS. Long Noncoding RNA and Cancer: A New Paradigm. *Cancer Res* 2017; **77**: 3965-3981 [PMID: 28701486 DOI: 10.1158/0008-5472.CAN-16-2634]

93   **You C**, Zhu K, Zhang Q, Yan J, Wang Y, Li J. ODNA: a manually curated database of noncoding RNAs associated with orthopedics. *Database (Oxford)* 2019; **2019** [PMID: 31781773 DOI: 10.1093/database/baz126]

94   **Venkatesh J**, Wasson MD, Brown JM, Fernando W, Marcato P. LncRNA-miRNA axes in breast cancer: Novel points of interaction for strategic attack. *Cancer Lett* 2021; **509**: 81-88 [PMID: 33848519 DOI: 10.1016/j.canlet.2021.04.002]

95   **Lanzillotti C**, De Mattei M, Mazziotta C, Taraballi F, Rotondo JC, Tognon M, Martini F. Long Non-coding RNAs and MicroRNAs Interplay in Osteogenic Differentiation of Mesenchymal Stem Cells. *Front Cell Dev Biol* 2021; **9**: 646032 [PMID: 33898434 DOI: 10.3389/fcell.2021.646032]

96   **Ahmad P**, Stoddart M J, Della Bella E. The role of noncoding RNAs in osteogenic differentiation of human periodontal ligament stem cells. *Craniomaxillofac Trauma Reconstr Open* 2021; **6**: 1-13 [DOI: 10.1177/2472751221999229]

97   **Li Z**, Jin C, Chen S, Zheng Y, Huang Y, Jia L, Ge W, Zhou Y. Long non-coding RNA MEG3 inhibits adipogenesis and promotes osteogenesis of human adipose-derived mesenchymal stem cells *via* miR-140-5p. *Mol Cell Biochem* 2017; **433**: 51-60 [PMID: 28382492 DOI: 10.1007/s11010-017-3015-z]

98   **Zhang Z**, Liu J, Zeng Z, Fan J, Huang S, Zhang L, Zhang B, Wang X, Feng Y, Ye Z, Zhao L, Cao D, Yang L, Pakvasa M, Liu B, Wagstaff W, Wu X, Luo H, Zhang J, Zhang M, He F, Mao Y, Ding H, Zhang Y, Niu C, Haydon RC, Luu HH, Lee MJ, Wolf JM, Shao Z, He TC. lncRNA Rmst acts as an important mediator of BMP9-induced osteogenic differentiation of mesenchymal stem cells (MSCs) by antagonizing Notch-targeting microRNAs. *Aging (Albany NY)* 2019; **11**: 12476-12496 [PMID: 31825894 DOI: 10.18632/aging.102583]

99   **Jia B**, Qiu X, Chen J, Sun X, Zheng X, Zhao J, Li Q, Wang Z. A feed-forward regulatory network lncPCAT1/miR-106a-5p/E2F5 regulates the osteogenic differentiation of periodontal ligament stem cells. *J Cell Physiol* 2019; **234**: 19523-19538 [PMID: 30997692 DOI: 10.1002/jcp.28550]

100   **Jia B**, Wang Z, Sun X, Chen J, Zhao J, Qiu X. Long noncoding RNA LINC00707 sponges miR-370-3p to promote osteogenesis of human bone marrow-derived mesenchymal stem cells through upregulating WNT2B. *Stem Cell Res Ther* 2019; **10**: 67 [PMID: 30795799 DOI: 10.1186/s13287-019-1161-9]

101   **Di Pietro L**, Barba M, Palacios D, Tiberio F, Prampolini C, Baranzini M, Parolini O, Arcovito A, Lattanzi W. Shaping modern human skull through epigenetic, transcriptional and post-transcriptional regulation of the RUNX2 master bone gene. *Sci Rep* 2021; **11**: 21316 [PMID: 34716352 DOI: 10.1038/s41598-021-00511-3]

102   **Li B**, Xu H, Han H, Song S, Zhang X, Ouyang L, Qian C, Hong Y, Qiu Y, Zhou W, Huang M, Zhuang W. Exosome-mediated transfer of lncRUNX2-AS1 from multiple myeloma cells to MSCs contributes to osteogenesis. *Oncogene* 2018; **37**: 5508-5519 [PMID: 29895968 DOI: 10.1038/s41388-018-0359-0]

103   **Léveillé N**, Baglio SR. Exosome-transferred lncRNAs at the core of cancer bone lesions. *Crit Rev Oncol Hematol* 2019; **139**: 125-127 [PMID: 30890387 DOI: 10.1016/j.critrevonc.2019.03.002]

104   **Wang C**, Li Y, Yu K, Jiang Z, Wang Y, Yang G. HOXA10 inhibit the osteogenic differentiation of periodontal ligament stem cells by regulating β-catenin localization and DKK1 expression. *Connect Tissue Res* 2021; **62**: 393-401 [PMID:

32299243 DOI: 10.1080/03008207.2020.1756271]

105   **Wang Y**, Chen W, Zhao L, Li Y, Liu Z, Gao H, Bai X, Wang B. Obesity regulates miR-467/HoxA10 axis on osteogenic differentiation and fracture healing by BMSC-derived exosome LncRNA H19. *J Cell Mol Med* 2021; **25**: 1712-1724 [PMID: 33471953 DOI: 10.1111/jcmm.16273]

106   **Behera J**, Kumar A, Voor MJ, Tyagi N. Exosomal lncRNA-H19 promotes osteogenesis and angiogenesis through mediating Angpt1/Tie2-NO signaling in CBS-heterozygous mice. *Theranostics* 2021; **11**: 7715-7734 [PMID: 34335960 DOI: 10.7150/thno.58410]

107   **Goyal B**, Yadav SRM, Awasthee N, Gupta S, Kunnumakkara AB, Gupta SC. Diagnostic, prognostic, and therapeutic significance of long non-coding RNA MALAT1 in cancer. *Biochim Biophys Acta Rev Cancer* 2021; **1875**: 188502 [PMID: 33428963 DOI: 10.1016/j.bbcan.2021.188502]

108   **Yang X**, Yang J, Lei P, Wen T. LncRNA MALAT1 shuttled by bone marrow-derived mesenchymal stem cells-secreted exosomes alleviates osteoporosis through mediating microRNA-34c/SATB2 axis. *Aging (Albany NY)* 2019; **11**: 8777-8791 [PMID: 31659145 DOI: 10.18632/aging.102264]

109   **Mo C**, Huang B, Zhuang J, Jiang S, Guo S, Mao X. LncRNA nuclear-enriched abundant transcript 1 shuttled by prostate cancer cells-secreted exosomes initiates osteoblastic phenotypes in the bone metastatic microenvironment *via* miR-205-5p/runt-related transcription factor 2/splicing factor proline- and glutamine-rich/polypyrimidine tract-binding protein 2 axis. *Clin Transl Med* 2021; **11**: e493 [PMID: 34459124 DOI: 10.1002/ctm2.493]

110   **Memczak S**, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, Maier L, Mackowiak SD, Gregersen LH, Munschauer M, Loewer A, Ziebold U, Landthaler M, Kocks C, le Noble F, Rajewsky N. Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 2013; **495**: 333-338 [PMID: 23446348 DOI: 10.1038/nature11928]

111   **Kristensen LS**, Andersen MS, Stagsted LVW, Ebbesen KK, Hansen TB, Kjems J. The biogenesis, biology and characterization of circular RNAs. *Nat Rev Genet* 2019; **20**: 675-691 [PMID: 31395983 DOI: 10.1038/s41576-019-0158-7]

112   **Fanale D**, Taverna S, Russo A, Bazan V. Circular RNA in Exosomes. *Adv Exp Med Biol* 2018; **1087**: 109-117 [PMID: 30259361 DOI: 10.1007/978-981-13-1426-1_9]

113   **Xie Q**, Wang Z, Bi X, Zhou H, Wang Y, Gu P, Fan X. Effects of miR-31 on the osteogenesis of human mesenchymal stem cells. *Biochem Biophys Res Commun* 2014; **446**: 98-104 [PMID: 24565840 DOI: 10.1016/j.bbrc.2014.02.058]

114   **Baglìo SR**, Devescovi V, Granchi D, Baldini N. MicroRNA expression profiling of human bone marrow mesenchymal stem cells during osteogenic differentiation reveals Osterix regulation by miR-31. *Gene* 2013; **527**: 321-331 [PMID: 23827457 DOI: 10.1016/j.gene.2013.06.021]

115   **Xie L**, Guan Z, Zhang M, Lyu S, Thuaksuban N, Kamolmattayakul S, Nuntanaranont T. Exosomal circLPAR1 Promoted Osteogenic Differentiation of Homotypic Dental Pulp Stem Cells by Competitively Binding to hsa-miR-31. *Biomed Res Int* 2020; **2020**: 6319395 [PMID: 33062690 DOI: 10.1155/2020/6319395]

116   **Zhuang C**, Huang X, Yu J, Gui Y. Circular RNA hsa_circ_0075828 promotes bladder cancer cell proliferation through activation of CREB1. *BMB Rep* 2020; **53**: 82-87 [PMID: 31072448 DOI: 10.5483/BMBRep.2020.53.2.059]

117   **Yuan W**, Peng S, Wang J, Wei C, Ye Z, Wang Y, Wang M, Xu H, Jiang S, Sun D, Dai C, Jiang L, Li X. Identification and characterization of circRNAs as competing endogenous RNAs for miRNA-mRNA in colorectal cancer. *PeerJ* 2019; **7**: e7602 [PMID: 31579577 DOI: 10.7717/peerj.7602]

118   **Zhi F**, Ding Y, Wang R, Yang Y, Luo K, Hua F. Exosomal hsa_circ_0006859 is a potential biomarker for postmenopausal osteoporosis and enhances adipogenic *vs* osteogenic differentiation in human bone marrow mesenchymal stem cells by sponging miR-431-5p. *Stem Cell Res Ther* 2021; **12**: 157 [PMID: 33648601 DOI: 10.1186/s13287-021-02214-y]

119   **Balandeh E**, Mohammadshafie K, Mahmoudi Y, Hossein Pourhanifeh M, Rajabi A, Bahabadi ZR, Mohammadi AH, Rahimian N, Hamblin MR, Mirzaei H. Roles of Non-coding RNAs and Angiogenesis in Glioblastoma. *Front Cell Dev Biol* 2021; **9**: 716462 [PMID: 34646821 DOI: 10.3389/fcell.2021.716462]

120   **Percival CJ**, Richtsmeier JT. Angiogenesis and intramembranous osteogenesis. *Dev Dyn* 2013; **242**: 909-922 [PMID: 23737393 DOI: 10.1002/dvdy.23992]

121   **Yin S**, Zhang W, Zhang Z, Jiang X. Recent Advances in Scaffold Design and Material for Vascularized Tissue-Engineered Bone Regeneration. *Adv Healthc Mater* 2019; **8**: e1801433 [PMID: 30938094 DOI: 10.1002/adhm.201801433]

122   **Jules J**, Zhang P, Ashley JW, Wei S, Shi Z, Liu J, Michalek SM, Feng X. Molecular basis of requirement of receptor activator of nuclear factor κB signaling for interleukin 1-mediated osteoclastogenesis. *J Biol Chem* 2012; **287**: 15728-15738 [PMID: 22416138 DOI: 10.1074/jbc.M111.296228]

123   **Atesok K**, Li R, Stewart DJ, Schemitsch EH. Endothelial progenitor cells promote fracture healing in a segmental bone defect model. *J Orthop Res* 2010; **28**: 1007-1014 [PMID: 20135674 DOI: 10.1002/jor.21083]

124   **Gao YS**, Zhang CQ. Cytotherapy of osteonecrosis of the femoral head: a mini review. *Int Orthop* 2010; **34**: 779-782 [PMID: 20361186 DOI: 10.1007/s00264-010-1009-5]

125   **Xu HJ**, Liao W, Liu XZ, Hu J, Zou WZ, Ning Y, Yang Y, Li ZH. Down-regulation of exosomal microRNA-224-3p derived from bone marrow-derived mesenchymal stem cells potentiates angiogenesis in traumatic osteonecrosis of the femoral head. *FASEB J* 2019; **33**: 8055-8068 [PMID: 30964699 DOI: 10.1096/fj.201801618RRR]

126   **Grosso A**, Burger MG, Lunger A, Schaefer DJ, Banfi A, Di Maggio N. It Takes Two to Tango: Coupling of Angiogenesis and Osteogenesis for Bone Regeneration. *Front Bioeng Biotechnol* 2017; **5**: 68 [PMID: 29164110 DOI: 10.3389/fbioe.2017.00068]

127   **Fish JE**, Santoro MM, Morton SU, Yu S, Yeh RF, Wythe JD, Ivey KN, Bruneau BG, Stainier DY, Srivastava D. miR-126 regulates angiogenic signaling and vascular integrity. *Dev Cell* 2008; **15**: 272-284 [PMID: 18694566 DOI: 10.1016/j.devcel.2008.07.008]

128   **Huang JH**, Xu Y, Yin XM, Lin FY. Exosomes Derived from miR-126-modified MSCs Promote Angiogenesis and Neurogenesis and Attenuate Apoptosis after Spinal Cord Injury in Rats. *Neuroscience* 2020; **424**: 133-145 [PMID: 31704348 DOI: 10.1016/j.neuroscience.2019.10.043]

129   **Wang ZF**, Liao F, Wu H, Dai J. Glioma stem cells-derived exosomal miR-26a promotes angiogenesis of microvessel endothelial cells in glioma. *J Exp Clin Cancer Res* 2019; **38**: 201 [PMID: 31101062 DOI: 10.1186/s13046-019-1181-4]

130   **Li C**, Li Y, Lu Y, Niu Z, Zhao H, Peng Y, Li M. miR-26 family and its target genes in tumorigenesis and development. *Crit Rev Oncol Hematol* 2021; **157**: 103124 [PMID: 33254041 DOI: 10.1016/j.critrevonc.2020.103124]

131   **Wu M**, Liu X, Li Z, Huang X, Guo H, Guo X, Yang X, Li B, Xuan K, Jin Y. SHED aggregate exosomes shuttled miR-26a promote angiogenesis in pulp regeneration *via* TGF-β/SMAD2/3 signalling. *Cell Prolif* 2021; **54**: e13074 [PMID: 34101281 DOI: 10.1111/cpr.13074]

132   **Zhang L**, Ouyang P, He G, Wang X, Song D, Yang Y, He X. Exosomes from microRNA-126 overexpressing mesenchymal stem cells promote angiogenesis by targeting the PIK3R2-mediated PI3K/Akt signalling pathway. *J Cell Mol Med* 2021; **25**: 2148-2162 [PMID: 33350092 DOI: 10.1111/jcmm.16192]

133   **Gangadaran P**, Rajendran RL, Lee HW, Kalimuthu S, Hong CM, Jeong SY, Lee SW, Lee J, Ahn BC. Extracellular vesicles from mesenchymal stem cells activates VEGF receptors and accelerates recovery of hindlimb ischemia. *J Control Release* 2017; **264**: 112-126 [PMID: 28837823 DOI: 10.1016/j.jconrel.2017.08.022]

134   **Pakravan K**, Babashah S, Sadeghizadeh M, Mowla SJ, Mossahebi-Mohammadi M, Ataei F, Dana N, Javan M. MicroRNA-100 shuttled by mesenchymal stem cell-derived exosomes suppresses *in vitro* angiogenesis through modulating the mTOR/HIF-1α/VEGF signaling axis in breast cancer cells. *Cell Oncol (Dordr)* 2017; **40**: 457-470 [PMID: 28741069 DOI: 10.1007/s13402-017-0335-7]

135   **Tian X**, Sun M, Wu H, Chen C, Li H, Qiu S, Wang T, Han J, Xiao Q, Chen K. Exosome-derived miR-let-7c promotes angiogenesis in multiple myeloma by polarizing M2 macrophages in the bone marrow microenvironment. *Leuk Res* 2021; **105**: 106566 [PMID: 33848709 DOI: 10.1016/j.leukres.2021.106566]

136   **Huang C**, Luo W, Wang Q, Ye Y, Fan J, Lin L, Shi C, Wei W, Chen H, Wu Y, Tang Y. Human mesenchymal stem cells promote ischemic repairment and angiogenesis of diabetic foot through exosome miRNA-21-5p. *Stem Cell Res* 2021; **52**: 102235 [PMID: 33601096 DOI: 10.1016/j.scr.2021.102235]

137   **Chen Z**, Wang H, Xia Y, Yan F, Lu Y. Therapeutic Potential of Mesenchymal Cell-Derived miRNA-150-5p-Expressing Exosomes in Rheumatoid Arthritis Mediated by the Modulation of MMP14 and VEGF. *J Immunol* 2018; **201**: 2472-2482 [PMID: 30224512 DOI: 10.4049/jimmunol.1800304]

138   **Wu D**, Kang L, Tian J, Wu Y, Liu J, Li Z, Wu X, Huang Y, Gao B, Wang H, Wu Z, Qiu G. Exosomes Derived from Bone Mesenchymal Stem Cells with the Stimulation of Fe_3O_4 Nanoparticles and Static Magnetic Field Enhance Wound Healing Through Upregulated miR-21-5p. *Int J Nanomedicine* 2020; **15**: 7979-7993 [PMID: 33116513 DOI: 10.2147/IJN.S275650]

139   **Cooper DR**, Wang C, Patel R, Trujillo A, Patel NA, Prather J, Gould LJ, Wu MH. Human Adipose-Derived Stem Cell Conditioned Media and Exosomes Containing *MALAT1* Promote Human Dermal Fibroblast Migration and Ischemic Wound Healing. *Adv Wound Care (New Rochelle)* 2018; **7**: 299-308 [PMID: 30263873 DOI: 10.1089/wound.2017.0775]

140   **Nan K**, Zhang Y, Zhang X, Li D, Zhao Y, Jing Z, Liu K, Shang D, Geng Z, Fan L. Exosomes from miRNA-378-modified adipose-derived stem cells prevent glucocorticoid-induced osteonecrosis of the femoral head by enhancing angiogenesis and osteogenesis *via* targeting miR-378 negatively regulated suppressor of fused (Sufu). *Stem Cell Res Ther* 2021; **12**: 331 [PMID: 34099038 DOI: 10.1186/s13287-021-02390-x]

141   **Ma JH**, Wang JJ, Zhang SX. The unfolded protein response and diabetic retinopathy. *J Diabetes Res* 2014; **2014**: 160140 [PMID: 25530974 DOI: 10.1155/2014/160140]

142   **Cao X**, Xue LD, Di Y, Li T, Tian YJ, Song Y. MSC-derived exosomal lncRNA SNHG7 suppresses endothelial-mesenchymal transition and tube formation in diabetic retinopathy *via* miR-34a-5p/XBP1 axis. *Life Sci* 2021; **272**: 119232 [PMID: 33600866 DOI: 10.1016/j.lfs.2021.119232]

143   **Boyle WJ**, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature* 2003; **423**: 337-342 [PMID: 12748652 DOI: 10.1038/nature01658]

144   **Novack DV**, Teitelbaum SL. The osteoclast: friend or foe? *Annu Rev Pathol* 2008; **3**: 457-484 [PMID: 18039135 DOI: 10.1146/annurev.pathmechdis.3.121806.151431]

145   **Lee Y**, Kim HJ, Park CK, Kim YG, Lee HJ, Kim JY, Kim HH. MicroRNA-124 regulates osteoclast differentiation. *Bone* 2013; **56**: 383-389 [PMID: 23867221 DOI: 10.1016/j.bone.2013.07.007]

146   **Weivoda MM**, Lee SK, Monroe DG. miRNAs in osteoclast biology. *Bone* 2021; **143**: 115757 [PMID: 33212320 DOI: 10.1016/j.bone.2020.115757]

147   **Clarke B**. Normal bone anatomy and physiology. *Clin J Am Soc Nephrol* 2008; **3** Suppl 3: S131-S139 [PMID: 18988698 DOI: 10.2215/CJN.04151206]

148   **Vidigal JA**, Ventura A. The biological functions of miRNAs: lessons from *in vivo* studies. *Trends Cell Biol* 2015; **25**: 137-147 [PMID: 25484347 DOI: 10.1016/j.tcb.2014.11.004]

149   **Lu J**, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. *Nature* 2005; **435**: 834-838 [PMID: 15944708 DOI: 10.1038/nature03702]

150   **Xu J**, Li D, Cai Z, Sun H, Su B, Qiu M, Ma R. Exosomal lncRNAs NONMMUT000375.2 and NONMMUT071578.2 derived from titanium particle treated RAW264.7 cells regulate osteogenic differentiation of MC3T3-E1 cells. *J Biomed Mater Res A* 2020; **108**: 2251-2262 [PMID: 32363719 DOI: 10.1002/jbm.a.36983]

151   **Sun W**, Zhao C, Li Y, Wang L, Nie G, Peng J, Wang A, Zhang P, Tian W, Li Q, Song J, Wang C, Xu X, Tian Y, Zhao D, Xu Z, Zhong G, Han B, Ling S, Chang YZ. Osteoclast-derived microRNA-containing exosomes selectively inhibit osteoblast activity. *Cell Discov* 2016; **2**: 16015 [PMID: 27462462 DOI: 10.1038/celldisc.2016.15]

152   **Borciani G**, Montalbano G, Baldini N, Cerqueni G, Vitale-Brovarone C, Ciapetti G. Co-culture systems of osteoblasts and osteoclasts: Simulating *in vitro* bone remodeling in regenerative approaches. *Acta Biomater* 2020; **108**: 22-45 [PMID: 32251782 DOI: 10.1016/j.actbio.2020.03.043]

153   **Cui Y**, Fu S, Sun D, Xing J, Hou T, Wu X. EPC-derived exosomes promote osteoclastogenesis through LncRNA-MALAT1. *J Cell Mol Med* 2019; **23**: 3843-3854 [PMID: 31025509 DOI: 10.1111/jcmm.14228]

154 **Wu P**, Mo Y, Peng M, Tang T, Zhong Y, Deng X, Xiong F, Guo C, Wu X, Li Y, Li X, Li G, Zeng Z, Xiong W. Emerging role of tumor-related functional peptides encoded by lncRNA and circRNA. *Mol Cancer* 2020; **19**: 22 [PMID: 32019587 DOI: 10.1186/s12943-020-1147-3]

155 **Batista PJ**. The RNA Modification N^6-methyladenosine and Its Implications in Human Disease. *Genomics Proteomics Bioinformatics* 2017; **15**: 154-163 [PMID: 28533023 DOI: 10.1016/j.gpb.2017.03.002]

156 **Wang W**, Qiao SC, Wu XB, Sun B, Yang JG, Li X, Zhang X, Qian SJ, Gu YX, Lai HC. Circ_0008542 in osteoblast exosomes promotes osteoclast-induced bone resorption through m6A methylation. *Cell Death Dis* 2021; **12**: 628 [PMID: 34145224 DOI: 10.1038/s41419-021-03915-1]

157 **Zhu L**, Li J, Gong Y, Wu Q, Tan S, Sun D, Xu X, Zuo Y, Zhao Y, Wei YQ, Wei XW, Peng Y. Exosomal tRNA-derived small RNA as a promising biomarker for cancer diagnosis. *Mol Cancer* 2019; **18**: 74 [PMID: 30940133 DOI: 10.1186/s12943-019-1000-8]

158 **Balatti V**, Nigita G, Veneziano D, Drusco A, Stein GS, Messier TL, Farina NH, Lian JB, Tomasello L, Liu CG, Palamarchuk A, Hart JR, Bell C, Carosi M, Pescarmona E, Perracchio L, Diodoro M, Russo A, Antenucci A, Visca P, Ciardi A, Harris CC, Vogt PK, Pekarsky Y, Croce CM. tsRNA signatures in cancer. *Proc Natl Acad Sci U S A* 2017; **114**: 8071-8076 [PMID: 28696308 DOI: 10.1073/pnas.1706908114]

159 **Zhu P**, Yu J, Zhou P. Role of tRNA-derived fragments in cancer: novel diagnostic and therapeutic targets tRFs in cancer. *Am J Cancer Res* 2020; **10**: 393-402 [PMID: 32195016]

160 **Fang S**, He T, Jiang J, Li Y, Chen P. Osteogenic Effect of tsRNA-10277-Loaded Exosome Derived from Bone Mesenchymal Stem Cells on Steroid-Induced Osteonecrosis of the Femoral Head. *Drug Des Devel Ther* 2020; **14**: 4579-4591 [PMID: 33149555 DOI: 10.2147/DDDT.S258024]

161 **Lin X**, Patil S, Gao YG, Qian A. The Bone Extracellular Matrix in Bone Formation and Regeneration. *Front Pharmacol* 2020; **11**: 757 [PMID: 32528290 DOI: 10.3389/fphar.2020.00757]

162 **Zhai P**, Peng X, Li B, Liu Y, Sun H, Li X. The application of hyaluronic acid in bone regeneration. *Int J Biol Macromol* 2020; **151**: 1224-1239 [PMID: 31751713 DOI: 10.1016/j.ijbiomac.2019.10.169]

163 **Zhang X**, Chen X, Hong H, Hu R, Liu J, Liu C. Decellularized extracellular matrix scaffolds: Recent trends and emerging strategies in tissue engineering. *Bioact Mater* 2022; **10**: 15-31 [PMID: 34901526 DOI: 10.1016/j.bioactmat.2021.09.014]

164 **Kara A**, Tamburaci S, Tihminlioglu F, Havitcioglu H. Bioactive fish scale incorporated chitosan biocomposite scaffolds for bone tissue engineering. *Int J Biol Macromol* 2019; **130**: 266-279 [PMID: 30797008 DOI: 10.1016/j.ijbiomac.2019.02.067]

165 **Xie X**, Wang W, Cheng J, Liang H, Lin Z, Zhang T, Lu Y, Li Q. Bilayer pifithrin-α loaded extracellular matrix/PLGA scaffolds for enhanced vascularized bone formation. *Colloids Surf B Biointerfaces* 2020; **190**: 110903 [PMID: 32120128 DOI: 10.1016/j.colsurfb.2020.110903]

166 **Fernandez-Piñeiro I**, Badiola I, Sanchez A. Nanocarriers for microRNA delivery in cancer medicine. *Biotechnol Adv* 2017; **35**: 350-360 [PMID: 28286148 DOI: 10.1016/j.biotechadv.2017.03.002]

167 **Tahmasebi A**, Enderami SE, Saburi E, Islami M, Yaslianifard S, Mahabadi JA, Ardeshirylajimi A, Soleimanifar F, Moghadam AS. Micro-RNA-incorporated electrospun nanofibers improve osteogenic differentiation of human-induced pluripotent stem cells. *J Biomed Mater Res A* 2020; **108**: 377-386 [PMID: 31654461 DOI: 10.1002/jbm.a.36824]

168 **Chen J**, Hendriks M, Chatzis A, Ramasamy SK, Kusumbe AP. Bone Vasculature and Bone Marrow Vascular Niches in Health and Disease. *J Bone Miner Res* 2020; **35**: 2103-2120 [PMID: 32845550 DOI: 10.1002/jbmr.4171]

169 **Hu X**, Xu Y, Zhong Z, Wu Y, Zhao J, Wang Y, Cheng H, Kong M, Zhang F, Chen Q, Sun J, Li Q, Jin J, Chen L, Wang C, Zhan H, Fan Y, Yang Q, Yu L, Wu R, Liang J, Zhu J, Jin Y, Lin Y, Yang F, Jia L, Zhu W, Chen J, Yu H, Zhang J, Wang J. A Large-Scale Investigation of Hypoxia-Preconditioned Allogeneic Mesenchymal Stem Cells for Myocardial Repair in Nonhuman Primates: Paracrine Activity Without Remuscularization. *Circ Res* 2016; **118**: 970-983 [PMID: 26838793 DOI: 10.1161/CIRCRESAHA.115.307516]

170 **Mohyeldin A**, Garzón-Muvdi T, Quiñones-Hinojosa A. Oxygen in stem cell biology: a critical component of the stem cell niche. *Cell Stem Cell* 2010; **7**: 150-161 [PMID: 20682444 DOI: 10.1016/j.stem.2010.07.007]

171 **Liu W**, Li L, Rong Y, Qian D, Chen J, Zhou Z, Luo Y, Jiang D, Cheng L, Zhao S, Kong F, Wang J, Xu T, Gong F, Huang Y, Gu C, Zhao X, Bai J, Wang F, Zhao W, Zhang L, Li X, Yin G, Fan J, Cai W. Hypoxic mesenchymal stem cell-derived exosomes promote bone fracture healing by the transfer of miR-126. *Acta Biomater* 2020; **103**: 196-212 [PMID: 31857259 DOI: 10.1016/j.actbio.2019.12.020]

172 **Liu L**, Liu Y, Feng C, Chang J, Fu R, Wu T, Yu F, Wang X, Xia L, Wu C, Fang B. Lithium-containing biomaterials stimulate bone marrow stromal cell-derived exosomal miR-130a secretion to promote angiogenesis. *Biomaterials* 2019; **192**: 523-536 [PMID: 30529871 DOI: 10.1016/j.biomaterials.2018.11.007]

173 **Liu D**, Li X, Li J, Yang J, Yokota H, Zhang P. Knee loading protects against osteonecrosis of the femoral head by enhancing vessel remodeling and bone healing. *Bone* 2015; **81**: 620-631 [PMID: 26416150 DOI: 10.1016/j.bone.2015.09.012]

174 **Wang X**, Li X, Li J, Zhai L, Liu D, Abdurahman A, Zhang Y, Yokota H, Zhang P. Mechanical loading stimulates bone angiogenesis through enhancing type H vessel formation and downregulating exosomal miR-214-3p from bone marrow-derived mesenchymal stem cells. *FASEB J* 2021; **35**: e21150 [PMID: 33161580 DOI: 10.1096/fj.202001080RR]

175 **Zheng R**, Zhang K, Tan S, Gao F, Zhang Y, Xu W, Wang H, Gu D, Zhu L, Li S, Chu H, Zhang Z, Liu L, Du M, Wang M. Exosomal circLPAR1 functions in colorectal cancer diagnosis and tumorigenesis through suppressing BRD4 *via* METTL3-eIF3h interaction. *Mol Cancer* 2022; **21**: 49 [PMID: 35164758 DOI: 10.1186/s12943-021-01471-y]

176 **Xie Y**, Li J, Li P, Li N, Zhang Y, Binang H, Zhao Y, Duan W, Chen Y, Wang Y, Du L, Wang C. RNA-Seq Profiling of Serum Exosomal Circular RNAs Reveals Circ-PNN as a Potential Biomarker for Human Colorectal Cancer. *Front Oncol* 2020; **10**: 982 [PMID: 32626660 DOI: 10.3389/fonc.2020.00982]

177 **Lv Q**, Deng J, Chen Y, Wang Y, Liu B, Liu J. Engineered Human Adipose Stem-Cell-Derived Exosomes Loaded with miR-21-5p to Promote Diabetic Cutaneous Wound Healing. *Mol Pharm* 2020; **17**: 1723-1733 [PMID: 32233440 DOI: 10.1021/acs.molpharmaceut.0c00177]

178 **Théry C**, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol* 2002; **2**: 569-579

[PMID: 12154376 DOI: 10.1038/nri855]

179 **Hu H**, Wang D, Li L, Yin H, He G, Zhang Y. Role of microRNA-335 carried by bone marrow mesenchymal stem cells-derived extracellular vesicles in bone fracture recovery. *Cell Death Dis* 2021; **12**: 156 [PMID: 33542183 DOI: 10.1038/s41419-021-03430-3]

180 **Colao IL**, Corteling R, Bracewell D, Wall I. Manufacturing Exosomes: A Promising Therapeutic Platform. *Trends Mol Med* 2018; **24**: 242-256 [PMID: 29449149 DOI: 10.1016/j.molmed.2018.01.006]

181 **Chen TS**, Arslan F, Yin Y, Tan SS, Lai RC, Choo AB, Padmanabhan J, Lee CN, de Kleijn DP, Lim SK. Enabling a robust scalable manufacturing process for therapeutic exosomes through oncogenic immortalization of human ESC-derived MSCs. *J Transl Med* 2011; **9**: 47 [PMID: 21513579 DOI: 10.1186/1479-5876-9-47]

182 **Davis C**, Dukes A, Drewry M, Helwa I, Johnson MH, Isales CM, Hill WD, Liu Y, Shi X, Fulzele S, Hamrick MW. MicroRNA-183-5p Increases with Age in Bone-Derived Extracellular Vesicles, Suppresses Bone Marrow Stromal (Stem) Cell Proliferation, and Induces Stem Cell Senescence. *Tissue Eng Part A* 2017; **23**: 1231-1240 [PMID: 28363268 DOI: 10.1089/ten.TEA.2016.0525]

REVIEW

Metabolic-epigenetic nexus in regulation of stem cell fate

Yi Liu, Di-Xin Cui, Yue Pan, Si-Han Yu, Li-Wei Zheng, Mian Wan

Yi Liu, Di-Xin Cui, Yue Pan, Si-Han Yu, Li-Wei Zheng, State Key Laboratory of Oral Diseases, National Clinical Research Center for Oral Diseases, Department of Pediatric Dentistry, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, Sichuan Province, China

Mian Wan, State Key Laboratory of Oral Diseases, National Clinical Research Center for Oral Diseases, Department of Cariology and Endodontics, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, Sichuan Province, China

Corresponding author: Mian Wan, DDS, PhD, Academic Research, Attending Doctor, Lecturer, Postdoctoral Fellow, State Key Laboratory of Oral Diseases, National Clinical Research Center for Oral Diseases, Department of Cariology and Endodontics, West China Hospital of Stomatology, Sichuan University, No. 14 People's South Road, Chengdu 610041, Sichuan Province, China. mianwan@scu.edu.cn

Abstract

Stem cell fate determination is one of the central questions in stem cell biology, and although its regulation has been studied at genomic and proteomic levels, a variety of biological activities in cells occur at the metabolic level. Metabolomics studies have established the metabolome during stem cell differentiation and have revealed the role of metabolites in stem cell fate determination. While metabolism is considered to play a biological regulatory role as an energy source, recent studies have suggested the nexus between metabolism and epigenetics because several metabolites function as cofactors and substrates in epigenetic mechanisms, including histone modification, DNA methylation, and microRNAs. Additionally, the epigenetic modification is sensitive to the dynamic metabolites and consequently leads to changes in transcription. The nexus between metabolism and epigenetics proposes a novel stem cell-based therapeutic strategy through manipulating metabolites. In the present review, we summarize the possible nexus between metabolic and epigenetic regulation in stem cell fate determination, and discuss the potential preventive and therapeutic strategies *via* targeting metabolites.

Key Words: Metabolism; Epigenetic regulation; Stem cell fate; Nexus effect

Core Tip: Stem cell fate can be regulated by metabolites. Recent studies have suggested that there is a nexus between metabolism and epigenetics, as several metabolites could function as cofactors and substrates in epigenetic mechanisms. We review many basic and preclinical studies, and the results support this view. This finding may provide a clue to further studies on the co-effects of metabolism and epigenetics in cell fate determination.

INTRODUCTION

Stem cells are specialized cells with a capacity for prolonged self-renewal and production of various lineage cells, which contribute to the development, maintenance and repair of organs, such as teeth, hair follicles, and liver. These long-lived cells produce proliferating progenitors that differentiate into functional cells. Disorder of this procedure results in hyperplasia, hypoplasia or dysfunction of the organs[1]. How such cell fate determination is regulated is one of the central questions in stem cell biology. High-throughput sequencing has been conducted to establish gene expression profiles of both embryonic and adult stem cells, which helps address the crucial genes in stem cell fate regulation[2]. Epigenetic mechanisms, including histone modification, DNA methylation, and microRNAs (miRNAs), have uncovered the post-transcriptional regulation associated with stem cell fate[3]. Parallel proteomics studies have expanded our understanding of stem cell biology through constructing protein expression profiles of various stem cell populations. Despite these findings, the molecular network that regulates stem cell fate, maintaining pluripotency or initiating differentiation, is not completely understood due to the expression differences between mRNA and protein, the inconsistency between protein expression and its function, or the discordance between gene expression and cellular phenotype[4].

Although genomics and proteomics discuss the biological events at the gene and protein levels, respectively, several biological activities in cells occur at the metabolic level, including cell signaling, energy transfer, and intercellular communication[5]. To establish the metabolome, the collection of all metabolites at a specific time, metabolomics has been developed as one of the important components in system biology. Metabolomics is considered to be a prospective approach in various areas of researches, such as development, pathology, diagnosis, and environmental science, since it elaborates what happens in cells[6]. To address what occurs during regulation of stem cell fate determination, metabolomic research has been conducted to construct metabolic profiles of embryonic stem cells and differentiated neurons and cardiomyocytes in mice. Stem cells are characterized by highly unsaturated metabolites that regulate cell differentiation through oxidative reactions, suggesting the vital role of metabolism in stem cell fate determination. Metabolism is considered to function as a major energy source during the process[5,7,8]. Recent studies have demonstrated that lipid metabolism provided 90% of acetyl-CoA in histone acetylation. S-adenosylmethionine (SAM), one of the methionine metabolism metabolites, functions as a methyl donor in histone as well as DNA methylation[9]. Additionally, the epigenetic modification could be sensitive to the dynamic change in the metabolites, leading to changes in transcription. These findings provide compelling evidence that establishes the nexus exists between metabolism and epigenetics and propose a novel stem cell-based therapeutic strategy through manipulating metabolites[10-12].

In the present review, we summarize the nexus between metabolic and epigenetic regulation in stem cell fate determination, along with potential preventive and therapeutic strategies targeting metabolites (Figures 1 and 2).

LIPID METABOLISM

Lipids, crucial in maintaining cellular homeostasis, is attached to epigenetic reprogramming of homeostasis[13]. Acetyl-CoA from lipid metabolism could promote histone acetylation and drive cellular growth. Hence, acetyl-CoA is a crucial indicator for cell growth and development. Furthermore, acetyl-CoA reduces the production of b-hydroxybutyrate, an inhibitor of histone deacetylases (HDACs), which functions as antiproliferative and prodifferentiative properties[14]. The relation between lipid metabolism and epigenetic modification of gene expression in different stem cells has been reported by several studies[15].
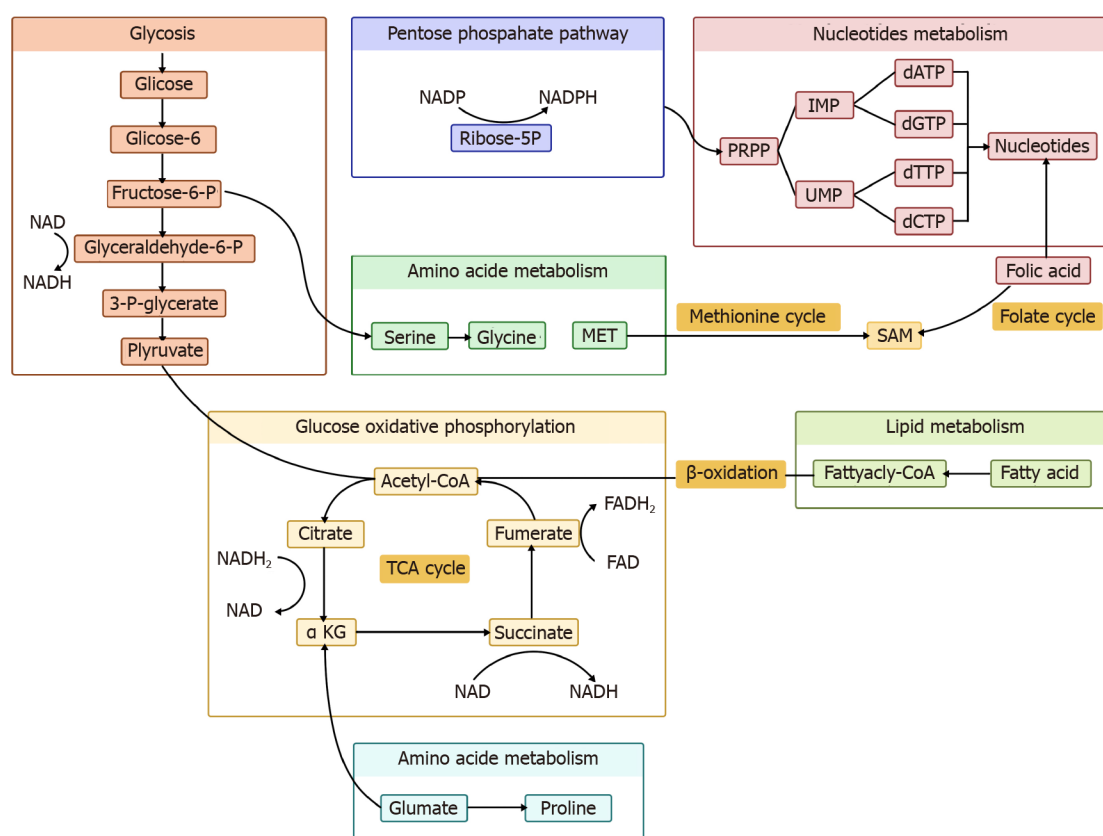
DOI: 10.4252/wjsc.v14.i7.490 **Copyright** ©The Author(s) 2022.

Figure 1 Schematic diagram of metabolic network. SAM: S-adenosylmethionine; TCA: Tricarboxylic acid; α-KG: α-ketoglutarate.

Lipid metabolism contributes 90% of acetyl. Chromatin structure opening occurs when histone acetylation is present, activating stem cell transcription[16]. This suggests that lipid availability regulates the pluripotency of stem cells and promotes cell differentiation[17]. Itokazu group's research on the importance of gangliosides in neural stem cells (NSCs) found that when the cellular histone deacetylase activity was inhibited by fatty acids, the levels of acetylated histone H3 and H4 on the *GM2/GD2* synthase gene increased, promoting neuronal differentiation of NSCs[18]. Ardah *et al*[19] and Boddeke *et al*[20] also showed that the increased level of saturated fatty acids promoted NSC differentiation into neurons. Murray *et al*[21] reported that butyrate promoted myogenic differentiation of satellite cells.

Cornacchia *et al*[22] showed that the level of H3K27Ac, H3K9Ac and H4K8Ac was elevated by activation of histone acetylation in human pluripotent stem cells, while histone deacetylases (HDAC), sirtuin 1 (SIRT1) and HDAC1were limited. Similar evidence has also been reported in animal studies[23, 24]. The level of H3K27ac decreased in the presence of low fatty acid metabolism in the gonads, leading to male differentiation-specific signal inhibition[25]. Acetyl-CoA production can be regulated by acetyl-CoA carboxylase , a rate-limiting enzyme whose activation limits the production of acetyl-CoA, thus promoting stem cell pluripotency. This is a traditional pathway in human as well as mouse embryonic stem cells (ESCs)[26,27]. These results show that lipid metabolism affects stem cell differentiation through histone acetylation modification.

AMINO ACID METABOLISM

Amino acids are one of the most fundamental substrates in cells, and are essential for metabolism of proteins, lipids and nucleotides. Previous reports have demonstrated that amino acid metabolism affected maintenance of stem cell pluripotency. In this review, we highlight the amino acids that influence stem cells critically.

Glutamine

Glutamine is the most abundant amino acid in metabolism, and is especially active in synthesis of nucleotides and fatty acids[28,29]. Glutamine changes α-ketoglutarate (α-KG) through deamination[30], which is a critical substrate for modification of proteins and DNA by demethylases. The mechanism is that α-KG acts as a substrate for Jumonji-C and ten-eleven translocation (TET), which regulate

DOI: 10.4252/wjsc.v14.i7.490 **Copyright** ©The Author(s) 2022.
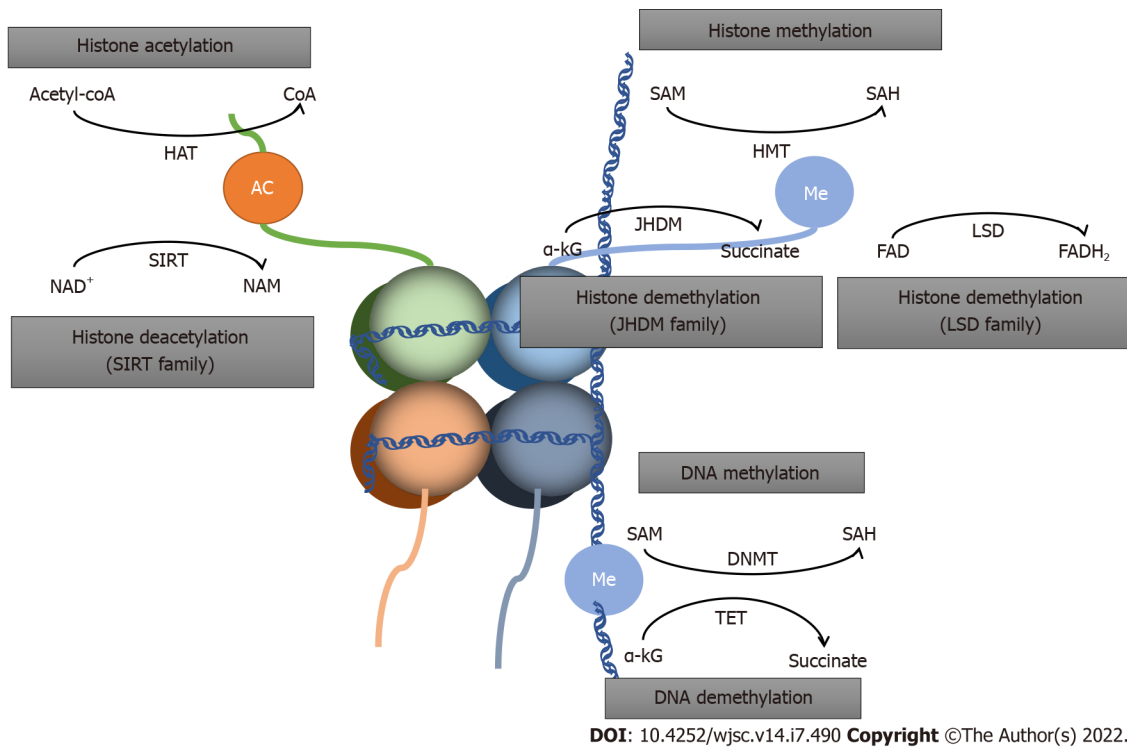
Figure 2 Schematic diagram of epigenetic metabolism. SAM: S-adenosylmethionine; SIRT: Sirtuin; α-KG: α-ketoglutarate; NAM: Nicotinamide; SAH: S-adenosylhomocysteine; HAT: Histone acetyltransferases; HMT: Histone methylases; JHDM: Jumonji-domain histone demethylase; LSD: Lysine-specific demethylase; DNMT: DNA methyltransferases; TET: Ten-eleven translocation.

demethylase interaction with histone and DNA, respectively. Demethylases are essential for stem cell pluripotency acquisition and maintenance. Many studies have highlighted the role of glutamine metabolism in the maintenance and differentiation of stem cells.

DNA methylation correlates with the repression of expression. α-KG positively regulates demethylation of DNA and promotes stem cell differentiation[31,32], and is key to the determination of stem cells fate as an appropriate balance between H3K9me2 acquisition and H3K27me3 depletion. Tischler *et al*[33], Xing *et al*[34] and Zylicz *et al*[35] came to similar conclusions in experiment on mouse primordial germ cell–like cells (PGCLCs). Okabe *et al*[36] have reported that histone H3K9me3 demethylation induced by an increase in α-KG activates transcription, leading to steatoblast cellular differentiation. Glutamine also regulates fetal oocyte differentiation through DNA demethylation enzyme TET1[37].

As DNA demethylation can lead to higher levels of 5-hydroxymethylcytosine, several recent studies have reported that α-KG fluctuations influence ESC differentiation[38]. The self-renewal of ESCs decreases with deficiency of glutamine, but can recover with α-KG supplementation[39]. Hepatic stellate cell (HSC) and effector T cell differentiation is also promoted by α-KG. A surprising finding is that α-KG can suppress tumor initiation and influence progression. These effects are inhibited by succinate and fumarate, providing a possible therapy for cancer[40].

Singh *et al*[41] have suggested that α-KG induced cell death, with degradation of hypoxia-inducible facor-1α and suppression of histone H3 (Lys 27) acetylation. The exact mechanism of histone acetylation regulated by α-KG still needs to be explored. Morris *et al*[42] has shown that α-KG was an effector of *p53*-mediated tumor suppression, whose accumulation in *p53*-deficient tumors can drive tumor cell differentiation and inhibit malignant progression. Ascorbate has a positive effect on HSC differentiation and suppresses leukemogenesis[43].

All these studies above highlight the importance of glutamine in cell fate determination.

Methionine

Methionine is an essential amino acid that plays an irreplaceable role in the synthesis of SAM. Methionine in the normal diet promotes production of SAM, which serves as a methyl donor for methyltransferases of histones and DNA[44]. The fluctuation of methionine and SAM levels regulates H3K4me3 formation and maintains the undifferentiated state of human ESCs/induced pluripotent stem cells (iPSCs)[45]. Kosti *et al*[46] have reported that limited methionine level was associated with neuronal differentiation, along with reduction of H3K27me3. Tang *et al*[47] have also provided evidence that reduced conversion of methionine to SAM lead to reduced ESC pluripotency. Zhang *et al*[48] have also found similar evidence that SAM played an important role in the differentiation of B cells into

plasmablasts, and SAM deficiency was accompanied by induction of H3K27me3. The theory may be an attractive option for improving therapeutic effectiveness in patients with systemic lupus erythematosus.

Fluctuation in the methionine cycle is related to cancer epigenetics. The increase in H3K4me3 and H3K27me3 level in cells treated with methionine in cancer stem cells parallels the increase in SAM to some extent[49,50]. This may provide a new therapy for cancer[51].

Taken together, these findings show that methionine has an important influence on stem cell fates.

Proline

Proline is a nonessential amino acid derived from glutamine metabolism. Pyrroline-5-carboxylate (P5C) is an intermediate product of both proline biosynthesis and catabolism. P5C is converted to proline by P5C reductase (Pycr1). Emerging evidence indicates that L-proline influences the epigenetic landscape of stem cells by regulating histones and DNA methylation[52,53]. L-Proline regulates H3K9 methylation and activates reprogramming of stem cells. Supplementation with L-proline increases DNA 5-methyl-cytosine and reduces of 5-hydroxy-methylcytosine, which promotes DNA methylation. It has recently emerged that hypermethylation lead to α-KG depletion, limiting the activity of TETs and Jumonji, and resulting in increased DNA and histone methylation .A study on mouse embryonic stem cell has shown that L-proline influenced the balance between self-renewal and differentiation[54]. Proline availability increases DNA and histone methylation, and is an essential procedure in embryonic-stem-to-mesenchymal like transition[55].

Proline is one of the most important amino acids in stem cell fate determination because of its epigenetic effects.

Glycine

Glycine takes part in one-carbon metabolism as a methyl group provider through the glycine cleavage system[56]. The glycine cleavage system is a multienzyme complex consisting of four individual components: glycine decarboxylase, amino methyltransferase, glycine cleavage system protein H, and dihydrolipoamide dehydrogenase[57]. It has been revealed that glycine influenced stem cell pluripotency by controlling the synthesis of SAM, thus promoting H3K4me3 modification, and open euchromatin[58]. This process is present in human and mouse PSCs[59].

NUCLEOTIDE METABOLISM

Noncoding RNA (ncRNA) is RNA that does not encode a protein. ncRNA is transcribed from the genome and exerts its effects at the RNA level. Global ncRNA abundance influences cell fate determ-ination and differentiation, and is important in embryonic development and its dysregulation causes cancer[60-62].

There are reports suggesting that long noncoding RNA (lncRNA) lnc13728 positively regulates expression of zinc finger BED-type containing 3 to promote the adipo-genic differentiation of human adipose-derived mesenchymal stem cells[63]. lncRNA has effects on hematopoietic cells in hematopoiesis regulation and the early stage of cell fate determination. Wu *et al*[64] have reported that, in hematopoietic stem cells and in differentiated lineage progenitors, lncRNA expression is given priority.

Griffiths and his colleagues have demonstrated that miRNA181a inhibition activated the early latent neurogenic gene to restore CA1 neurons, providing a positive clinical outcome in survivors of forebrain ischemia[65]. Zhang *et al*[66] have shown that miR-124 inhibited pancreatic progenitor cell proliferation to maintain a quiescent state, thus determining the fate of pancreatic progenitor cells. In cancer cells, miRNA might be a preferential pathway in cell reprograming. It has been reported that glucose transporter type 1 (GLUT1), GLUT3 and GLUT4 were overexpressed in most cancers. miR-122 regulates lipid levels in liver. miR-185 and miR-342 inhibit migration and invasion of prostate cancer cells, which could be a therapeutic option for prostate cancer[63]. He *et al*[67] have shown that miR-146a from exosomes had an effect on β-cell dedifferentiation, which provide a new therapy for type 2 diabetes[68]. High expression of miR-130a can increase osteogenic differentiation of bone marrow mesenchymal stem cells, which could be a potential therapy for age-related bone loss[69,70]. Huang *et al*[71] have reported that miR-330-5p negatively regulated differentiation of mesenchymal stem cells.

In summary, ncRNA plays an essential role in stem cell fate determination and could act as a breakthrough point in disease therapy. However, we still have a long way to go to understand the whole regulatory network of ncRNA.

GLUCOSE METABOLISM

Glucose and oxygen are important regulatory elements that help direct stem cell fate. In the undifferen-tiated state, stem cells, and their artificially reprogrammed equivalent iPSCs, are characterized by

limited oxidative capacity and active anaerobic glycolysis. The importance of optimizing glucose metabolism during nuclear reprogramming by epigenetic regulation has been demonstrated in several studies.

Glycolysis

Glycolysis is defined as a cytosolic redox reaction that transform a single glucose molecule into two pyruvate molecules accompanied by generation of two net ATP and two reduced NADH molecules. Although glycolysis is not as energetically efficient as complete oxidation, this pathway can occur in the absence of oxygen and enables a fast rate of ATP production, which may also be the reason why some highly proliferating cell types typically utilize glycolysis.

High glycolytic flux could be frequently observed in various stem cell populations and is critical for the acquisition and maintenance of cell pluripotency[72]. Li *et al*[73] have shown that GLIS family zinc finger 1 could directly bind to and open chromatin structure at glycolysis-related genes to promote glycolysis. Higher glycolytic flux subsequently upregulates cellular acetyl-CoA and lactate levels, leading to increased acetylation of H3K27 and pluripotency gene loci.

Glycolytic flux can be influenced by several factors, including epigenetic regulators and environmental conditions. For example, NAD-dependent histone deacetylase SIRT6 has been proved to act as a key regulator of glucose homeostasis, and its absence favors the metabolic profile of anaerobic glycolysis, which may activate gene reprogramming and pluripotency maintenance[74]. The epigenetic modifications are essential for the cell fate decisions in NSCs as well. High glucose levels increase H3K14 acetylation level, which can lead to premature neurogenetic differentiation of NSCs, providing a promising target for intervention in fetal neurodevelopment deficits[75]. Protein glycosylation is one of the most diverse and complicated co- and post-translational modifications, regulating self-renewal, pluripotency, and differentiation of stem cells through epigenetic mechanisms by histone modification and DNA methylation[76]. Glycolytic flux can also be regulated by oxygen. Glycolysis increases at 5% oxygen and acetylation of H3K9 and H3K27 is elevated, while H3K27 trimethylation is downregulated, leading to a more open chromatin structure and altered fate of human PSCs[77,78].

In summary, glycolysis is the dominant metabolic phenotype that controls stem cell fate.

Glucose oxidative phosphorylation

Glucose oxidative phosphorylation is another critical pathway for maintaining bioenergetic homeostasis as a bridge between the tricarboxylic acid (TCA) cycle and ATP synthesis. Oxidative phosphorylation is a more efficient pathway for ATP production compared to glycolysis, producing 36 ATP molecules per glucose. Oxidative phosphorylation promotes stem cell differentiation. Uittenbogaard *et al*[79] have provided evidence that enhancing oxidative phosphorylation can trigger neuronal differentiation by generating H3K27ac. Oxidative phosphorylation also mediates hematopoiesis stem cell differentiation toward definitive hematopoiesis through actyl-CoA metabolism[80].

Several TCA-cycle-related metabolic intermediates like NADH, FADH, fumarate and succinate are reported to contribute to epigenetic regulation of transcription and be connected with stem cell fate.

NADH: NAD$^+$ is a coenzyme that serves as a co-substrate for sirtuins, an HDAC family, and catalyzes deacetylation of histone lysine; a crucial protein post-translational modification[81-83].

NAD/NADH ratio can dictate the fate and function of different cell types. Increased NAD$^+$ production is required for cell differentiation[84]. Bmal1 regulates primary myoblast proliferation and differentiation through increasing cytosolic NAD$^+$. Reduced NAD$^+$ level prevents the differentiation of preadipocytes[85]. Okabe *et al*[36] have confirmed that high NAD$^+$ levels upregulated the TCA cycle, increasing α-KG and contributing to histone H3K9 demethylation and transcriptional activation. Zhu *et al*[85] have demonstrated that increasing cytosolic NAD levels could restore hypoxic cell proliferation and myofiber formation in Bmal1-deficient myoblasts, influencing oxygen-dependent myoblast cell fate. The effect of NAD/NADH ratio on stem cell fate is caused by generation of L-2-hydroxyglutaric acid, an analog of α-KG that regulates histone and DNA methylation by competitive inhibition of Jumonji-domain histone demethylase (JHDM) and TETs. There are reports revealing that increased NAD$^+$ levels delay aging-related phenotypes, which may provide new therapeutic option for type 2 diabetes and heart failure[86,87]. Besides, NAD$^+$ is a cosubstrate of Sirtuins, potentially regulating T cells, and could provide a therapeutic option for immune-related diseases[88].

In summary, NAD$^+$ plays a key role in a diverse array of biological processes.

FADH: FAD, the oxidized form of FADH2, is a cofactor of human lysine-specific demethylase-1 (LSD1), and plays a pivotal role during early embryonic development and differentiation of ESCs and cancer stem cells[89-92]. LSD1 catalyzes the demethylation of mono- and dimethylated K4 or K9 on histone H3 *via* the FAD-dependent enzymatic oxidation[93]. Recent studies have found that LSD1 inhibition can enhance death in rhabdomyosarcoma cells[94]. Decreased expression of LSD1 is involved in the programmed oocyte death by autophagy in perinatal mice through promotion of H3K4me2 expression [95]. FAD also regulates NSC proliferation through modulation of histone methylation by affecting the action of LSD1. In addition, LSD1 is highly expressed in a few aggressive cancer types and is closely related with differentiation, proliferation, migration and invasion of cancer cells and poor prognosis.

Succinate: Succinate accumulation can decrease α-KG/succinate ratio, leading to inhibition of TET and JHDM enzymes and delayed differentiation of primed human PSCs. This effect can be reversed when the α-KG/succinate ratio increases[32,86]. Accumulation of succinate, resulting in genetic and epigenetic changes like histone hypermethylation, may lead to transformation of normal cells to cancerous cells[96, 97] . Wong *et al*'s study in colorectal cancer cells showed that promoting accumulation of succinate upregulated DNA methylation and stem cell features[98]. AA6 is a novel compound succinic acid, identified as an inhibitor of α-KG dehydrogenase, which can increase the α-KG level in diabetic human cardiac mesenchymal cells and in the heart of high-fat diet, leading to DNA demethylation, and has beneficial effects of cardiac mesenchymal stem cells protection in diabetes[99].

Fumarate: Fumarate is reported to inhibit α-KG-dependent dioxygenases involved in DNA and histone demethylation. Laukka *et al*[100] have shown that fumarate downregulates global 5-hydroxymethyl-cytosine level in neuroblastoma cells *via* TET inhibition. Furthermore, Sharda *et al*[101] have reported that fumarate promotes monomer-to-dimer transition of malic enzyme 2 to enhance mitobiogenesis, linking metabolism to mitobiogenesis. Aberrant accumulation of fumarate may mediate epigenetic reprogramming. Some studies have reported the link between fumarate accumulation, epigenetic changes, and tumorigenesis. Accumulation of fumarate, inhibiting Tet-mediated demethylation, induces epithelial-to-mesenchymal transition; a phenotypic switch associated with cancer initiation, invasion and metastasis[102]. This implies that fumarate accumulation contributes to the aggressive features tumors[103].

Pentose phosphate pathway

The pentose phosphate pathway (PPP) is another glucose metabolism pathway, divided into oxidative and nonoxidative arms, producing NADH and ribose-5-phosphate and/or xylulose-5-phosphate that influence the regulation of transcription[104]. NADPH production in the pathway is involved in folate metabolism[105]. Previous studies have reported that regulation of the PPP resulted in iPSC reprogramming[106]. The PPP actively provides energy and metabolic intermediates for proliferation and pluripotency in cancer cells, ESCs and iPSCs[107,108]. Intracellular pH increase selectively activates catalysis, enhancing PPP flux, leading to nucleotide upregulation, increased NADPH/NADP$^+$ratio, and cell proliferation[109].

It remains to be elucidated whether PPP is linked to stem cell epigenetic remodeling.

OTHER POTENTIAL INFLUENTIAL FACTORS

Structure

The structure of scaffolds can affect stem cell metabolism. Three-dimensional graphene foam has better properties than two-dimensional foam for NSC differentiation. However, the possible mechanism needs to be explored[110].

Micronutrients

Vitamin C: Vitamin C is a crucial micronutrient that may be involved in stem cell pluripotency by activating H3K36 and H3K9 demethylases through Jumonji-C function[111]. A study using human PGCLCs also indicated the pathway[112,113]. Micronutrients influence stem cells specification.

Folic acid: Folic acid is first metabolized to dihydrofolate and then to tetrahydrofolate, taking part in DNA synthesis, influencing DNA and histone methylation[105]. Several studies have elucidated the role of folate metabolism in regulating of the epigenetic landscape of stem cells[114,115]. Li *et al*[116] have shown that folic acid deficiency in NSCs decreased cell proliferative capacity but increased apoptosis. Kasulanati *et al*[117] in a study of ESCs have provided more evidence for the effect of folic acid on PSC pluripotency. Pei *et al*[118] in a study of mouse ESCs have demonstrated that under folate deficiency conditions, H2AK119ub1 increases, and expression of neural tube closure-associated genes decreases. This suggests a possible mechanism for neural tube defects. Xie *et al*[119] have shown that folate inhibition can activate histone modification of monomethylation at lysine 4 of histone H3 transcription, suggesting that epigenetic regulation varies for different histone modifications.

Crosslinking: Horitani *et al*[120] have reported that glucose along with triglyceride increased metabolic stress spikes in mice, resulting in demethylation of H3K27me3, and expression of senescence-like phenotypes in bone marrow stem/progenitor cells. This could provide a therapeutic method for patients with cardiovascular disease and type 2 diabetes.

CONCLUSION

This review summarizes the recent studies about the metabolic–epigenetic nexus and provides compelling evidence that metabolism regulates stem cell fate determination through epigenetic mechanisms, such as histone acetylation, histone methylation and DNA methylation, in a variety of physical and pathological phenomena. The latest studies have also suggested that potential manipulation of metabolites held great promise in developing novel preventive, diagnostic and therapeutic strategies for a variety of diseases, which still requires further study prior to application in clinic settings.

There are still some essential questions. For example, what is the outcome of the regulation of metabolism on the epigenetic and transcriptional procedures of stem cells. The interplay of metabolism and epigenetics also brings out the complexity in environmental exposures studies, as the method of cell metabolism and potential transgenerational inheritance has been changed[15,121].

Furthermore, there were still some limitations. Most studies have attached importance to the level of enzymatic activity in cells, and we must accept that there is a difference between the measured and actual values[15]. All researches were conducted under experimental and not physiological conditions, and it is not hard to conclude that there might be some variation.

In summary, when it comes to the mechanism of stem cell fate determination, there is indeed interplay between metabolism and epigenetics. We need more accurate data acquisition and more realistic simulation as well as more specific mechanisms. The development of new technologies makes it easier to measure cellular metabolic status, and the accumulation of past studies supports our further exploration in this field.

FOOTNOTES

ORCID number: Yi Liu 0000-0003-4038-6051; Di-Xin Cui 0000-0002-8102-9504; Yue Pan 0000-0001-8197-869X; Si-Han Yu 0000-0002-7740-2660; Li-Wei Zheng 0000-0002-0467-1720; Mian Wan 0000-0002-3373-2836.

REFERENCES

1   **Ohara TE**, Colonna M, Stappenbeck TS. Adaptive differentiation promotes intestinal villus recovery. *Dev Cell* 2022; **57**: 166-179.e6 [PMID: 35016013 DOI: 10.1016/j.devcel.2021.12.012]

2   **Neves de Oliveira BH**, Dalmaz C, Zeidán-Chuliá F. Network-Based Identification of Altered Stem Cell Pluripotency and Calcium Signaling Pathways in Metastatic Melanoma. *Med Sci (Basel)* 2018; **6** [PMID: 29518019 DOI: 10.3390/medsci6010023]

3   **Tian Q**, Gao S, Zhou X, Zheng L, Zhou Y. Histone Acetylation in the Epigenetic Regulation of Bone Metabolism and Related Diseases. *Stem Cells Int* 2021; **2021**: 8043346 [PMID: 34326880 DOI: 10.1155/2021/8043346]

4   **Dvir S**, Argoetti A, Lesnik C, Roytblat M, Shriki K, Amit M, Hashimshony T, Mandel-Gutfreund Y. Uncovering the RNA-binding protein landscape in the pluripotency network of human embryonic stem cells. *Cell Rep* 2021; **35**: 109198 [PMID: 34077720 DOI: 10.1016/j.celrep.2021.109198]

5   **Döhla J**, Kuuluvainen E, Gebert N, Amaral A, Englund JI, Gopalakrishnan S, Konovalova S, Nieminen AI, Salminen ES,

Torregrosa Muñumer R, Ahlqvist K, Yang Y, Bui H, Otonkoski T, Käkelä R, Hietakangas V, Tyynismaa H, Ori A, Katajisto P. Metabolic determination of cell fate through selective inheritance of mitochondria. *Nat Cell Biol* 2022; **24**: 148-154 [PMID: 35165416 DOI: 10.1038/s41556-021-00837-0]

6   **Yang Y**, Fan TW, Lane AN, Higashi RM. Chloroformate derivatization for tracing the fate of Amino acids in cells and tissues by multiple stable isotope resolved metabolomics (mSIRM). *Anal Chim Acta* 2017; **976**: 63-73 [PMID: 28576319 DOI: 10.1016/j.aca.2017.04.014]

7   **Sun Z**, Zhao J, Yu H, Zhang C, Li H, Zeng Z, Zhang J. Metabolomics in Stem Cell Biology Research. *Methods Mol Biol* 2019; **1975**: 321-330 [PMID: 31062317 DOI: 10.1007/978-1-4939-9224-9_15]

8   **Tencerova M**, Rendina-Ruedy E, Neess D, Færgeman N, Figeac F, Ali D, Danielsen M, Haakonsson A, Rosen CJ, Kassem M. Metabolic programming determines the lineage-differentiation fate of murine bone marrow stromal progenitor cells. *Bone Res* 2019; **7**: 35 [PMID: 31754546 DOI: 10.1038/s41413-019-0076-5]

9   **Ferrari A**, Longo R, Silva R, Mitro N, Caruso D, De Fabiani E, Crestani M. Epigenome modifiers and metabolic rewiring: New frontiers in therapeutics. *Pharmacol Ther* 2019; **193**: 178-193 [PMID: 30125527 DOI: 10.1016/j.pharmthera.2018.08.008]

10   **D'Aniello C**, Cermola F, Patriarca EJ, Minchiotti G. Metabolic-Epigenetic Axis in Pluripotent State Transitions. *Epigenomes* 2019; **3** [PMID: 34968225 DOI: 10.3390/epigenomes3030013]

11   **Verdikt R**, Allard P. Metabolo-epigenetics: the interplay of metabolism and epigenetics during early germ cells development. *Biol Reprod* 2021; **105**: 616-624 [PMID: 34132770 DOI: 10.1093/biolre/ioab118]

12   **Li L**, Chen K, Wu Y, Xiang G, Liu X. Epigenome-Metabolome-Epigenome signaling cascade in cell biological processes. *J Genet Genomics* 2022; **49**: 279-286 [PMID: 34648996 DOI: 10.1016/j.jgg.2021.09.006]

13   **Tsogtbaatar E**, Landin C, Minter-Dykhouse K, Folmes CDL. Energy Metabolism Regulates Stem Cell Pluripotency. *Front Cell Dev Biol* 2020; **8**: 87 [PMID: 32181250 DOI: 10.3389/fcell.2020.00087]

14   **Prasad KN**. Butyric acid: a small fatty acid with diverse biological functions. *Life Sci* 1980; **27**: 1351-1358 [PMID: 7003281 DOI: 10.1016/0024-3205(80)90397-5]

15   **Fawal MA**, Davy A. Impact of Metabolic Pathways and Epigenetics on Neural Stem Cells. *Epigenet Insights* 2018; **11**: 2516865718820946 [PMID: 30627699 DOI: 10.1177/2516865718820946]

16   **McDonnell E**, Crown SB, Fox DB, Kitir B, Ilkayeva OR, Olsen CA, Grimsrud PA, Hirschey MD. Lipids Reprogram Metabolism to Become a Major Carbon Source for Histone Acetylation. *Cell Rep* 2016; **17**: 1463-1472 [PMID: 27806287 DOI: 10.1016/j.celrep.2016.10.012]

17   **Johnson MT**, Mahmood S, Patel MS. Intermediary metabolism and energetics during murine early embryogenesis. *J Biol Chem* 2003; **278**: 31457-31460 [PMID: 12788927 DOI: 10.1074/jbc.R300002200]

18   **Itokazu Y**, Wang J, Yu RK. Gangliosides in Nerve Cell Specification. *Prog Mol Biol Transl Sci* 2018; **156**: 241-263 [PMID: 29747816 DOI: 10.1016/bs.pmbts.2017.12.008]

19   **Ardah MT**, Parween S, Varghese DS, Emerald BS, Ansari SA. Saturated fatty acid alters embryonic cortical neurogenesis through modulation of gene expression in neural stem cells. *J Nutr Biochem* 2018; **62**: 230-246 [PMID: 30317068 DOI: 10.1016/j.jnutbio.2018.09.006]

20   **Balasubramaniyan V**, Boddeke E, Bakels R, Küst B, Kooistra S, Veneman A, Copray S. Effects of histone deacetylation inhibition on neuronal differentiation of embryonic mouse neural stem cells. *Neuroscience* 2006; **143**: 939-951 [PMID: 17084985 DOI: 10.1016/j.neuroscience.2006.08.082]

21   **Murray RL**, Zhang W, Liu J, Cooper J, Mitchell A, Buman M, Song J, Stahl CH. Tributyrin, a Butyrate Pro-Drug, Primes Satellite Cells for Differentiation by Altering the Epigenetic Landscape. *Cells* 2021; **10** [PMID: 34943981 DOI: 10.3390/cells10123475]

22   **Cornacchia D**, Zhang C, Zimmer B, Chung SY, Fan Y, Soliman MA, Tchieu J, Chambers SM, Shah H, Paull D, Konrad C, Vincendeau M, Noggle SA, Manfredi G, Finley LWS, Cross JR, Betel D, Studer L. Lipid Deprivation Induces a Stable, Naive-to-Primed Intermediate State of Pluripotency in Human PSCs. *Cell Stem Cell* 2019; **25**: 120-136.e10 [PMID: 31155483 DOI: 10.1016/j.stem.2019.05.001]

23   **Palmer NO**, Fullston T, Mitchell M, Setchell BP, Lane M. SIRT6 in mouse spermatogenesis is modulated by diet-induced obesity. *Reprod Fertil Dev* 2011; **23**: 929-939 [PMID: 21871212 DOI: 10.1071/RD10326]

24   **Hayashi Y**, Matsui Y. Metabolic Control of Germline Formation and Differentiation in Mammals. *Sex Dev* 2022; 1-16 [PMID: 35086109 DOI: 10.1159/000520662]

25   **Xu Y**, Xie J. Etomoxir regulates the differentiation of male germ cells by specifically reducing H3K27ac level. *BMC Dev Biol* 2021; **21**: 5 [PMID: 33517883 DOI: 10.1186/s12861-020-00237-x]

26   **Liu K**, Cao J, Shi X, Wang L, Zhao T. Cellular metabolism and homeostasis in pluripotency regulation. *Protein Cell* 2020; **11**: 630-640 [PMID: 32643102 DOI: 10.1007/s13238-020-00755-1]

27   **Wang L**, Zhang T, Wang L, Cai Y, Zhong X, He X, Hu L, Tian S, Wu M, Hui L, Zhang H, Gao P. Fatty acid synthesis is critical for stem cell pluripotency *via* promoting mitochondrial fission. *EMBO J* 2017; **36**: 1330-1347 [PMID: 28377463 DOI: 10.15252/embj.201695417]

28   **Smith RJ**, Wilmore DW. Glutamine nutrition and requirements. *JPEN J Parenter Enteral Nutr* 1990; **14**: 94S-99S [PMID: 2119461 DOI: 10.1177/014860719001400412]

29   **Curi R**, Newsholme P, Procopio J, Lagranha C, Gorjão R, Pithon-Curi TC. Glutamine, gene expression, and cell function. *Front Biosci* 2007; **12**: 344-357 [PMID: 17127303 DOI: 10.2741/2068]

30   **Baksh SC**, Finley LWS. Metabolic Coordination of Cell Fate by α-Ketoglutarate-Dependent Dioxygenases. *Trends Cell Biol* 2021; **31**: 24-36 [PMID: 33092942 DOI: 10.1016/j.tcb.2020.09.010]

31   **Zhang Z**, He C, Zhang L, Zhu T, Lv D, Li G, Song Y, Wang J, Wu H, Ji P, Liu G. Alpha-ketoglutarate affects murine embryo development through metabolic and epigenetic modulations. *Reproduction* 2019; **158**: 123-133 [PMID: 31158818 DOI: 10.1530/REP-19-0018]

32   **TeSlaa T**, Chaikovsky AC, Lipchina I, Escobar SL, Hochedlinger K, Huang J, Graeber TG, Braas D, Teitell MA. α-Ketoglutarate Accelerates the Initial Differentiation of Primed Human Pluripotent Stem Cells. *Cell Metab* 2016; **24**: 485-

493 [PMID: 27476976 DOI: 10.1016/j.cmet.2016.07.002]

33   **Tischler J**, Gruhn WH, Reid J, Allgeyer E, Buettner F, Marr C, Theis F, Simons BD, Wernisch L, Surani MA. Metabolic regulation of pluripotency and germ cell fate through α-ketoglutarate. *EMBO J* 2019; **38** [PMID: 30257965 DOI: 10.15252/embj.201899518]

34   **Xing M**, Wang N, Zeng H, Zhang J. α-ketoglutarate promotes the specialization of primordial germ cell-like cells through regulating epigenetic reprogramming. *J Biomed Res* 2020; **35**: 36-46 [PMID: 32994387 DOI: 10.7555/JBR.34.20190160]

35   **Zylicz JJ**, Dietmann S, Günesdogan U, Hackett JA, Cougot D, Lee C, Surani MA. Chromatin dynamics and the role of G9a in gene regulation and enhancer silencing during early mouse development. *Elife* 2015; **4** [PMID: 26551560 DOI: 10.7554/eLife.09571]

36   **Okabe K**, Nawaz A, Nishida Y, Yaku K, Usui I, Tobe K, Nakagawa T. NAD+ Metabolism Regulates Preadipocyte Differentiation by Enhancing α-Ketoglutarate-Mediated Histone H3K9 Demethylation at the PPARγ Promoter. *Front Cell Dev Biol* 2020; **8**: 586179 [PMID: 33330464 DOI: 10.3389/fcell.2020.586179]

37   **Yamaguchi S**, Hong K, Liu R, Shen L, Inoue A, Diep D, Zhang K, Zhang Y. Tet1 controls meiosis by regulating meiotic gene expression. *Nature* 2012; **492**: 443-447 [PMID: 23151479 DOI: 10.1038/nature11709]

38   **El-Habr EA**, Dubois LG, Burel-Vandenbos F, Bogeas A, Lipecka J, Turchi L, Lejeune FX, Coehlo PL, Yamaki T, Wittmann BM, Fareh M, Mahfoudhi E, Janin M, Narayanan A, Morvan-Dubois G, Schmitt C, Verreault M, Oliver L, Sharif A, Pallud J, Devaux B, Puget S, Korkolopoulou P, Varlet P, Ottolenghi C, Plo I, Moura-Neto V, Virolle T, Chneiweiss H, Junier MP. A driver role for GABA metabolism in controlling stem and proliferative cell state through GHB production in glioma. *Acta Neuropathol* 2017; **133**: 645-660 [PMID: 28032215 DOI: 10.1007/s00401-016-1659-5]

39   **Vardhana SA**, Arnold PK, Rosen BP, Chen Y, Carey BW, Huangfu D, Carmona Fontaine C, Thompson CB, Finley LWS. Glutamine independence is a selectable feature of pluripotent stem cells. *Nat Metab* 2019; **1**: 676-687 [PMID: 31511848 DOI: 10.1038/s42255-019-0082-3]

40   **Klysz D**, Tai X, Robert PA, Craveiro M, Cretenet G, Oburoglu L, Mongellaz C, Floess S, Fritz V, Matias MI, Yong C, Surh N, Marie JC, Huehn J, Zimmermann V, Kinet S, Dardalhon V, Taylor N. Glutamine-dependent α-ketoglutarate production regulates the balance between T helper 1 cell and regulatory T cell generation. *Sci Signal* 2015; **8**: ra97 [PMID: 26420908 DOI: 10.1126/scisignal.aab2610]

41   **Singh K**, Krug L, Basu A, Meyer P, Treiber N, Vander Beken S, Wlaschek M, Kochanek S, Bloch W, Geiger H, Maity P, Scharffetter-Kochanek K. Alpha-Ketoglutarate Curbs Differentiation and Induces Cell Death in Mesenchymal Stromal Precursors with Mitochondrial Dysfunction. *Stem Cells* 2017; **35**: 1704-1718 [PMID: 28398002 DOI: 10.1002/stem.2629]

42   **Morris JP 4th**, Yashinskie JJ, Koche R, Chandwani R, Tian S, Chen CC, Baslan T, Marinkovic ZS, Sánchez-Rivera FJ, Leach SD, Carmona-Fontaine C, Thompson CB, Finley LWS, Lowe SW. α-Ketoglutarate links p53 to cell fate during tumour suppression. *Nature* 2019; **573**: 595-599 [PMID: 31534224 DOI: 10.1038/s41586-019-1577-5]

43   **Cimmino L**, Dolgalev I, Wang Y, Yoshimi A, Martin GH, Wang J, Ng V, Xia B, Witkowski MT, Mitchell-Flack M, Grillo I, Bakogianni S, Ndiaye-Lobry D, Martín MT, Guillamot M, Banh RS, Xu M, Figueroa ME, Dickins RA, Abdel-Wahab O, Park CY, Tsirigos A, Neel BG, Aifantis I. Restoration of TET2 Function Blocks Aberrant Self-Renewal and Leukemia Progression. *Cell* 2017; **170**: 1079-1095.e20 [PMID: 28823558 DOI: 10.1016/j.cell.2017.07.032]

44   **Liu G**, Yang W, Zhang X, Peng T, Zou Y, Zhang T, Wang H, Liu X, Tao LZ. Cystathionine beta-lyase is crucial for embryo patterning and the maintenance of root stem cell niche in Arabidopsis. *Plant J* 2019; **99**: 536-555 [PMID: 31002461 DOI: 10.1111/tpj.14343]

45   **Van Winkle LJ**, Ryznar R. One-Carbon Metabolism Regulates Embryonic Stem Cell Fate Through Epigenetic DNA and Histone Modifications: Implications for Transgenerational Metabolic Disorders in Adults. *Front Cell Dev Biol* 2019; **7**: 300 [PMID: 31824950 DOI: 10.3389/fcell.2019.00300]

46   **Kosti A**, de Araujo PR, Li WQ, Guardia GDA, Chiou J, Yi C, Ray D, Meliso F, Li YM, Delambre T, Qiao M, Burns SS, Lorbeer FK, Georgi F, Flosbach M, Klinnert S, Jenseit A, Lei X, Sandoval CR, Ha K, Zheng H, Pandey R, Gruslova A, Gupta YK, Brenner A, Kokovay E, Hughes TR, Morris QD, Galante PAF, Tiziani S, Penalva LOF. The RNA-binding protein SERBP1 functions as a novel oncogenic factor in glioblastoma by bridging cancer metabolism and epigenetic regulation. *Genome Biol* 2020; **21**: 195 [PMID: 32762776 DOI: 10.1186/s13059-020-02115-y]

47   **Tang S**, Fang Y, Huang G, Xu X, Padilla-Banks E, Fan W, Xu Q, Sanderson SM, Foley JF, Dowdy S, McBurney MW, Fargo DC, Williams CJ, Locasale JW, Guan Z, Li X. Methionine metabolism is essential for SIRT1-regulated mouse embryonic stem cell maintenance and embryonic development. *EMBO J* 2017; **36**: 3175-3193 [PMID: 29021282 DOI: 10.15252/embj.201796708]

48   **Zhang M**, Iwata S, Hajime M, Ohkubo N, Todoroki Y, Miyata H, Ueno M, Hao H, Zhang T, Fan J, Nakayamada S, Yamagata K, Tanaka Y. Methionine Commits Cells to Differentiate Into Plasmablasts Through Epigenetic Regulation of BTB and CNC Homolog 2 by the Methyltransferase EZH2. *Arthritis Rheumatol* 2020; **72**: 1143-1153 [PMID: 31961063 DOI: 10.1002/art.41208]

49   **Altundag Ö**, Canpinar H, Çelebi-Saltik B. Methionine affects the expression of pluripotency genes and protein levels associated with methionine metabolism in adult, fetal, and cancer stem cells. *J Cell Biochem* 2022; **123**: 406-416 [PMID: 34783058 DOI: 10.1002/jcb.30180]

50   **Wang Z**, Yip LY, Lee JHJ, Wu Z, Chew HY, Chong PKW, Teo CC, Ang HY, Peh KLE, Yuan J, Ma S, Choo LSK, Basri N, Jiang X, Yu Q, Hillmer AM, Lim WT, Lim TKH, Takano A, Tan EH, Tan DSW, Ho YS, Lim B, Tam WL. Methionine is a metabolic dependency of tumor-initiating cells. *Nat Med* 2019; **25**: 825-837 [PMID: 31061538 DOI: 10.1038/s41591-019-0423-5]

51   **Jung J**, Kim LJ, Wang X, Wu Q, Sanvoranart T, Hubert CG, Prager BC, Wallace LC, Jin X, Mack SC, Rich JN. Nicotinamide metabolism regulates glioblastoma stem cell maintenance. *JCI Insight* 2017; **2** [PMID: 28515364 DOI: 10.1172/jci.insight.90019]

52   **Washington JM**, Rathjen J, Felquer F, Lonic A, Bettess MD, Hamra N, Semendric L, Tan BS, Lake JA, Keough RA, Morris MB, Rathjen PD. L-Proline induces differentiation of ES cells: a novel role for an amino acid in the regulation of pluripotent cells in culture. *Am J Physiol Cell Physiol* 2010; **298**: C982-C992 [PMID: 20164384 DOI:

10.1152/ajpcell.00498.2009]

53   **Casalino L**, Comes S, Lambazzi G, De Stefano B, Filosa S, De Falco S, De Cesare D, Minchiotti G, Patriarca EJ. Control of embryonic stem cell metastability by L-proline catabolism. *J Mol Cell Biol* 2011; **3**: 108-122 [PMID: 21307025 DOI: 10.1093/jmcb/mjr001]

54   **Shparberg RA**, Glover HJ, Morris MB. Modeling Mammalian Commitment to the Neural Lineage Using Embryos and Embryonic Stem Cells. *Front Physiol* 2019; **10**: 705 [PMID: 31354503 DOI: 10.3389/fphys.2019.00705]

55   **D'Aniello C**, Patriarca EJ, Phang JM, Minchiotti G. Proline Metabolism in Tumor Growth and Metastatic Progression. *Front Oncol* 2020; **10**: 776 [PMID: 32500033 DOI: 10.3389/fonc.2020.00776]

56   **Tian S**, Feng J, Cao Y, Shen S, Cai Y, Yang D, Yan R, Wang L, Zhang H, Zhong X, Gao P. Glycine cleavage system determines the fate of pluripotent stem cells *via* the regulation of senescence and epigenetic modifications. *Life Sci Alliance* 2019; **2** [PMID: 31562192 DOI: 10.26508/Lsa.201900413]

57   **Narisawa A**, Komatsuzaki S, Kikuchi A, Niihori T, Aoki Y, Fujiwara K, Tanemura M, Hata A, Suzuki Y, Relton CL, Grinham J, Leung KY, Partridge D, Robinson A, Stone V, Gustavsson P, Stanier P, Copp AJ, Greene ND, Tominaga T, Matsubara Y, Kure S. Mutations in genes encoding the glycine cleavage system predispose to neural tube defects in mice and humans. *Hum Mol Genet* 2012; **21**: 1496-1503 [PMID: 22171071 DOI: 10.1093/hmg/ddr585]

58   **Kidder BL**, Hu G, Zhao K. KDM5B focuses H3K4 methylation near promoters and enhancers during embryonic stem cell self-renewal and differentiation. *Genome Biol* 2014; **15**: R32 [PMID: 24495580 DOI: 10.1186/gb-2014-15-2-r32]

59   **Zhang J**, Ratanasirintrawoot S, Chandrasekaran S, Wu Z, Ficarro SB, Yu C, Ross CA, Cacchiarelli D, Xia Q, Seligson M, Shinoda G, Xie W, Cahan P, Wang L, Ng SC, Tintara S, Trapnell C, Onder T, Loh YH, Mikkelsen T, Sliz P, Teitell MA, Asara JM, Marto JA, Li H, Collins JJ, Daley GQ. LIN28 Regulates Stem Cell Metabolism and Conversion to Primed Pluripotency. *Cell Stem Cell* 2016; **19**: 66-80 [PMID: 27320042 DOI: 10.1016/j.stem.2016.05.009]

60   **Wang Y**, Hussein AM, Somasundaram L, Sankar R, Detraux D, Mathieu J, Ruohola-Baker H. microRNAs Regulating Human and Mouse Naïve Pluripotency. *Int J Mol Sci* 2019; **20** [PMID: 31766734 DOI: 10.3390/ijms20235864]

61   **Essig K**, Kronbeck N, Guimaraes JC, Lohs C, Schlundt A, Hoffmann A, Behrens G, Brenner S, Kowalska J, Lopez-Rodriguez C, Jemielity J, Holtmann H, Reiche K, Hackermüller J, Sattler M, Zavolan M, Heissmeyer V. Roquin targets mRNAs in a 3'-UTR-specific manner by different modes of regulation. *Nat Commun* 2018; **9**: 3810 [PMID: 30232334 DOI: 10.1038/s41467-018-06184-3]

62   **Beilharz TH**, See MM, Boag PR. 3'-UTRs and the Control of Protein Expression in Space and Time. *Adv Exp Med Biol* 2019; **1203**: 133-148 [PMID: 31811633 DOI: 10.1007/978-3-030-31434-7_5]

63   **Alamoudi AA**, Alnoury A, Gad H. miRNA in tumour metabolism and why could it be the preferred pathway for energy reprograming. *Brief Funct Genomics* 2018; **17**: 157-169 [PMID: 29028873 DOI: 10.1093/bfgp/elx023]

64   **Wu Z**, Gao S, Zhao X, Chen J, Keyvanfar K, Feng X, Kajigaya S, Young NS. Long noncoding RNAs of single hematopoietic stem and progenitor cells in healthy and dysplastic human bone marrow. *Haematologica* 2019; **104**: 894-906 [PMID: 30545929 DOI: 10.3324/haematol.2018.208926]

65   **Griffiths BB**, Ouyang YB, Xu L, Sun X, Giffard RG, Stary CM. Postinjury Inhibition of miR-181a Promotes Restoration of Hippocampal CA1 Neurons after Transient Forebrain Ischemia in Rats. *eNeuro* 2019; **6** [PMID: 31427401 DOI: 10.1523/eneuro.0002-19.2019]

66   **Zhang Z**, Zhai W, Liang J, Chen Z, Ma M, Zhao Y, Liang Y, Li X, Teng CB. Mutual inhibitions between epidermal growth factor receptor signaling and miR-124a control pancreatic progenitor proliferation. *J Cell Physiol* 2019; **234**: 12978-12988 [PMID: 30537082 DOI: 10.1002/jcp.27967]

67   **He Q**, Song J, Cui C, Wang J, Hu H, Guo X, Yang M, Wang L, Yan F, Liang K, Liu Z, Liu F, Sun Z, Dong M, Hou X, Chen L. Mesenchymal stem cell-derived exosomal miR-146a reverses diabetic β-cell dedifferentiation. *Stem Cell Res Ther* 2021; **12**: 449 [PMID: 34380570 DOI: 10.1186/s13287-021-02371-0]

68   **Soltani A**, Jafarian A, Allameh A. The Predominant microRNAs in β-cell Clusters for Insulin Regulation and Diabetic Control. *Curr Drug Targets* 2020; **21**: 722-734 [PMID: 31886749 DOI: 10.2174/1389450121666191230145848]

69   **Lin Z**, He H, Wang M, Liang J. MicroRNA-130a controls bone marrow mesenchymal stem cell differentiation towards the osteoblastic and adipogenic fate. *Cell Prolif* 2019; **52**: e12688 [PMID: 31557368 DOI: 10.1111/cpr.12688]

70   **Chesi A**, Wagley Y, Johnson ME, Manduchi E, Su C, Lu S, Leonard ME, Hodge KM, Pippin JA, Hankenson KD, Wells AD, Grant SFA. Genome-scale Capture C promoter interactions implicate effector genes at GWAS loci for bone mineral density. *Nat Commun* 2019; **10**: 1260 [PMID: 30890710 DOI: 10.1038/s41467-019-09302-x]

71   **Huang W**, Li K, Liu A, Yang Z, Hu C, Chen D, Wang H. miR3305p inhibits H2O2induced adipogenic differentiation of MSCs by regulating RXRγ. *Int J Mol Med* 2018; **42**: 2042-2052 [PMID: 30015907 DOI: 10.3892/ijmm.2018.3773]

72   **Cho YM**, Kwon S, Pak YK, Seol HW, Choi YM, Park DJ, Park KS, Lee HK. Dynamic changes in mitochondrial biogenesis and antioxidant enzymes during the spontaneous differentiation of human embryonic stem cells. *Biochem Biophys Res Commun* 2006; **348**: 1472-1478 [PMID: 16920071 DOI: 10.1016/j.bbrc.2006.08.020]

73   **Li L**, Chen K, Wang T, Wu Y, Xing G, Chen M, Hao Z, Zhang C, Zhang J, Ma B, Liu Z, Yuan H, Long Q, Zhou Y, Qi J, Zhao D, Gao M, Pei D, Nie J, Ye D, Pan G, Liu X. Glis1 facilitates induction of pluripotency *via* an epigenome-metabolome-epigenome signalling cascade. *Nat Metab* 2020; **2**: 882-892 [PMID: 32839595 DOI: 10.1038/s42255-020-0267-9]

74   **Sanhueza Salas LF**, García-Venzor A, Beltramone N, Capurro C, Toiber D, Silberman DM. Metabolic Imbalance Effect on Retinal Müller Glial Cells Reprogramming Capacity: Involvement of Histone Deacetylase SIRT6. *Front Genet* 2021; **12**: 769723 [PMID: 34804128 DOI: 10.3389/fgene.2021.769723]

75   **Ji S**, Zhou W, Li X, Liu S, Wang F, Zhao T, Ji G, Du J, Hao A. Maternal hyperglycemia disturbs neocortical neurogenesis *via* epigenetic regulation in C57BL/6J mice. *Cell Death Dis* 2019; **10**: 211 [PMID: 30824686 DOI: 10.1038/s41419-019-1438-z]

76   **Sheikh MA**, Emerald BS, Ansari SA. Stem cell fate determination through protein O-GlcNAcylation. *J Biol Chem* 2021; **296**: 100035 [PMID: 33154167 DOI: 10.1074/jbc.REV120.014915]

77   **Lees JG**, Cliff TS, Gammilonghi A, Ryall JG, Dalton S, Gardner DK, Harvey AJ. Oxygen Regulates Human Pluripotent Stem Cell Metabolic Flux. *Stem Cells Int* 2019; **2019**: 8195614 [PMID: 31236115 DOI: 10.1155/2019/8195614]

78   **Moussaieff A**, Rouleau M, Kitsberg D, Cohen M, Levy G, Barasch D, Nemirovski A, Shen-Orr S, Laevsky I, Amit M, Bomze D, Elena-Herrmann B, Scherf T, Nissim-Rafinia M, Kempa S, Itskovitz-Eldor J, Meshorer E, Aberdam D, Nahmias Y. Glycolysis-mediated changes in acetyl-CoA and histone acetylation control the early differentiation of embryonic stem cells. *Cell Metab* 2015; **21**: 392-402 [PMID: 25738455 DOI: 10.1016/j.cmet.2015.02.002]

79   **Uittenbogaard M**, Brantner CA, Chiaramello A. Epigenetic modifiers promote mitochondrial biogenesis and oxidative metabolism leading to enhanced differentiation of neuroprogenitor cells. *Cell Death Dis* 2018; **9**: 360 [PMID: 29500414 DOI: 10.1038/s41419-018-0396-1]

80   **Oburoglu L**, Mansell E, Canals I, Sigurdsson V, Guibentif C, Soneji S, Woods NB. Pyruvate metabolism guides definitive lineage specification during hematopoietic emergence. *EMBO Rep* 2022; **23**: e54384 [PMID: 34914165 DOI: 10.15252/embr.202154384]

81   **Shvedunova M**, Akhtar A. Modulation of cellular processes by histone and non-histone protein acetylation. *Nat Rev Mol Cell Biol* 2022; **23**: 329-349 [PMID: 35042977 DOI: 10.1038/s41580-021-00441-y]

82   **Imai S**, Guarente L. NAD+ and sirtuins in aging and disease. *Trends Cell Biol* 2014; **24**: 464-471 [PMID: 24786309 DOI: 10.1016/j.tcb.2014.04.002]

83   **Williams EO**, Taylor AK, Bell EL, Lim R, Kim DM, Guarente L. Sirtuin 1 Promotes Deacetylation of Oct4 and Maintenance of Naive Pluripotency. *Cell Rep* 2016; **17**: 809-820 [PMID: 27732856 DOI: 10.1016/j.celrep.2016.09.046]

84   **Chiarugi A**, Dölle C, Felici R, Ziegler M. The NAD metabolome--a key determinant of cancer cell biology. *Nat Rev Cancer* 2012; **12**: 741-752 [PMID: 23018234 DOI: 10.1038/nrc3340]

85   **Zhu P**, Hamlish NX, Thakkar AV, Steffeck AWT, Rendleman EJ, Khan NH, Waldeck NJ, DeVilbiss AW, Martin-Sandoval MS, Mathews TP, Chandel NS, Peek CB. BMAL1 drives muscle repair through control of hypoxic NAD$^+$ regeneration in satellite cells. *Genes Dev* 2022; **36**: 149-166 [PMID: 35115380 DOI: 10.1101/gad.349066.121]

86   **Chakrabarty RP**, Chandel NS. Mitochondria as Signaling Organelles Control Mammalian Stem Cell Fate. *Cell Stem Cell* 2021; **28**: 394-408 [PMID: 33667360 DOI: 10.1016/j.stem.2021.02.011]

87   **Lopaschuk GD**, Karwi QG, Tian R, Wende AR, Abel ED. Cardiac Energy Metabolism in Heart Failure. *Circ Res* 2021; **128**: 1487-1513 [PMID: 33983836 DOI: 10.1161/CIRCRESAHA.121.318241]

88   **Hamaidi I**, Kim S. Sirtuins are crucial regulators of T cell metabolism and functions. *Exp Mol Med* 2022; **54**: 207-215 [PMID: 35296782 DOI: 10.1038/s12276-022-00739-7]

89   **Sun G**, Alzayady K, Stewart R, Ye P, Yang S, Li W, Shi Y. Histone demethylase LSD1 regulates neural stem cell proliferation. *Mol Cell Biol* 2010; **30**: 1997-2005 [PMID: 20123967 DOI: 10.1128/MCB.01116-09]

90   **Gao L**, Alumkal J. Epigenetic regulation of androgen receptor signaling in prostate cancer. *Epigenetics* 2010; **5**: 100-104 [PMID: 20160483 DOI: 10.4161/epi.5.2.10778]

91   **Tu WJ**, McCuaig RD, Tan AHY, Hardy K, Seddiki N, Ali S, Dahlstrom JE, Bean EG, Dunn J, Forwood J, Tsimbalyuk S, Smith K, Yip D, Malik L, Prasanna T, Milburn P, Rao S. Targeting Nuclear LSD1 to Reprogram Cancer Cells and Reinvigorate Exhausted T Cells *via* a Novel LSD1-EOMES Switch. *Front Immunol* 2020; **11**: 1228 [PMID: 32612611 DOI: 10.3389/fimmu.2020.01228]

92   **Li Z**, Ding L, Li Z, Wang Z, Suo F, Shen D, Zhao T, Sun X, Wang J, Liu Y, Ma L, Zhao B, Geng P, Yu B, Zheng Y, Liu H. Development of the triazole-fused pyrimidine derivatives as highly potent and reversible inhibitors of histone lysine specific demethylase 1 (LSD1/KDM1A). *Acta Pharm Sin B* 2019; **9**: 794-808 [PMID: 31384539 DOI: 10.1016/j.apsb.2019.01.001]

93   **Metzger E**, Wissmann M, Yin N, Müller JM, Schneider R, Peters AH, Günther T, Buettner R, Schüle R. LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature* 2005; **437**: 436-439 [PMID: 16079795 DOI: 10.1038/nature04020]

94   **Schulz-Fincke J**, Hau M, Barth J, Robaa D, Willmann D, Kürner A, Haas J, Greve G, Haydn T, Fulda S, Lübbert M, Lüdeke S, Berg T, Sippl W, Schüle R, Jung M. Structure-activity studies on N-Substituted tranylcypromine derivatives lead to selective inhibitors of lysine specific demethylase 1 (LSD1) and potent inducers of leukemic cell differentiation. *Eur J Med Chem* 2018; **144**: 52-67 [PMID: 29247860 DOI: 10.1016/j.ejmech.2017.12.001]

95   **He M**, Zhang T, Zhu Z, Qin S, Wang H, Zhao L, Zhang X, Hu J, Wen J, Cai H, Xin Q, Guo Q, Lin L, Zhou B, Zhang H, Xia G, Wang C. LSD1 contributes to programmed oocyte death by regulating the transcription of autophagy adaptor SQSTM1/p62. *Aging Cell* 2020; **19**: e13102 [PMID: 32074399 DOI: 10.1111/acel.13102]

96   **Burnichon N**, Brière JJ, Libé R, Vescovo L, Rivière J, Tissier F, Jouanno E, Jeunemaitre X, Bénit P, Tzagoloff A, Rustin P, Bertherat J, Favier J, Gimenez-Roqueplo AP. SDHA is a tumor suppressor gene causing paraganglioma. *Hum Mol Genet* 2010; **19**: 3011-3020 [PMID: 20484225 DOI: 10.1093/hmg/ddq206]

97   **Nazar E**, Khatami F, Saffar H, Tavangar SM. The Emerging Role of Succinate Dehyrogenase Genes (SDHx) in Tumorigenesis. *Int J Hematol Oncol Stem Cell Res* 2019; **13**: 72-82 [PMID: 31372201]

98   **Wong CC**, Xu J, Bian X, Wu JL, Kang W, Qian Y, Li W, Chen H, Gou H, Liu D, Yat Luk ST, Zhou Q, Ji F, Chan LS, Shirasawa S, Sung JJ, Yu J. In Colorectal Cancer Cells With Mutant KRAS, SLC25A22-Mediated Glutaminolysis Reduces DNA Demethylation to Increase WNT Signaling, Stemness, and Drug Resistance. *Gastroenterology* 2020; **159**: 2163-2180.e6 [PMID: 32814111 DOI: 10.1053/j.gastro.2020.08.016]

99   **Spallotta F**, Cencioni C, Atlante S, Garella D, Cocco M, Mori M, Mastrocola R, Kuenne C, Guenther S, Nanni S, Azzimato V, Zukunft S, Kornberger A, Sürün D, Schnütgen F, von Melchner H, Di Stilo A, Aragno M, Braspenning M, van Criekinge W, De Blasio MJ, Ritchie RH, Zaccagnini G, Martelli F, Farsetti A, Fleming I, Braun T, Beiras-Fernandez A, Botta B, Collino M, Bertinaria M, Zeiher AM, Gaetano C. Stable Oxidative Cytosine Modifications Accumulate in Cardiac Mesenchymal Cells From Type2 Diabetes Patients: Rescue by α-Ketoglutarate and TET-TDG Functional Reactivation. *Circ Res* 2018; **122**: 31-46 [PMID: 29158345 DOI: 10.1161/CIRCRESAHA.117.311300]

100   **Laukka T**, Mariani CJ, Ihantola T, Cao JZ, Hokkanen J, Kaelin WG Jr, Godley LA, Koivunen P. Fumarate and Succinate Regulate Expression of Hypoxia-inducible Genes *via* TET Enzymes. *J Biol Chem* 2016; **291**: 4256-4265 [PMID: 26703470 DOI: 10.1074/jbc.M115.688762]

101   **Wang YP**, Sharda A, Xu SN, van Gastel N, Man CH, Choi U, Leong WZ, Li X, Scadden DT. Malic enzyme 2 connects the Krebs cycle intermediate fumarate to mitochondrial biogenesis. *Cell Metab* 2021; **33**: 1027-1041.e8 [PMID:

33770508 DOI: 10.1016/j.cmet.2021.03.003]

102   **De Craene B**, Berx G. Regulatory networks defining EMT during cancer initiation and progression. *Nat Rev Cancer*
        2013; **13**: 97-110 [PMID: 23344542 DOI: 10.1038/nrc3447]

103   **Sciacovelli M**, Frezza C. Metabolic reprogramming and epithelial-to-mesenchymal transition in cancer. *FEBS J* 2017;
        284: 3132-3144 [PMID: 28444969 DOI: 10.1111/febs.14090]

104   **Krüger A**, Grüning NM, Wamelink MM, Kerick M, Kirpy A, Parkhomchuk D, Bluemlein K, Schweiger MR, Soldatov A,
        Lehrach H, Jakobs C, Ralser M. The pentose phosphate pathway is a metabolic redox sensor and regulates transcription
        during the antioxidant response. *Antioxid Redox Signal* 2011; **15**: 311-324 [PMID: 21348809 DOI:
        10.1089/ars.2010.3797]

105   **Chen L**, Zhang Z, Hoshino A, Zheng HD, Morley M, Arany Z, Rabinowitz JD. NADPH production by the oxidative
        pentose-phosphate pathway supports folate metabolism. *Nat Metab* 2019; **1**: 404-415 [PMID: 31058257]

106   **Ishida T**, Nakao S, Ueyama T, Harada Y, Kawamura T. Metabolic remodeling during somatic cell reprogramming to
        induced pluripotent stem cells: involvement of hypoxia-inducible factor 1. *Inflamm Regen* 2020; **40**: 8 [PMID: 32426078
        DOI: 10.1186/s41232-020-00117-8]

107   **Varum S**, Rodrigues AS, Moura MB, Momcilovic O, Easley CA 4th, Ramalho-Santos J, Van Houten B, Schatten G.
        Energy metabolism in human pluripotent stem cells and their differentiated counterparts. *PLoS One* 2011; **6**: e20914
        [PMID: 21698063 DOI: 10.1371/journal.pone.0020914]

108   **Walvekar AS**, Laxman S. Methionine at the Heart of Anabolism and Signaling: Perspectives From Budding Yeast. *Front
        Microbiol* 2019; **10**: 2624 [PMID: 31798560 DOI: 10.3389/fmicb.2019.02624]

109   **Man CH**, Mercier FE, Liu N, Dong W, Stephanopoulos G, Jiang L, Jung Y, Lin CP, Leung AYH, Scadden DT. Proton
        export alkalinizes intracellular pH and reprograms carbon metabolism to drive normal and malignant cell growth. *Blood*
        2022; **139**: 502-522 [PMID: 34610101 DOI: 10.1182/blood.2021011563]

110   **Fang Q**, Zhang Y, Chen X, Li H, Cheng L, Zhu W, Zhang Z, Tang M, Liu W, Wang H, Wang T, Shen T, Chai R. Three-
        Dimensional Graphene Enhances Neural Stem Cell Proliferation Through Metabolic Regulation. *Front Bioeng Biotechnol*
        2019; **7**: 436 [PMID: 31998703 DOI: 10.3389/fbioe.2019.00436]

111   **Chen J**, Liu H, Liu J, Qi J, Wei B, Yang J, Liang H, Chen Y, Chen J, Wu Y, Guo L, Zhu J, Zhao X, Peng T, Zhang Y,
        Chen S, Li X, Li D, Wang T, Pei D. H3K9 methylation is a barrier during somatic cell reprogramming into iPSCs. *Nat
        Genet* 2013; **45**: 34-42 [PMID: 23202127 DOI: 10.1038/ng.2491]

112   **Li Z**, Fang F, Zhao Q, Li H, Xiong C. Supplementation of vitamin C promotes early germ cell specification from human
        embryonic stem cells. *Stem Cell Res Ther* 2019; **10**: 324 [PMID: 31730021 DOI: 10.1186/s13287-019-1427-2]

113   **Zhitkovich A**. Nuclear and Cytoplasmic Functions of Vitamin C. *Chem Res Toxicol* 2020; **33**: 2515-2526 [PMID:
        33001635 DOI: 10.1021/acs.chemrestox.0c00348]

114   **Zhang Y**, Kato H, Sato H, Yamaza H, Hirofuji Y, Han X, Masuda K, Nonaka K. Folic acid-mediated mitochondrial
        activation for protection against oxidative stress in human dental pulp stem cells derived from deciduous teeth. *Biochem
        Biophys Res Commun* 2019; **508**: 850-856 [PMID: 30528238 DOI: 10.1016/j.bbrc.2018.11.169]

115   **Srivastava AC**, Thompson YG, Singhal J, Stellern J, Srivastava A, Du J, O'Connor TR, Riggs AD. Elimination of human
        folypolyglutamate synthetase alters programming and plasticity of somatic cells. *FASEB J* 2019; **33**: 13747-13761
        [PMID: 31585510 DOI: 10.1096/fj.201901721R]

116   **Li Z**, Li W, Zhou D, Zhao J, Ma Y, Huang L, Dong C, Wilson JX, Huang G. Alleviating Oxidative Damage-Induced
        Telomere Attrition: a Potential Mechanism for Inhibition by Folic Acid of Apoptosis in Neural Stem Cells. *Mol Neurobiol*
        2022; **59**: 590-602 [PMID: 34741234 DOI: 10.1007/s12035-021-02623-3]

117   **Kasulanati S**, Venkatesan V. Understanding pluripotency under folic acid deficiency using embryonic stem cells as an in
        vitro model. *Med Hypotheses* 2018; **111**: 24-26 [PMID: 29406990 DOI: 10.1016/j.mehy.2017.12.019]

118   **Pei P**, Cheng X, Yu J, Shen J, Li X, Wu J, Wang S, Zhang T. Folate deficiency induced H2A ubiquitination to lead to
        downregulated expression of genes involved in neural tube defects. *Epigenetics Chromatin* 2019; **12**: 69 [PMID:
        31722724 DOI: 10.1186/s13072-019-0312-7]

119   **Xie Q**, Li C, Song X, Wu L, Jiang Q, Qiu Z, Cao H, Yu K, Wan C, Li J, Yang F, Huang Z, Niu B, Jiang Z, Zhang T.
        Folate deficiency facilitates recruitment of upstream binding factor to hot spots of DNA double-strand breaks of rRNA
        genes and promotes its transcription. *Nucleic Acids Res* 2017; **45**: 2472-2489 [PMID: 27924000 DOI:
        10.1093/nar/gkw1208]

120   **Horitani K**, Iwasaki M, Kishimoto H, Wada K, Nakano M, Park H, Adachi Y, Motooka D, Okuzaki D, Shiojima I.
        Repetitive spikes of glucose and lipid induce senescence-like phenotypes of bone marrow stem cells through H3K27me3
        demethylase-mediated epigenetic regulation. *Am J Physiol Heart Circ Physiol* 2021; **321**: H920-H932 [PMID: 34533398
        DOI: 10.1152/ajpheart.00261.2021]

121   **Cantor JR**, Abu-Remaileh M, Kanarek N, Freinkman E, Gao X, Louissaint A Jr, Lewis CA, Sabatini DM. Physiologic
        Medium Rewires Cellular Metabolism and Reveals Uric Acid as an Endogenous Inhibitor of UMP Synthase. *Cell* 2017;
        169: 258-272.e17 [PMID: 28388410 DOI: 10.1016/j.cell.2017.03.023]

Stem cell therapy for insulin-dependent diabetes: Are we still on the road?

Lu Yang, Zhu-Meng Hu, Fang-Xu Jiang, Wei Wang

Lu Yang, Zhu-Meng Hu, Fang-Xu Jiang, Wei Wang, Department of Endocrinology, Xiang'an Hospital of Xiamen University, School of Medicine, Xiamen University, Xiamen 361100, Fujian Province, China

Fang-Xu Jiang, School of Biomedical Science, University of Western Australia, Nedlands 6009, Australia

Fang-Xu Jiang, School of Health and Medical Sciences, Edith Cowan University, Perth 6000, Australia

Corresponding author: Wei Wang, MD, Doctor, Department of Endocrinology, Xiang'an Hospital of Xiamen University, School of Medicine, Xiamen University, No. 2000 Xiang'an East Road, Xiang'an District, Xiamen 361100, Fujian Province, China. wwei19742007@hotmail.com

Abstract

In insulin-dependent diabetes, the islet β cells do not produce enough insulin and the patients must receive exogenous insulin to control blood sugar. However, there are still many deficiencies in exogenous insulin supplementation. Therefore, the replacement of destroyed functional β cells with insulin-secreting cells derived from functional stem cells is a good idea as a new therapeutic idea. This review introduces the development schedule of mouse and human embryonic islets. The differences between mouse and human pancreas embryo development were also listed. Accordingly to the different sources of stem cells, the important research achievements on the differentiation of insulin-secreting β cells of stem cells and the current research status of stem cell therapy for diabetes were reviewed. Stem cell replacement therapy is a promising treatment for diabetes, caused by defective insulin secretion, but there are still many problems to be solved, such as the biosafety and reliability of treatment, the emergence of tumors during treatment, untargeted differentiation and autoimmunity, *etc.* Therefore, further understanding of stem cell therapy for insulin is needed.

Key Words: Diabetes mellitus; Stem cell therapy; Transplantation; β cell; Differentiation

Core Tip: Diabetes mellitus is one of the major health problems. Although traditional treatments such as exogenous insulin injection can relieve diabetes to a certain extent, they have failed to achieve a radical cure. Stem cell replacement therapy is a promising treatment for diabetes. So in this review, we introduce the development schedule of mouse and human embryonic islets and summarize the important research progressions in stem cell therapy for diabetes.

INTRODUCTION

Diabetes mellitus is one of the major health problems now. This disease currently affects more than 425 million individuals and the prevalence of it is a year by year (http://www.idf.org/diabetesatlas). About 537 million adults (20-79 years) worldwide are living with diabetes. This number is predicted to rise to 643 million by 2030 and 783 million by 2045. Among all patients with diabetes, about 10% individuals suffer from type 1 diabetes mellitus (T1DM), a kind of diabetes that is caused by the autoimmune destruction of insulin-secreting β cells in the pancreas, and about 90% of those are affected by type 2 diabetes mellitus (T2DM) due to the insulin resistance in key metabolic tissues and the dysfunction of pancreatic insulin-secreting β cells[1,2]. Because both types of patients require exogenous insulin supplementation to regulate blood sugar, they are collectively call insulin-dependent diabetes[3].

Although treatment can be effective, it often induces hypoglycemia and complex complications[4,5]. Therefore, β cells replacement therapy had transplant in 1966[6] and the development of schemes for islet isolation[7], and the first clinical islet transplantation was carried out in 1977[8]. However, both pancreas and islet transplantation are severely limited affected by donor tissue sources and immunosuppressive demand[9,10].

Compared with the limitations of human donor-derived β cells, the differentiation of insulin β cells from pluripotent stem cells (PSCs) may be a more suitable method. Embryonic stem cells were first isolated by Evans and Kaufman in 1981[11], and human embryonic stem cells were first cultured in 1998 by Thomson *et al*[12]. In 2007, Voltarelli *et al*[13] conducted a phase I/II trial of T1DM patients with hematopoietic stem cells (one kind of adult stem cells) transplantation, which showed that autologous stem cells transplantation could produce insulin. In this review, we will summarize the specific process of differentiation of definitive endoderm (DE) into insulin-secreting β cells in the existing literature, and discuss the advances of promising stem cell therapy for insulin-dependent diabetes in recent years, and finally, explore its future development direction.

PANCREAS DEVELOPMENT: FROM MULTIPOTENT PANCREAS PROGENITORS TO ENDOCRINE CELLS

The pancreas is an important organ with both endocrine and digestive functions. Because human materials are difficult to obtain, most researchers currently use mouse models to explore pancreatic embryonic development. In this review, mouse embryonic age is converted into human embryonic age, and the possible process of human pancreatic embryonic development is described from front to back on the basis of mouse model according to the timeline.

The timeline of human embryonic development is based on age estimates until 60 d post conception when identifiable human characteristics become apparent and then the name changes from embryo to fetus[14].

Under the activation of epidermal growth factor, fibroblast growth factor 1, 7 and 10 (FGF1, FGF7and FGF10), multipotent pancreas progenitors (MPPs) proliferate in a large amount[15]. The levels of FGF7 and FGF10 parallel MPPs expansion[16], and they activate the proliferation of human pancreatic epithelial cells *in vitro*[17]. During 6-7 wpc, pancreatic trunk and tip domains are formed from the foregut[18], and the former will continue to differentiate into ductal cells and endocrine cells, while the latter will differentiate into acinar cells. Progenitors in the tip domain have the ability to develop into all pancreatic epithelial cells initially, including acinar, ductal, and endocrine cells. The cells remaining at the distal end of the epithelial structure then undergo acinar differentiation, while those near the apical end become bipotent progenitors (endocrine/duct), also known as sex determining region Y-box 9 (SOX9+) bipotent progenitors. Acinar apical cells are isolated from the trunk by the antagonistic relationship between Nirenberg and Kim homeobox factor 6.1 (NKX6.1) and pancreas transcription

factor 1A (PTF1A). PTF1A is gradually localized only in tip cells, while NKX6.1, SOX9 and hepatocyte nuclear factor 1 homeobox B (HNF1B) are located in the trunk domain, whilst SOX9, NKX6.1 are limited to ductal lineages ultimately. Under the mediation of FGF7, FGF 10, lamimin-1, and WNT-activating ligands, the ducts and acini form the exocrine part of the pancreas[19]. Acini secrete digestive enzymes such as trypsin, chymotrypsin, lipase and amylase, and their differentiation is regulated by a series of transcription factors, including PTF1A and MIST1[20], While the molecular mechanisms by which ducts are regulated is not completely clear, it is thought that HNF1B and HNF6 are active in ductal cells[19]. Ductal cells are polar and ciliated, forming tubular networks and secreting bicarbonates and mucins[21].

During 7-8 wpc, insulin+ cells are first detected in human pancreas[18]. At around 8 wpc, the left ventral bud degenerate and the right ventral bud fuse with the dorsal bud due to the gut rotation movement[22]. The expression of transcription factor neurogenin 3 (NGN3) increases with the appearance of human fetal β cells around 8 wpc, but the expression of NGN3 in human fetus is transient and reaches its peak around 10-14 wpc, and cannot be detected after 35 wpc[23]. Around e9.5, a small group of cells in the thickened DE epithelium begin to express the basic helix-loop-helix transcription factor NGN3[24]. Studies have shown that these NGN3+ cells are islet progenitors because SOX9+ bipotent progenitors depend on the regulation of NGN3 to differentiate into endocrine or ductal cells, and cells expressing NGN3 produce all islet lineage cells[18]. The evidence to this effect are as follows: islet cells are not observed in NGN3 knockout mice[24]. Mice with *NGN3* gene defects failed to develop all endocrine cells and died of diabetes 1-3 d after birth[24]. Genealogical tracing experiments showed that NGN3+ cells could produce all pancreatic endocrine cells[25].

In adult pancreas, purified NGN3+ cells activated by partial ductal ligation could differentiate into all islet cell types after being injected into embryonic pancreas *in vitro*[26]. In human, biallelic mutations of NGN3 caused permanent neonatal diabetes mellitus[27]. The efficiency of endocrine cells induced by NGN3 was low. Only 3%-4.5% of SOX9+ progenitors express NGN3 at the peak, which means that in order to produce a sufficient number of islet cells in the human body, it takes a long time for endocrine cells to be induced, and it is still unknown why only some SOX9+ cells activate the expression of NGN3 [23].

After the expression of NGN3, pro-endocrine cells trigger the expression of downstream endocrine transcription factor genes. These include pancreatic and duodenal homeobox 1 (PDX1), NKX6.1, paired box protein 4 and 6 (PAX4 and PAX6), neurogenic differentiation 1, aristaless related homeobox, regulatory factor X6, NKX2.2 *etc.*, expressed around 8 wpc[28]. These progenitors migrate into mesenchyme and form islets composed of α, β, δ, pancreatic polypeptide (PP) and ε cells. Glucagon, insulin, somatostatin (SST), PPY and ghrelin are produced respectively. Insulin acts on peripheral tissues such as liver, muscle, and adipose tissue to increase glucose utilization and lower blood glucose, while glucagon increases blood glucose concentration by acting on liver, brain, adipose tissue, and heart [29]. These two hormones are the key to maintaining blood glucose homeostasis. There is a close paracrine regulatory loop between α and β cells. For example, β cells secrete urocortin 3 to stimulate the release of SST, and SST inhibits the secretion of glucagon from α cells[30]; α cells also produce ghrelin to inhibit insulin secretion and stimulate their own glucagon secretion[31]. Around 8.5 wpc, the expression of glucagon or SST can be probed; at 9 wpc, cells with polypeptide+ or ghrelin+ appear[18,32]. Not all cells with the positive expression of NGN3 are identical in their developmental potential, and we do not fully understand how NGN3+ cells decide to differentiate into specific endocrine subtypes yet.

Although most researchers currently use mouse models to explore pancreatic embryonic development due to the lack of human materials, we cannot ignore the differences between mouse and human pancreatic embryogenesis. The following outlines the differences between human and mouse pancreatic embryogenesis that are currently known (Table 1).

IMPORTANT ACHIEVEMENTS IN DIFFERENTIATION OF PLURIPOTENT STEM CELLS INTO INSULIN-SECRETING B CELLS

After fertilization, mammalian embryos undergo a series of cellular divisions to form morulae and are transformed into blastocyst by further cell division. The cells at the outer layer of the blastocyst differentiate into the trophoblast, forming the majority of the placenta that sustains nutrient supply to the embryo, and the inner cell mass (ICM) cells located inside the blastocyst maintain pluripotency and produce all cell types of the extraembryonic tissues and the embryos. In 1981, Evans and Kaufman[11] demonstrated that mouse ICM cells could be isolated and cultured *in vitro* without losing their pluripotency. Because they could mimic the differentiation ability of ICM cells, the cultured cells were called embryonic stem cells. Embryonic stem cells are a class of cells isolated from early embryos that could proliferate and self-renew indefinitely. Whether *in vivo* or *in vitro*, embryonic stem cells can be induced to differentiate into almost all types of cells in the inner, middle and outer germ layers. If embryonic stem cells are transplanted into patients, it is possible to replace a variety of damaged cells, and restore the corresponding function.

Table 1 The differences between human and mouse pancreatic embryogenesis

Differences	Mice	Human	Ref.
Morphological change	Early separation of foregut from notochord: e8.75	Delayed separation of foregut from notochord: 4-5 wpc	[58,59]
	Early formation of tip and trunk of pancreass: e14-e15	The tip and trunk pancreas form late: 6-8 wpc	[18,60, 61]
	Late islet formation: Endocrine cells do not aggregate until birth to form islets	Islet formation is early: formation begins at 12 wpc	[62,63]
Expression of transcription factors	PDX1: Early expression, the current intestinal and notochord is still in contact with the expression	PDX1: Late expression, delayed until the foregut and notochord separated from each other	[58,59]
	NKX2.2: When it was confined to NGN3+ progenitor cells, it was widely expressed in mouse MPPs up to e13	NKX2.2: This expression does not appear until the cells have differentiated into endocrine lineage in human	[18,64]
	SOX17: It was not present in mouse pancreatic epithelial cells	SOX17: Markers specific to the endoderm of the human islet	[65]
Endocrine cell formation	α cells: e8.5	α cells: 8-9 wpc	[25,62, 66]
	β cells: e10.5-e.12.5	β cells: 6 wpc	
	δ cells: e14.5	δ cells: 10 wpc	
	PP cells: e10.5-e.12.5	PP cells: 17 wpc	

Embryonic stem cells were the first type of stem cell used to induce β cell *in vitro*. In 2001, Lumelsky *et al*[33] induced mouse embryonic stem cells to differentiate into insulin+ cells by "five-stage differentiation protocol" for the first time. In 2005, D'Amour *et al*[34] also designed a "five-stage differentiation protocol" to induce embryonic stem cells to differentiate into insulin producing cells (IPCs). This method mainly simulated the process of pancreatic development in vivo. In 2008, Kroon *et al*[35] designed a "four-stage differentiation protocol" based on the "five-stage differentiation protocol" of D'Amour's team. The result showed that after pancreatic endoderm derived from human ESCs was transplanted into mice *in vitro*, the team obtained IPCs that were matured, functional and responsive to the changes of glucose level in the environment, and its function was similar to that of human mature islets. In 2014, Pagliuca *et al*[36] induced human ESCs to differentiate into insulin-secreting β cells for the first time.

Induced pluripotent stem cells are autologous pluripotent stem cells with multipotent differentiation potential generated by reprogramming somatic cells. In 2006, Takahashi *et al*[37] reprogrammed mouse somatic cells into pluripotent stem cells by adding four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) for the first time. They showed that pluripotent stem cells can be directly induced by the addition of only a few defined factors. Until now, the classical induction pattern of four transcription factors has been broken, and the number of added transcription factors has been reduced from four to two or even one[38]. Since ectopic expression of c-Myc can cause tumorigenicity of progeny, generation of pluripotent stem cells with minimal factors may reduce the risk of treatment. The study found that neural stem cells expressed endogenous levels of Sox2 and c-Myc higher than embryonic stem cells in adult mice, and that exogenous Oct4 together with either Klf4 or c-Myc is sufficient to induce pluripotent stem cells. In 2016, Zhu *et al*[39] reprogrammed skin cells to transform into endodermal progenitors using drugs and genetic molecules for the first time. After adding four other molecules, the endodermal progenitors were transformed into pancreatic precursors and they developed into fully functional pancreatic β cells. These cells protect mice from diabetes and are capable of producing different doses of insulin in response to the changes in blood glucose levels.

Adult stem cells are a kind of undifferentiated cells that exist in differentiated tissues. They have the characteristics of self-renewal and multi-differentiation potential, and they exist in a variety of tissues and organs. Bone marrow mesenchymal stem cells[40], adipose mesenchymal stem cells[41,42] and pancreatic mesenchymal stem cells[43,44] are widely studied. In 2013, Lima *et al*[44] induced pancreatic mesenchymal stem cells to differentiate into induced pluripotent stem cells (iPSCs) by adenovirus transfection of PDX1, NGN3, macrophage-activating factor A and PAX4.

THE PROGRESSIONS IN STEM CELL THERAPY FOR INSULIN-INDEPENDENT DIABETES

At present, among many methods for treating diabetes, islet replacement therapy can be regarded as an effective treatment method to relieve diabetes, especially for insulin dependent type 1 diabetes. However, there are two problems in islet donors[45,46]: (1) islet donors are in short supply, and recipients may face huge costs for surgery and postoperative follow-up treatment; and (2) the most critical is the existence of organ immune rejection. The emergence of stem cell therapy provides a new

way to solve these problems.

As mentioned above, stem cells are produced in various ways and from various sources. Different types of stem cells have different applications and clinical limitations (Table 2). Embryonic stem cells (ESCs, derived from the ICM of pre-implanted embryo) have limitations such as high tumorigenic risk, obvious host immune rejection and ethical controversy[36,38,39]. Therefore, the clinical application of ESCs is not clear. The biological characteristics of IPSCs (derived from embryonic gonadal ridge or postnatal testes) are highly similar to ESCs, and their biggest advantage lie in: by obtaining specific IPSCs from diabetic patients themselves, immune rejection and ethical controversies in ESCs transplantation can be effectively avoided[41]. IPSCs-derived β cells have been considered as a potential alternative source of β cells for T1DM[47] (Figure 1). However, IPSCs technology still faces the following problems[48-50]: (1) high genetic variability between individual cell lines can result in immature function of derived β cells. This mutation has been found to be repairable using genome editing tools such as CRISPR-Cas9, *etc*[51]. To target diabetes caused by single-gene disease in 2020 the study demonstrated that CRISPR-Cas9 correction of diabetes-induced gene variants enhances differentiation of autologous SC-cells, corrects glucose in diabetic mice, and alleviates endoplasmic reticulum and mitochondrial stress in β cells, thereby protecting pancreatic β cells[52]; (2) IPSCs have very low differentiation efficiency *in vitro* and may become cancerous. In 2022, Chinese scholar Deng Hongkui's research group[53] adjusted the *in vitro* differentiation scheme and realized efficient induction of differentiation, thus making it possible to prepare functional mature islet cells on a large scale *in vitro*. The research team transplanted islet cells differentiated from IPSCs into diabetic money model, which effectively reversed diabetes in mice, and no tumor-causing phenomenon was observed in all transplanted mice during the observation period of up to 48 wk. And this year, Chinese researchers have found[54] that the bromine-containing domain and the additional terminal domain family protein inhibitor I-BET151 can effectively promote the amplification of PPs. These expandable islet progenitors (ePPs) maintain the islet progenitor status for a long time and have the ability to efficiently differentiate into functional mature islet β cells (ePP-β). In particular, ePP-β cells can be transplanted *in vivo* to rapidly improve diabetes in mice, and thus have great potential in cell transplantation therapy; and (3) There is transplantation immune rejection. Studies have shown that using *CRISPR* gene editing to knock out the *β2-microglobulin* gene could eliminate all HLA class I molecules, or deleting double alleles of HLA-A and HLAB, leaving only one allele of HLA-C. This allows the IPSCs-induced β cells to avoid T and NK cell attack after transplantation[55]. Mesenchymal stem cells (MSCs) are adult stem cells (derived from postnatal tissues) with self-replicating ability and multidirectional differentiation potential. Among them, human bone marrow MSCs, umbilical cord blood mesenchymal stem cells and adipose tissue MSCs are widely used. In recent years, MSC based clinical trials in patients with T1DM and T2DM diabetes were conducted, and in 2014, the first islet organ transplantation from MSC was performed in T1D patients to evaluate the efficacy and safety of stem cells in the treatment of type 1 diabetes[47]. In 2018, it was found that MSCS (ASC) in adipose tissue can effectively reduce blood glucose, improve insulin sensitivity, improve islet β cell function and reduce fat deposition in liver of type 2 diabetic mice[56].

ViaCyte's research and development focuses on the targeted differentiation of pluripotent stem cells into PPs, which are encapsulated and trialed to treat type 1 diabetes. These pancreatic progenitor cells encased in biomaterial "envelopes" (cysts) require further differentiation *in vivo* to develop into islet cells for optimal implant size for therapeutic effect and long-term viability of transplanted cells[54]. In July 2021, the company released the latest clinical data of its VC-02 islet cell replacement therapy for type 1 diabetes: implanted islet progenitor cells produced endogenous insulin in patients with clinical manifestation of increased glucose reactive C-peptide levels, with time delay, and decreased HbA1C. This led to the further development of stem cell therapy for diabetes. ViaCyte, in partnership with CRISPR Therapeutics, is currently developing immune avoidant stem cell lines that combine the two strategies. Methods designed to induce immune protection may produce cells that cannot be recognized and thus cleared by the immune system[57], which will further improve the safety of stem cell-derived islets transplanted into diabetic patients and effectively improve their postoperative quality of life. Unlike ViaCyte, Vertex is designed to grow fully differentiated islet cells in the laboratory and transplant them directly into patients without encapsulation. In February 2021, the FDA approved the application for clinical trial of a new drug (VX-880) for the treatment of type 1 diabetes with stem cells. In October, Vertex's clinical trial showed that the first patient treated with its stem-derived islet cells not only resumed insulin production 90 d after treatment, but also reduced daily insulin use by 91 percent.

CONCLUSION

In the past 20 years, researchers have made great progresses on how to induce stem cells to differentiate into pancreatic cells, and then insulin-secreting β cells. We also have a further understanding of the treatment of diabetes by islet transplantation. However, although some studies have been able to induce stem cells to differentiate into insulin-secreting β cells, these cells are cells with immature phenotypes, which are different from normal human β cells. Moreover, these studies have low differentiation

Table 2 Advantages and disadvantages of different types of stem cell therapy for diabetes

Cell types	Advantages	Disadvantages	Ref.
Embryonic stem cell	High degree of differentiation	ESCs is weak in directional differentiation and difficult to induce	[35, 36]
		There are ethical issues: ESCs are usually allogeneic	
		Teratoma, immune rejection and gene mutation may occur after transplantation	
Induced pluripotent stem cell	IPSC technology does not use embryonic or egg cells, so ethical problems are less likely	At present, the differentiation scheme of induced pluripotent stem cells is not mature, and the induction efficiency is low, the stability is poor, and the cost is high	[39]
	Proprietary stem cells can be made from a patient's own cells, so there is less immune rejection	The use of virus vectors poses security problems	
Adult stem cell	It is easy to achieve targeted differentiation, and some studies have shown that adult stem cells can be used to treat diabetes	The direction of differentiation is limited, not omnipotent	[43, 44]
		After transplantation, the ability of induced differentiated cells to secrete insulin was usually lower than that of normal islet β cells, and the cell survival rate was also lower	
		The efficiency of inducing differentiation at different stages is still low based on reprogramming and small molecule screening	

IPSC: Induced pluripotent stem cell.

DOI: 10.4252/wjsc.v14.i7.503 **Copyright** ©The Author(s) 2022.
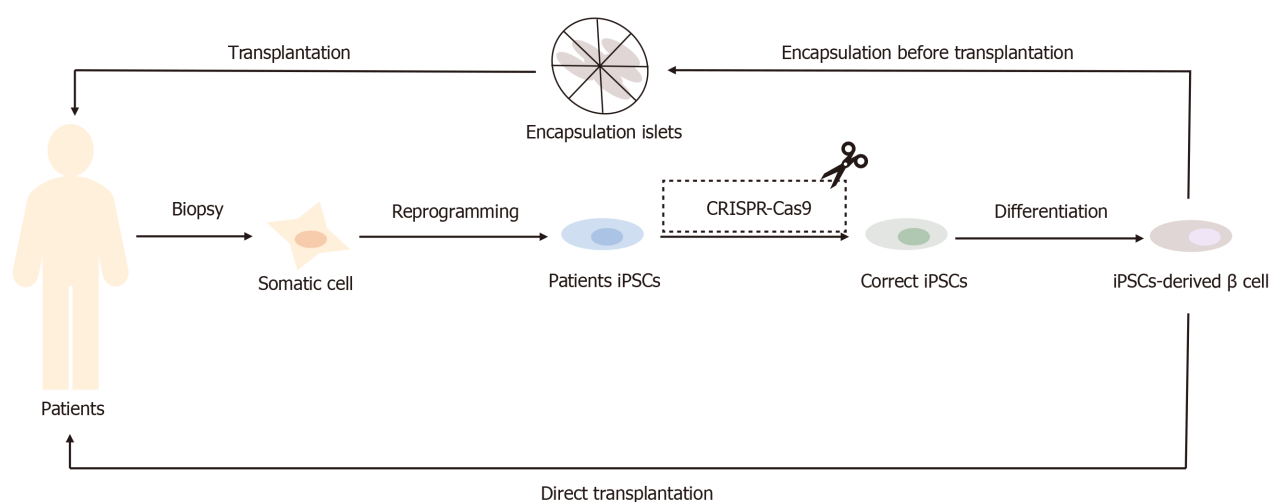
Figure 1 Application of induced pluripotent stem cells in the treatment of diabetes mellitus. The In insulin-dependent diabetes patients, induced pluripotent stem cells (IPSCs)-derived β cells can be induced by autologous IPSCs and then directly or indirectly transplanted back into the body after encapsulation to achieve the effect of diabetes treatment. For patients with monogenic diabetes, such as Wolfram syndrome patients, IPSCs-derived β cells with correct coding can also be obtained after *CRISPR-Cas9* gene modification technology and then transplanted. IPSCs: Induced pluripotent stem cells.

efficiencies, thus stem cells cannot fully develop into insulin-secreting β cells.

Therefore, the selection of stem cells, the identification of maturation inducing factors in vivo, and the improvement methods of culture efficiency are all problems that need to be solved. In addition, there are other difficulties to overcome as follows: (1) the evaluation method of stem cells derived endocrine cells function; (2) the selection of surface antigens of progenitors in specific differentiation stage and the formulation of cell purification methods; and (3) the appearances of tumor, untargeted differentiation and autoimmunity during the treatment with stem cells, as well as the biosafety and reliability of the treatment.

On the whole, the application of genomics, epigenomics, proteomics and other methods to characterize the differentiation products and the cooperation with biotechnology and pharmaceutical departments are conducive to promoting the development of progenitors in specific stages to mature insulin-secreting β cells. In the near future, stem cell replacement therapy may be clinically applicable to diabetes.

FOOTNOTES

REFERENCES

1   **Halban PA**, Polonsky KS, Bowden DW, Hawkins MA, Ling C, Mather KJ, Powers AC, Rhodes CJ, Sussel L, Weir GC. β-cell failure in type 2 diabetes: postulated mechanisms and prospects for prevention and treatment. *Diabetes Care* 2014; **37**: 1751-1758 [PMID: 24812433 DOI: 10.2337/dc14-0396]

2   **Jiang FX**, Morahan G. Multipotent pancreas progenitors: Inconclusive but pivotal topic. *World J Stem Cells* 2015; **7**: 1251-1261 [PMID: 26730269 DOI: 10.4252/wjsc.v7.i11.1251]

3   **Yalow RS**, Berson SA. Immunoassay of endogenous plasma insulin in man. *J Clin Invest* 1960; **39**: 1157-1175 [PMID: 13846364 DOI: 10.1172/JCI104130]

4   **Magliano DJ**, Harding JL, Shaw JE. Glycemic control and excess mortality in type 1 diabetes. *N Engl J Med* 2015; **372**: 880 [PMID: 25714171 DOI: 10.1056/NEJMc1415677]

5   **Khunti K**, Davies M, Majeed A, Thorsted BL, Wolden ML, Paul SK. Hypoglycemia and risk of cardiovascular disease and all-cause mortality in insulin-treated people with type 1 and type 2 diabetes: a cohort study. *Diabetes Care* 2015; **38**: 316-322 [PMID: 25492401 DOI: 10.2337/dc14-0920]

6   **Kelly WD**, Lillehei RC, Merkel FK, Idezuki Y, Goetz FC. Allotransplantation of the pancreas and duodenum along with the kidney in diabetic nephropathy. *Surgery* 1967; **61**: 827-837 [PMID: 5338113]

7   **Ricordi C**, Lacy PE, Scharp DW. Automated islet isolation from human pancreas. *Diabetes* 1989; **38** Suppl 1: 140-142 [PMID: 2642838 DOI: 10.2337/diab.38.1.s140]

8   **Najarian JS**, Sutherland DE, Matas AJ, Steffes MW, Simmons RL, Goetz FC. Human islet transplantation: a preliminary report. *Transplant Proc* 1977; **9**: 233-236 [PMID: 405770]

9   **Kandaswamy R**, Stock PG, Gustafson SK, Skeans MA, Urban R, Fox A, Israni AK, Snyder JJ, Kasiske BL. OPTN/SRTR 2018 Annual Data Report: Pancreas. *Am J Transplant* 2020; **20** Suppl s1: 131-192 [PMID: 31898415 DOI: 10.1111/ajt.15673]

10   **Balamurugan AN**, Naziruddin B, Lockridge A, Tiwari M, Loganathan G, Takita M, Matsumoto S, Papas K, Trieger M, Rainis H, Kin T, Kay TW, Wease S, Messinger S, Ricordi C, Alejandro R, Markmann J, Kerr-Conti J, Rickels MR, Liu C, Zhang X, Witkowski P, Posselt A, Maffi P, Secchi A, Berney T, O'Connell PJ, Hering BJ, Barton FB. Islet product characteristics and factors related to successful human islet transplantation from the Collaborative Islet Transplant Registry (CITR) 1999-2010. *Am J Transplant* 2014; **14**: 2595-2606 [PMID: 25278159 DOI: 10.1111/ajt.12872]

11   **Evans MJ**, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981; **292**: 154-156 [PMID: 7242681 DOI: 10.1038/292154a0]

12   **Thomson JA**, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science* 1998; **282**: 1145-1147 [PMID: 9804556 DOI: 10.1126/science.282.5391.1145]

13   **Voltarelli JC**, Couri CE, Stracieri AB, Oliveira MC, Moraes DA, Pieroni F, Coutinho M, Malmegrim KC, Foss-Freitas MC, Simões BP, Foss MC, Squiers E, Burt RK. Autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. *JAMA* 2007; **297**: 1568-1576 [PMID: 17426276 DOI: 10.1001/jama.297.14.1568]

14   **O'Rahilly R**, Müller F. Developmental stages in human embryos: revised and new measurements. *Cells Tissues Organs* 2010; **192**: 73-84 [PMID: 20185898 DOI: 10.1159/000289817]

15   **Bonfanti P**, Nobecourt E, Oshima M, Albagli-Curiel O, Laurysens V, Stangé G, Sojoodi M, Heremans Y, Heimberg H, Scharfmann R. Ex Vivo Expansion and Differentiation of Human and Mouse Fetal Pancreatic Progenitors Are Modulated

by Epidermal Growth Factor. *Stem Cells Dev* 2015; **24**: 1766-1778 [PMID: 25925840 DOI: 10.1089/scd.2014.0550]

16   **Benazra M**, Lecomte MJ, Colace C, Müller A, Machado C, Pechberty S, Bricout-Neveu E, Grenier-Godard M, Solimena M, Scharfmann R, Czernichow P, Ravassard P. A human beta cell line with drug inducible excision of immortalizing transgenes. *Mol Metab* 2015; **4**: 916-925 [PMID: 26909308 DOI: 10.1016/j.molmet.2015.09.008]

17   **Ye F**, Duvillié B, Scharfmann R. Fibroblast growth factors 7 and 10 are expressed in the human embryonic pancreatic mesenchyme and promote the proliferation of embryonic pancreatic epithelial cells. *Diabetologia* 2005; **48**: 277-281 [PMID: 15690149 DOI: 10.1007/s00125-004-1638-6]

18   **Jennings RE**, Berry AA, Kirkwood-Wilson R, Roberts NA, Hearn T, Salisbury RJ, Blaylock J, Piper Hanley K, Hanley NA. Development of the human pancreas from foregut to endocrine commitment. *Diabetes* 2013; **62**: 3514-3522 [PMID: 23630303 DOI: 10.2337/db12-1479]

19   **Jacobson EF**, Tzanakakis ES. Human pluripotent stem cell differentiation to functional pancreatic cells for diabetes therapies: Innovations, challenges and future directions. *J Biol Eng* 2017; **11**: 21 [PMID: 28680477 DOI: 10.1186/s13036-017-0066-3]

20   **Reichert M**, Rustgi AK. Pancreatic ductal cells in development, regeneration, and neoplasia. *J Clin Invest* 2011; **121**: 4572-4578 [PMID: 22133881 DOI: 10.1172/JCI57131]

21   **Benitez CM**, Goodyer WR, Kim SK. Deconstructing pancreas developmental biology. *Cold Spring Harb Perspect Biol* 2012; **4** [PMID: 22587935 DOI: 10.1101/cshperspect.a012401]

22   **Sarkar A**, Hochedlinger K. The sox family of transcription factors: versatile regulators of stem and progenitor cell fate. *Cell Stem Cell* 2013; **12**: 15-30 [PMID: 23290134 DOI: 10.1016/j.stem.2012.12.007]

23   **Salisbury RJ**, Blaylock J, Berry AA, Jennings RE, De Krijger R, Piper Hanley K, Hanley NA. The window period of NEUROGENIN3 during human gestation. *Islets* 2014; **6**: e954436 [PMID: 25322831 DOI: 10.4161/19382014.2014.954436]

24   **Gradwohl G**, Dierich A, LeMeur M, Guillemot F. neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *Proc Natl Acad Sci U S A* 2000; **97**: 1607-1611 [PMID: 10677506 DOI: 10.1073/pnas.97.4.1607]

25   **Johansson KA**, Dursun U, Jordan N, Gu G, Beermann F, Gradwohl G, Grapin-Botton A. Temporal control of neurogenin3 activity in pancreas progenitors reveals competence windows for the generation of different endocrine cell types. *Dev Cell* 2007; **12**: 457-465 [PMID: 17336910 DOI: 10.1016/j.devcel.2007.02.010]

26   **Xu X**, D'Hoker J, Stangé G, Bonné S, De Leu N, Xiao X, Van de Casteele M, Mellitzer G, Ling Z, Pipeleers D, Bouwens L, Scharfmann R, Gradwohl G, Heimberg H. Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. *Cell* 2008; **132**: 197-207 [PMID: 18243096 DOI: 10.1016/j.cell.2007.12.015]

27   **Rubio-Cabezas O**, Jensen JN, Hodgson MI, Codner E, Ellard S, Serup P, Hattersley AT. Permanent Neonatal Diabetes and Enteric Anendocrinosis Associated With Biallelic Mutations in NEUROG3. *Diabetes* 2011; **60**: 1349-1353 [PMID: 21378176 DOI: 10.2337/db10-1008]

28   **Jennings RE**, Berry AA, Strutt JP, Gerrard DT, Hanley NA. Human pancreas development. *Development* 2015; **142**: 3126-3137 [PMID: 26395141 DOI: 10.1242/dev.120063]

29   **Campbell JE**, Drucker DJ. Islet α cells and glucagon--critical regulators of energy homeostasis. *Nat Rev Endocrinol* 2015; **11**: 329-338 [PMID: 25850661 DOI: 10.1038/nrendo.2015.51]

30   **van der Meulen T**, Donaldson CJ, Cáceres E, Hunter AE, Cowing-Zitron C, Pound LD, Adams MW, Zembrzycki A, Grove KL, Huising MO. Urocortin3 mediates somatostatin-dependent negative feedback control of insulin secretion. *Nat Med* 2015; **21**: 769-776 [PMID: 26076035 DOI: 10.1038/nm.3872]

31   **Chuang JC**, Sakata I, Kohno D, Perello M, Osborne-Lawrence S, Repa JJ, Zigman JM. Ghrelin directly stimulates glucagon secretion from pancreatic alpha-cells. *Mol Endocrinol* 2011; **25**: 1600-1611 [PMID: 21719535 DOI: 10.1210/me.2011-1001]

32   **Riedel MJ**, Asadi A, Wang R, Ao Z, Warnock GL, Kieffer TJ. Immunohistochemical characterisation of cells co-producing insulin and glucagon in the developing human pancreas. *Diabetologia* 2012; **55**: 372-381 [PMID: 22038519 DOI: 10.1007/s00125-011-2344-9]

33   **Lumelsky N**, Blondel O, Laeng P, Velasco I, Ravin R, McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* 2001; **292**: 1389-1394 [PMID: 11326082 DOI: 10.1126/science.1058866]

34   **D'Amour KA**, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol* 2006; **24**: 1392-1401 [PMID: 17053790 DOI: 10.1038/nbt1259]

35   **Kroon E**, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazer S, Young H, Richardson M, Smart NG, Cunningham J, Agulnick AD, D'Amour KA, Carpenter MK, Baetge EE. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nat Biotechnol* 2008; **26**: 443-452 [PMID: 18288110 DOI: 10.1038/nbt1393]

36   **Pagliuca FW**, Millman JR, Gürtler M, Segel M, Van Dervort A, Ryu JH, Peterson QP, Greiner D, Melton DA. Generation of functional human pancreatic β cells in vitro. *Cell* 2014; **159**: 428-439 [PMID: 25303535 DOI: 10.1016/j.cell.2014.09.040]

37   **Takahashi K**, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663-676 [PMID: 16904174 DOI: 10.1016/j.cell.2006.07.024]

38   **Kim JB**, Zaehres H, Wu G, Gentile L, Ko K, Sebastiano V, Araúzo-Bravo MJ, Ruau D, Han DW, Zenke M, Schöler HR. Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. *Nature* 2008; **454**: 646-650 [PMID: 18594515 DOI: 10.1038/nature07061]

39   **Zhu S**, Russ HA, Wang X, Zhang M, Ma T, Xu T, Tang S, Hebrok M, Ding S. Human pancreatic beta-like cells converted from fibroblasts. *Nat Commun* 2016; **7**: 10080 [PMID: 26733021 DOI: 10.1038/ncomms10080]

40   **Karnieli O**, Izhar-Prato Y, Bulvik S, Efrat S. Generation of insulin-producing cells from human bone marrow mesenchymal stem cells by genetic manipulation. *Stem Cells* 2007; **25**: 2837-2844 [PMID: 17615265 DOI: 10.1634/stemcells.2007-0164]

41   **Chandra V**, G S, Phadnis S, Nair PD, Bhonde RR. Generation of pancreatic hormone-expressing islet-like cell aggregates from murine adipose tissue-derived stem cells. *Stem Cells* 2009; **27**: 1941-1953 [PMID: 19544426 DOI: 10.1002/stem.117]

42   **Zuk PA**, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001; **7**: 211-228 [PMID: 11304456 DOI: 10.1089/107632701300062859]

43   **Guo XR**, Wang XL, Li MC, Yuan YH, Chen Y, Zou DD, Bian LJ, Li DS. PDX-1 mRNA-induced reprogramming of mouse pancreas-derived mesenchymal stem cells into insulin-producing cells in vitro. *Clin Exp Med* 2015; **15**: 501-509 [PMID: 25345580 DOI: 10.1007/s10238-014-0319-0]

44   **Lima MJ**, Muir KR, Docherty HM, Drummond R, McGowan NW, Forbes S, Heremans Y, Houbracken I, Ross JA, Forbes SJ, Ravassard P, Heimberg H, Casey J, Docherty K. Suppression of epithelial-to-mesenchymal transitioning enhances *ex vivo* reprogramming of human exocrine pancreatic tissue toward functional insulin-producing β-like cells. *Diabetes* 2013; **62**: 2821-2833 [PMID: 23610058 DOI: 10.2337/db12-1256]

45   **Nakamura T**, Fujikura J, Anazawa T, Ito R, Ogura M, Okajima H, Uemoto S, Inagaki N. Long-term outcome of islet transplantation on insulin-dependent diabetes mellitus: An observational cohort study. *J Diabetes Investig* 2020; **11**: 363-372 [PMID: 31390159 DOI: 10.1111/jdi.13128]

46   **Bottino R**, Knoll MF, Knoll CA, Bertera S, Trucco MM. The Future of Islet Transplantation Is Now. *Front Med (Lausanne)* 2018; **5**: 202 [PMID: 30057900 DOI: 10.3389/fmed.2018.00202]

47   **Chen S**, Du K, Zou C. Current progress in stem cell therapy for type 1 diabetes mellitus. *Stem Cell Res Ther* 2020; **11**: 275 [PMID: 32641151 DOI: 10.1186/s13287-020-01793-6]

48   **Araki R**, Uda M, Hoki Y, Sunayama M, Nakamura M, Ando S, Sugiura M, Ideno H, Shimada A, Nifuji A, Abe M. Negligible immunogenicity of terminally differentiated cells derived from induced pluripotent or embryonic stem cells. *Nature* 2013; **494**: 100-104 [PMID: 23302801 DOI: 10.1038/nature11807]

49   **Tanosaki S**, Tohyama S, Fujita J, Someya S, Hishiki T, Matsuura T, Nakanishi H, Ohto-Nakanishi T, Akiyama T, Morita Y, Kishino Y, Okada M, Tani H, Soma Y, Nakajima K, Kanazawa H, Sugimoto M, Ko MSH, Suematsu M, Fukuda K. Fatty Acid Synthesis Is Indispensable for Survival of Human Pluripotent Stem Cells. *iScience* 2020; **23**: 101535 [PMID: 33083764 DOI: 10.1016/j.isci.2020.101535]

50   **Yabe SG**, Fukuda S, Nishida J, Takeda F, Nashiro K, Okochi H. Induction of functional islet-like cells from human iPS cells by suspension culture. *Regen Ther* 2019; **10**: 69-76 [PMID: 30623004 DOI: 10.1016/j.reth.2018.11.003]

51   **Kyttälä A**, Moraghebi R, Valensisi C, Kettunen J, Andrus C, Pasumarthy KK, Nakanishi M, Nishimura K, Ohtaka M, Weltner J, Van Handel B, Parkkonen O, Sinisalo J, Jalanko A, Hawkins RD, Woods NB, Otonkoski T, Trokovic R. Genetic Variability Overrides the Impact of Parental Cell Type and Determines iPSC Differentiation Potential. *Stem Cell Reports* 2016; **6**: 200-212 [PMID: 26777058 DOI: 10.1016/j.stemcr.2015.12.009]

52   **Maxwell KG**, Augsornworawat P, Velazco-Cruz L, Kim MH, Asada R, Hogrebe NJ, Morikawa S, Urano F, Millman JR. Gene-edited human stem cell-derived β cells from a patient with monogenic diabetes reverse preexisting diabetes in mice. *Sci Transl Med* 2020; **12** [PMID: 32321868 DOI: 10.1126/scitranslmed.aax9106]

53   **Du Y**, Liang Z, Wang S, Sun D, Wang X, Liew SY, Lu S, Wu S, Jiang Y, Wang Y, Zhang B, Yu W, Lu Z, Pu Y, Zhang Y, Long H, Xiao S, Liang R, Zhang Z, Guan J, Wang J, Ren H, Wei Y, Zhao J, Sun S, Liu T, Meng G, Wang L, Gu J, Wang T, Liu Y, Li C, Tang C, Shen Z, Peng X, Deng H. Human pluripotent stem-cell-derived islets ameliorate diabetes in non-human primates. *Nat Med* 2022; **28**: 272-282 [PMID: 35115708 DOI: 10.1038/s41591-021-01645-7]

54   **Ma X**, Lu Y, Zhou Z, Li Q, Chen X, Wang W, Jin Y, Hu Z, Chen G, Deng Q, Shang W, Wang H, Fu H, He X, Feng XH, Zhu S. Human expandable pancreatic progenitor-derived β cells ameliorate diabetes. *Sci Adv* 2022; **8**: eabk1826 [PMID: 35196077 DOI: 10.1126/sciadv.abk1826]

55   **Xu H**, Wang B, Ono M, Kagita A, Fujii K, Sasakawa N, Ueda T, Gee P, Nishikawa M, Nomura M, Kitaoka F, Takahashi T, Okita K, Yoshida Y, Kaneko S, Hotta A. Targeted Disruption of HLA Genes *via* CRISPR-Cas9 Generates iPSCs with Enhanced Immune Compatibility. *Cell Stem Cell* 2019; **24**: 566-578.e7 [PMID: 30853558 DOI: 10.1016/j.stem.2019.02.005]

56   **Wang M**, Song L, Strange C, Dong X, Wang H. Therapeutic effects of adipose stem cells from diabetic mice for the treatment of type 2 diabetes. *Mol Ther* 2018; **26**: 1921-1930 [PMID: 30005867 DOI: 10.1016/j.ymthe.2018.06.013]

57   **de Klerk E**, Hebrok M. Stem Cell-Based Clinical Trials for Diabetes Mellitus. *Front Endocrinol (Lausanne)* 2021; **12**: 631463 [PMID: 33716982 DOI: 10.3389/fendo.2021.631463]

58   **Lorberbaum DS**, Docherty FM, Sussel L. Animal Models of Pancreas Development, Developmental Disorders, and Disease. *Adv Exp Med Biol* 2020; **1236**: 65-85 [PMID: 32304069 DOI: 10.1007/978-981-15-2389-2_3]

59   **Alvarez Fallas ME**, Pedraza-Arevalo S, Cujba AM, Manea T, Lambert C, Morrugares R, Sancho R. Stem/progenitor cells in normal physiology and disease of the pancreas. *Mol Cell Endocrinol* 2021; **538**: 111459 [PMID: 34543699 DOI: 10.1016/j.mce.2021.111459]

60   **Nair G**, Hebrok M. Islet formation in mice and men: lessons for the generation of functional insulin-producing β-cells from human pluripotent stem cells. *Curr Opin Genet Dev* 2015; **32**: 171-180 [PMID: 25909383 DOI: 10.1016/j.gde.2015.03.004]

61   **Napolitano T**, Avolio F, Silvano S, Forcisi S, Pfeifer A, Vieira A, Navarro-Sanz S, Friano ME, Ayachi C, Garrido-Utrilla A, Atlija J, Hadzic B, Becam J, Sousa-De-Veiga A, Plaisant MD, Balaji S, Pisani DF, Mondin M, Schmitt-Kopplin P, Amri EZ, Collombat P. Gfi1 Loss Protects against Two Models of Induced Diabetes. *Cells* 2021; **10** [PMID: 34831029 DOI: 10.3390/cells10112805]

62   **Piper K**, Brickwood S, Turnpenny LW, Cameron IT, Ball SG, Wilson DI, Hanley NA. Beta cell differentiation during early human pancreas development. *J Endocrinol* 2004; **181**: 11-23 [PMID: 15072563 DOI: 10.1677/joe.0.1810011]

63   **Perez-Frances M**, Abate MV, Baronnier D, Scherer PE, Fujitani Y, Thorel F, Herrera PL. Adult pancreatic islet endocrine cells emerge as fetal hormone-expressing cells. *Cell Rep* 2022; **38**: 110377 [PMID: 35172145 DOI: 10.1016/j.celrep.2022.110377]

64   **Ali EA**, Sardar S, Yassin MA. Priapism in lymphoproliferative disorders: A systematic review. *Hematol Oncol Stem Cell Ther* 2021 [PMID: 34157311 DOI: 10.1038/s41598-021-91774-3]

65   **Shaw-Smith C**, De Franco E, Lango Allen H, Batlle M, Flanagan SE, Borowiec M, Taplin CE, van Alfen-van der Velden

J, Cruz-Rojo J, Perez de Nanclares G, Miedzybrodzka Z, Deja G, Wlodarska I, Mlynarski W, Ferrer J, Hattersley AT, Ellard S. GATA4 mutations are a cause of neonatal and childhood-onset diabetes. *Diabetes* 2014; **63**: 2888-2894 [PMID: 24696446 DOI: 10.2337/db14-0061]

66   **Panzer JK**, Caicedo A. Targeting the Pancreatic α-Cell to Prevent Hypoglycemia in Type 1 Diabetes. *Diabetes* 2021; **70**: 2721-2732 [PMID: 34872936 DOI: 10.2337/dbi20-0048]

Prodigious therapeutic effects of combining mesenchymal stem cells with magnetic nanoparticles

Ejlal Abu-El-Rub, Ramada R Khasawneh, Fatimah Almahasneh

Ejlal Abu-El-Rub, Fatimah Almahasneh, Department of Physiology and Pathophysiology, Yarmouk University, Irbid 21163, Jordan

Ramada R Khasawneh, Department of Anatomy and Histology, Yarmouk University, Irbid 21163, Jordan

Corresponding author: Ramada R Khasawneh, PhD, Associate Professor, Department of Anatomy and Histology, Yarmouk University, Shafiq Irshidat St, Irbid 21163, Jordan. ramada@yu.edu.jo

Abstract

Mesenchymal stem cells (MSCs) have gained wide-ranging reputation in the medical research community due to their promising regenerative abilities. MSCs can be isolated from various resources mostly bone marrow, Adipose tissues and Umbilical cord. Huge advances have been achieved in comprehending the possible mechanisms underlying the therapeutic functions of MSCs. Despite the proven role of MSCs in repairing and healing of many disease modalities, many hurdles hinder the transferring of these cells in the clinical settings. Among the most reported problems encountering MSCs therapy *in vivo* are loss of tracking signal post-transplantation, insufficient migration, homing and engraftment post-infusion, and undesirable differentiation at the site of injury. Magnetic nano-particles (MNPs) have been used widely for various biomedical applications. MNPs have a metallic core stabilized by an outer coating material and their magnetic properties can be modulated by an external magnetic field. These magnetic properties of MNPs were found to enhance the quality of diagnostic imaging procedures and can be used to create a carrying system for targeted delivery of therapeutic substances mainly drug, genes and stem cells. Several studies highlighted the advantageous outcomes of combining MSCs with MNPs in potentiating their tracking, monitoring, homing, engraftment and differentiation. In this review, we will discuss the role of MNPs in promoting the therapeutic profile of MSCs which may improve the success rate of MSCs transplantation and solve many challenges that delay their clinical applicability.

Key Words: Mesenchymal stem cells; Magnetic nanoparticles; Tracking; Homing; Migration; Differentiation

Core Tip: Mesenchymal stem cells (MSCs) have been thoroughly investigated in many disease models and they showed great therapeutic potential. Despite the confirmed therapeutic abilities of MSCs, many challenges still exist which hinder the transfer of these cells to the treatment guidelines. The incorporation of magnetic nanoparticles (MNPs) with MSCs has been reported to increase the therapeutic outcomes of MSCs by solving major challenges that impede their long–term regenerative effects. MNPs are able to improve the ability to track and deliver MSCs and to increase their migration, homing, survival and differentiation *in vitro* and *in vivo*. This may help increase the success rate of MSCs transplantation and thus increase the chance to include these cells in the treatment guidelines used in different clinical settings.

INTRODUCTION

Mesenchymal stem cells (MSCs) are the mostly investigated stem cells due to their enchanting, wide-range therapeutic and regenerative potential[1]. Since their discovery by Friedenstein in 1970, MSCs have been thoroughly analyzed and characterized to discover the mechanistic explanations for their therapeutic abilities[2]. MSCs are easily reached stem cells and can be isolated from many sources including bone marrow (BM), adipose tissues and umbilical cord (UC)[3]. These cells are extensively studied compared to other types of stem cells because they are ethically benign and have low teratogenic tendency[3]. In addition, MSCs have an acceptable safety profile and less likely to cause serious side effects[3]. MSCs beneficial effects have been linked primarily to the ability of MSCs to secrete a cocktail of therapeutically active paracrine factors[4]. These paracrine factors secreted by MSCs can attenuate many pathological processes including apoptosis, necrosis, fibrosis, and inflammation and initiate repairing mechanisms in the damaged organs[4]. MSCs immunomodulatory functions also contribute strongly to their curative potential[5]. Moreover, MSCs can exert actual regeneration of the injured tissues by adopting the intrinsic machinery and differentiating to many functional cell types such as osteocytes, chondrocytes, adipocytes, and cardiomyocytes-like cells[6]. Endogenous or exogenous MSCs must migrate and home in the damaged tissues in order to gain their therapeutic benefits[3]. After homing in the damaged tissues, MSCs should endure the harsh microenvironment that may present[7]. Despite the numerous studies that highlighted the therapeutic efficiency of MSCs, many serious obstacles encumber the shift of MSCs from bench to bedside and delay their presence in the treatment guidelines[8]. The most reported post-transplantation challenges that researchers bump into when they use MSCs in clinical studies: (1) The disparities in the differentiation potential between *in vitro* and *in vivo*[9]; (2) The shift in their immunological characteristics and cytokines secretion profile under different stress microenvironments that may exist at the site of injury mainly Hypoxia and inflammation[5]; (3) The poor homing and migratory abilities of administered MSCs which may vary based on the route of injection and microenvironment status[10]; and (4) The loss of signal emitted from labelled cells due to the leakage of contrast agent after being injected, leads to difficulties in tracking and monitoring of these cells[11].

Magnetic nanoparticles (MNPs) have gained great attention among the medical researchers due to their unique biochemical and physical characteristics, their intrinsic biocompatibility and being biodegradable through normal cellular pathways which make them suitable for wide range of biomedical applications[12]. The intrinsic magnetic field elicited by the MNPs, which can be modulated externally by an applied magnetic field , is the basis for using these MNPs as contrast agents for biomedical imaging[13], biomarkers and biosensors[14], and targeted drug[15], cell and gene delivery [16]. Combining MNPs with stem cells was found to enhance their therapeutic performance and solve many challenges that hamper their regenerative potential and delay their clinical applications[17]. There are many types of MNPs that have been fabricated, but the most non-toxic and non- immunogenic MNPs that have been used with MSCs are iron oxide nanoparticles (IONs) such as magnetite (Fe_3O_4) or its oxidized form maghemite (γ-Fe_2O_3)[18-20]. These iron oxides based MNPs can be synthesized with different particles' diameters such as Superparamagnetic iron oxide (SPIO) nanoparticles (50–200 nm diameter)[21] and ultra-small SPIO (USPIO) nanoparticles (around 35 nm diameter)[22] and different types of stabilizing non-toxic coating substrates such as dextran, polyethylene glycol, and Silica[23]. In general, the uptake of MNPs by MSCs is mediated mostly through endocytosis. MNPs usually are engulfed by MSCs to form endosomes, which then transformed into Mature multivesicular endosomes (MVEs). The MVEs then combined with lysosomes and get digested and decomposed into Fe^{3+}. The free iron released into the cytoplasm of MSCs modified many cellular pathways to induce and promote their survival, migration, homing, anti-apoptosis and anti-inflammatory, and differentiation. These

magnetized MSCs can be further modulated and guided to enhance their therapeutic outcomes by external magnetic fields. The internalization of MNPs inside MSCs can be also achieved by passive diffusion if their particle size is small and by using MNPs that bind specific cell surface immune marker found on MSCs. The prodigious power of using MNPs with MSCs to potentiate their tracking, migration and homing, differentiation and regenerative abilities will be the focus of this review.

MNPs AS A CONTRAST AGENT TO TRACK MSCs

The use of MSCs in the clinical settings requires more accurate tracking methods of MSCs after transplantation to determine their destinations, survival and final differentiated fates[24]. To visualize transplanted MSCs using imaging modalities importantly the computed tomography, positron emission tomography, and magnetic resonance imaging (MRI), these cells must be labelled with contrast agents [25-28]. The problem with the traditional contrast agents is the high leakage rate which causes the loss of emitted signal after short time course[29]. The contrast features of MNPs and their high safety profile encouraged many researchers to use them for labeling MSCs prior to injection[18]. MSCs labelled with MNPs have less leaking tendency and do not affect their stemness[30], rate of proliferation and the differentiation potential beside providing higher contrast-to-noise ratio for effective imaging[31,32].

IONs are the most commonly used MNPs for labelling and tracking MSCs due to their non-toxic and non-immunogenic features, high spatial resolution and penetration depth, and the non-ionizing radiation characteristics[33]. Superparamagnetic iron oxide nanoparticles (SPIONs), ultra-small SPIO-poly (acrylic acid) (USPIO-PAA)[34], glucosamine-modified USPIO-PAA (USPIO-PAA-GlcN)[35], and microgel iron oxide (MGIO)[36] are the most studied MNPs for MSCs labelling and tracking by multiple imaging methods. Using SPIONs for stem cell labeling and tracking is a relatively new application. Recently, ferumoxytol (Feraheme®, AMAG Pharmaceuticals), an ultrasmall SPION used clinically as an MRI contrast agent[37]. Ferumoxytol colloidal particle size is less than 50 nm and can be phagocytized efficiently by the MSCs–which have an inherited phagocytosis property- and can then be imaged and tracked by MRI[37]. FeraTrack™, a dextran coated SPIONs, have a positive surface charge making it cell penetrable through a vesicular endocytosis route[38]. FeraTrack™ has gained utility as a biocompatible MRI contrast agent for cell tracking purposes due to their high safety profile[38]. Mesentier-Louro *et al* [39] used FeraTrack to track BM-MSCs at site of injury in a rodent model of optical nerve injury[39]. They reported that the after injecting FeraTrack™ labeled MSCs intravitreously, they migrated to the site of optical nerve injury and remained there for up to 18 wk which is suitable to monitor their integration with the host tissues at the site of injury using MRI. The incorporation of cationic compounds such as poly-l-lysine and protamine onto the surface coating of SPIONs can enhance labeling of MSCs by promoting interactions with the negatively charged cell surface[39]. Guldris *et al* [35] studied the contrast characteristics of SPIOs and USPIOs coated with PAA, and USPIO-PAA-GlcN as labeling agents for MSCs *in vitro*[35]. A portion of these MNPs was cultured with MSCs for 24 h at a concentration of 100 µg mL–1. In the second group, the conditions were maintained, but polylysine (PLL) was used to promote particle uptake. The study found that in the absence of PLL, SPIO-PAA showed a very low and non-homogeneous labeling efficiency. USPIO-PAA and USPIO-PAA-GlcN showed little to no internalization by MSCs, while combining USPIO-PAA-GlcN with polylysine enhances their biocompatibility with MSCs and increases the detection sensitivity by MRI in both *in vitro* and *in vivo* experiments[35]. Studies also reported that using an external pulsed magnetic field opened channels in the cell membrane and increased the uptake of SPIONs by MSCs which intensified the contrast signal[40,41]. Interestingly, Ngen and Artemov[42] developed a dual-contrast agent by combining SPIONs and gadolinium chelate to monitor and track MSCs[42]. This dual contrast agent generates powerful positive contrast and increases the signal gain[42]. Furthermore, this dual-contrast agent was also able to distinguish between dead and live cells at the site of injury. This helps in estimating the percentage of MSCs survival rate, as Gadolinium dependent positive contrast is expunged in the live cells, whereas enhanced contrast level found in dead cells[42].

MGIO particles were studied to track human fetal MSCs through using 1.5T MRI[43]. MGIO particles were found to achieve high detection sensitivity with low cellular toxicity through a simple incubation protocol, which makes them useful for cellular tracking using standard MRI scanners[43]. These results were similar to that reported by Mailänder *et al*[44] using adult BM-MSCs[44]. The tracking efficacy achieved by MGIO was higher than that achieved with USPIO particles and the larger polystyrene particles[43]. Extracellular vesicles (EVs) are secreted lipid bilayered vesicles containing enzymes, nucleic acids and lipoproteins that are involved in intercellular communication. MSCs can activate various repairing machineries by secreting EVs[45]. SPIONs were also used to facilitate the labelling and imaging of EVs derived from MSCs. Dabrowska *et al*[46] labeled these EVs derived from MSCs using the fluorescent lipophilic stain PKH26 and SPION nanoparticles conjugated with rhodamine (Molday ION Rhodamine B™) which was found to be highly biocompatible with EVs to be imaged using MRI [46]. The prospective use of MNPs in MSCs tracking is highly encouraging. MRI and MNPs are complementary and provide integrated information, like tracking and monitoring MSCs transplanting and engulfing overtime, and this will provide more information to guide further therapy.

MNPs TO ENHANCE THE HOMING OF TRANSPLANTED MSCs

Most of MSCs curative applications require injecting these cells directly to the injured tissues or delivering them intravenously, which requires their migration and homing in the damaged tissues[47, 48]. MSCs homing is one of the major challenges in clinical settings because only a small percentage of delivered MSCs reaches the desired injury site and integrates with host tissues, while the majority of the administrated cells are trapped in the draining organs and get washed.

Recently, MNPs have been used to improve the homing percentage of transplanted MSCs at the site of injury[49]. Among all nanoparticles, SPIONs are the most extensively used nanomaterials to increase MSCs homing tendency without affecting their viability, proliferation and differentiation[31,32]. MSCs labeled with SPIONs exhibit enhanced homing due to magnetic attraction[50]. Several research groups have investigated the homing and tracking of MSCs after being labelled with SPIONs. Meng *et al*[51] used SPIONs and green fluorescent protein (GFP) reporter gene to create a double labelling of Wharton's Jelly human umbilical cord-derived MSCs (WJ-MSCs)[51]. These cells were injected to a nude mouse with cutaneous tissue injury. In this work, they used 25 µg/mL of SPION, and they divided the nude mice into three groups: The first group treated with WJ-MSCs only, the second group treated with GFP/SPIONs-positive WJ-MSCs, and the third group treated with SPIONs/GFP-positive WJ-MSCs and exposed to an external magnetic field (0.5T)[51]. In all three groups, MSCs were injected subcutaneously. The results showed a remarkable increase the migration abilities of GFP/SPIONs-labeled WJ-MSCs *in vivo* without changing their inherited characteristics. The employment of a non-invasive external magnetic field provides a rapid guided homing of WJ-MSCs to the targeted injury site. Yun *et al* [48] used 15 µg/mL Rhodamine B, which was added to SPIONs to label MSCs that were injected to mouse model of wounded olfactory bulb. The Rhodamine B/SPIONs-labelled MSCs showed an improved homing by upregulating various homing factors mainly CXCR4 and CXCR4-SDF-1[51]. Yun *et al*[48] also used a magnetic field of 0.32 T to direct the Rhodamine B/SPIONs-labelled MSCs rapidly to the site of injury. Based on many studies, SPIONs enhance the MSCs homing by stimulating the expression of chemokine receptors mainly CXCR4-SDF-1α signaling[48].

A recent study by Braniste *et al*[52] in which they created a semiconductor nanoparticle by combining nanometer scale GaN thin layers with a sacrificial zinc ferrite core ($ZnFe_2O_4$)[52]. Braniste *et al*[52] incubated MSCs with (10 mg/mL) semiconductor nanoparticles and applied a remote magnetic field to control the direction of their movement. They found that these semiconductor nanoparticles were effectual to redistribute and rearrange MSCs according to the remote magnetic field intensity, thus enhanced the long term tracking and monitoring of the injected cells *in vivo*[52].

Silva *et al*[53] fabricated gold and maghemite nanoparticles that were functionalized with 2,3-dimercaptosuccinic acid (DMSA) (Au-DMSA and γ-Fe2O3-DMSA)[53]. These nanoparticles were incubated with human MSCs and these labelled MSCs were inoculated through intranasal route and tracked using standard computed microtomography. Despite the high biocompatibility of these nanoparticles with MSCs, γ-Fe2O3-DMSA and Au-DMSA based contrast was not strong enough for tracking MSCs *in vivo* by standard computed microtomography[53]. An innovative iron-doped hydroxyapatite nanoparticles (FeHA NPs) were prepared by Panseri *et al*[54] and were found to be superior to SPIONs in improving the survival of MSCs due to rapid degradation and lower resulting intracellular iron content[54]. The unique magnetic properties of FeHA NPs make them a suitable carrier for delivering MSCs to the injury site and other therapeutically active products such as drugs, growth factors, and miRNA[54].

Moayeri *et al*[55] used a poly-L-lysine hydrobromide coated SPIONs to label adipose-derived stem cells (ADSC-SPION/PLL)[55]. These labeled ADSCs were injected in the medial forebrain bundle in a rat model of Parkinson's disease (PD), and simultaneously an external magnetic field were placed on the top of rat skull for 2 wk[55]. The results of this study showed a significant improvement in the migration and homing of these labeled ADSCs in the damaged sites of substantia nigra[55]. These abovementioned studies provided strong evidence about the importance of these non-toxic and biocompatible MNPs in potentiating the homing percentage of transplanted MSCs which may improve the successful rate of MSCs transplantation in different disease models.

MNPs TO IMPROVE THE MIGRATION ABILITIES OF TRANSPLANTED MSCs

Migration and subsequent engraftment following the infusion of MSCs are essential to enkindle the regenerative power of MSCs. The desultory, undirected movement of MSCs and poor accumulation at the injured site can hinder their therapeutic abilities. It has been found that MNPs can improve the migratory features of MSCs and directed them to the target site[56]. Dextran-coated iron oxide nanoparticles have been reported by Chung *et al*[57] to boost MSCs migration and the subsequent transdifferentiation into dopaminergic like neurons in a mouse model of PD[57].

Li *et al*[33] also examined the *in vitro* migration of rat BM-MSCs to an injury site in the presence or absence of polydopamine (PDA)-capped Fe_3O_4 (Fe_3O_4@PDA) superparticles[33]. The results showed a significant difference in the number of migrated cells between control MSCs and MSCs labeled with

these superparticles[33]. Iron oxide nanoparticles were also found to increase the number of MSCs in the S-phase, their proliferation index, migration ability and secretion of vascular endothelial growth factor [47]. This suggests that labeling with iron oxide nanoparticles increased MSCs migration, while the cell cycle progression was unaffected. It was also demonstrated that labeling MSCs with Fe_3O_4@PDA NPs increase their migration towards laser burn injury sites in a living rat model, as well as their expression of CXCR4[47]. The latter could explain the increased migration ability of labeled MSCs. Indeed, previous studies had showed that the migration process is heavily dependent on the interaction between SDF-1α and CXCR4, and the internalization of magnetic iron oxide nanoparticles elevates CXCR4 levels in MSCs[58,59]. Furthermore, SPIONs have been found to activate the hepatocyte growth factor/tyrosine-protein kinase Met pathway in MSCs to regulate their migratory and engraftment properties[60].

MNPs TO POTENTIATE THE DIFFERENTIATION AND SURVIVAL OF TRANSPLANTED MSCs

The superparamagnetic properties of MNPs are not only suitable for improving the homing and migration properties of MSCs, studies found that MNPs can potentiate the MSCs survival and differentiation[61,62]. Several studies demonstrated a substantial enhancement of MSCs differentiation when these cells are combined with magnetic iron oxide nanoparticles, magnetic field and a specialized differentiation medium. MNPs improve the engraftment of MSCs at the injury site which is an essential step to adopt the cellular and molecular machinery required to initiate the differentiation to committed cell type[63-66]. MNPs can be also used to enhance the quality of MSCs cryopreservation and survival after thawing these cells[67]. Naseroleslami *et al*[68] transplanted a SPIONs-labelled human-derived MSCs (hAMSCs) in a rat model of isoproterenol-induced myocardial injury[68]. They reported that SPIONs-labeled hAMSCs produce a remarkable activation of cardiac repair machinery in the presence of magnetic field through suppressing nuclear factor-kappaB/mitogen-activated protein kinases dependent inflammation[68]. Zhang *et al*[69] reconstructed a Fe_3O_4 MNPs by adding graphene oxide (GO) to generate Fe_3O_4@GO magnetic nanocomposites (MNCs) that were loaded with bone morphogenetic protein-2 (BMP2)[69]. This Fe_3O_4@GO MNCs were able to mitigate the cell damage caused by oxidative stress and through delivering BMP2, they also improved the osteogenic differentiation abilities of MSCs[69]. Wang *et al*[70] created a magnetic lanthanum-doped HA/CS scaffolds (MLaHA/CS)[70]. They found after placing the MLaHA/CS scaffolds into rats with calvarial defects, it significantly enhances the recruitment of endogenous MSCs and facilitated regeneration of new bone matrix[70]. The dose of internalized MNPs found to have a great influence on the preferential differentiation of MSCs. When less than 10 pgFe/cell was used, the differentiation of MSCs into chondrocytes, adipocytes or osteocytes using citrate-coated maghemite nanoparticles was similar to that of control unlabeled cells[71]. On the other hand, when higher dose of 30 to 60 pgFe/cell was used, the chondrogenesis was significantly turned off while the adipogenesis and osteogenesis were turned on. Intriguingly, the source of MSCs may also govern their response to certain MNPs[72]. Labusca *et al*[73] showed some discrepancies in the response of ADSCs and WJ-MSCs to uncoated MNPs, with average size of 20 nm[73]. When external magnetic field was applied, the chondrogenic differentiation was more pronounced in the ADSCs cell culture but not in WJ-MSCs cell culture[73]. The possible explanation for these findings was the presence of an active senescent protective mechanism in WJ-MSCs. Fan *et al*[74] studied the differences in intracellular iron content, labeling efficiency, cell viability, and Adipogenic and osteogenic differentiation potentials between AD-MSCs and BM-MSCs after labelling them with SPIOs. They found that SPIO-labeled AD-MSCs and SPIO-labelled BM-MSCs were similar in their labeling efficiency, intracellular iron level, survival, proliferation, differentiation potentials, and MRI imaging[74]. Since the presence of an external magnetic field can dictate the differentiation fate of MSCs, the same group of investigators, Labusca *et al*[75], also studied the effect of duration, intensity and frequency of magnetic field on the differentiation abilities of ADSCs labeled with MNPs[75]. These scientists revealed that using an intermittent low intensity magnetic field (0.5 MT) for short time (2 d) triggered their differentiation to adipocytes, while applying intermittent high intensity magnetic field (21.6 MT) for short time (2 d) or continuous low intensity magnetic field (0.5 MT) for longer time (7 d) activated the osteogenic machinery[75]. Wang *et al*[76] injected SPION-labeled ADSCs in a rat model of stress urinary incontinence. These magnetically labeled MSCs found to have a high survival rate post-transplantation and efficiently enhanced the repairing process of the non-functional sphincter[76]. In the similar context, Xu *et al*[77] showed that UC-MSCs labelled with SPIONs can tolerate the inflammatory microenvironment in mouse model of sepsis by enhancing their immunomodulatory abilities and the expression of heme oxygenase-1 and tumor necrosis factor receptor-associated factor (TRAF1)[77]. These findings highlighted the advantageous outcomes of incorporating MNPs with MSCs therapy which may ultimately potentiate the success rate of MSCs transplantation and increase the chance to shift these cells toward bedside. Future studies should be designed to extensively investigate the long-term efficacy and safety of these MNPs labeled MSCs, and in parallel clinical trials must be conducted to reveal the translational possibilities of these MNPs–labeled MSCs. Table 1 summarizes the different studies that

Table 1 Summary of studies that used magnetic nanoparticles to improve the transplantation characteristics of mesenchymal stem cells

No.	Ref.	Magnetic nanoparticle	Source of MSCs	Application	Outcomes
1	Maggio *et al*[78], 2016	Iron MNP with poly(epsilon-lysine) dendrons exposing carboxybetaine residue (CB-MNP)	hBM-MSCs	Viability and differentiation	Survival, Adipogenic and osteogenic differentiation were significantly improved
2	Hu *et al*[79], 2021	3D printing Magnetic nanoparticles scaffold made from Ferumoxytol (γ-Fe_2O_3 @PSC) and polylysine	AD-MSCs	Bone tissue engineering and Osteogenesis	Upregulated the MAPK signaling and PI3K-Akt signaling and increased the levels of RUNX2, ALP and SMAD 1/5/8 which promoted the Osteogenic differentiation
3	Huang *et al*[80], 2017	Magnetic nanoparticle composite scaffold formulated using the magnetic nanoparticles Fe_2O_3, Nano-hydroxyapatite and l-polylactic acid	BM-MSCs	Osteogenic differentiation of MSCs	The expression of type I collagen gene increased in MSCs with noticeable enhancement in their Osteogenic differentiation without toxic effects
4	Andrzejewska *et al* [30], 2019	Molday ION Rhodamine B™	hBM-MSCs	Tracking of transplanted MSCs	Basic hBM-MSC characteristics and functions might be affected by labeling. Molday ION Rhodamine B™ labeling had a better profile than other vital stains
5	Kono *et al*[81], 2021	Magnetic anionic liposome/atelocollagen complexes	mBM-MSCs	Sarcopenia mouse model	Magnetized MSCs have higher retention rate in the skeletal muscles after their local injection with significant enhancement in their immunomodulation abilities marked by upregulating IL-6 and IL-10 and downregulating TNF-α and IL-1β in the inflamed skeletal muscle which may be useful for effective Sarcopenia treatment
6	Guldris *et al*[35], 2017	(1) SPIO-PAA; (2) USPIO-PAA; and (3) USPIO-PAA-GlcN	Rat MSCs	Cell tracking by MRI	SPIO-PAA combined with polylysine showed non-homogeneous cell internalization. USPIO-PAA showed no uptake. USPIO-PAA-GlcN featured high cellular uptake, bio-compatibility, and sensitive *in vitro* and *in vivo*
7	Lee *et al*[36], 2010	MGIO	Primary endothelial progenitor cells	*In vivo* tracking of stem cells after transplantation	MGIO is an efficient label for the studying of relaxation induced by magnetic particles and cellular tracking by MRI
8	Thu *et al*[37], 2012	Self-assembling ferumoxytol-HPF nanocomplexes	(1) Hematopoietic stem cells; (2) Bone marrow stromal cells; and (3) Neural stem cells	Cell tracking by MRI	HPF labeling facilitates the monitoring of infused or implanted cells by MRI
9	Unterweger *et al* [82], 2017	Dextran-coated SPION^{Dex}	Human endothelial and monocytic cells	MRI imaging	SPION^{Dex} are extremely safe and represents a promising candidate for further clinical development
10	Han *et al*[83], 2021	3D-printed poly(lactic-co-glycolic acid) scaffolds coated with IONPs	rBM-MSCs	Rat Calvarial bone defect model to investigate Osteogenic differentiation	Increased the adhered cell number, and promoted cell spreading by upregulating the expression of integrin α1 and β1 and their downstream signaling molecules FAK and ERK1/2. ALP levels and Osteogenesis also significantly increased
11	Lee *et al*[43], 2009	MGIOs	Human fetal mesenchymal stem cells	MSC tracking by MRI	The use of M600 particles may be useful for cellular tracking using MRI
12	Mailänder *et al* [44], 2008	Carboxylated superpara-magnetic iron oxide particles	MSC	Monitor trafficking of transplanted MSCs cells by MRI without transfection agents	Feasibility and efficiency of labeling MSC with SPIONs was determined
13	Dabrowska *et al* [46], 2018	Superparamagnetic iron oxide nanoparticles conjugated with	Human bone marrow MSCs EVs	Imaging of EVs	Molday ION is biocompatible with EVs. Labeling did not interfere with

	rhodamine (Molday ION Rhodamine B™)			the capability of EVs to re-enter hBM-MSCs. IONs have magnetic properties useful for imaging by MRI
14	Li *et al*[59], 2019	Fe$_3$O$_4$@PDA	Rat bone marrow-derived MSCs	Migration and homing of MSCs	Iron oxide nanoparticles increased the expression of CXCR4 in MSCs and improved their homing and ant-inflammatory abilities
15	Yun *et al*[48], 2018	SPIONs with rhodamine B	Mouse bone marrow-derived MSCs	Enhanced homing effect in a model of olfactory injury	SPIONs-labeled MSCs produced better homing effects of MSCs *in vivo*
16	Meng *et al*[51], 2017	SPIONs (Molday ION Rhodamine B™)	WJ-MSCs	Gene carrying into cutaneous injury sites	Exposure to an external magnetic field increases transportation of SPIONs-labeled WJ-MSCs *in vivo*
17	Braniste *et al*[52], 2020	ZnFe$_2$O nanoparticles based on iron covered with a chemically stable crystalline GaN film	Rat bone marrow MSCs	Long term monitoring of tracked MSCs	These nanoparticles are compatible with MSCs. Increasing concentrations of nanoparticles inhibit proliferation of MSCs. GaN growth on zinc ferrite nanoparticles increases the chemical stability of the material
18	Silva *et al*[53], 2016	Gold and maghemite nanoparticles functionalized with DMSA: (1) Au-DMSA; and (2) γ-Fe2O3-DMSA	Dental pulp derived MSCs	Tracking of MSCs *in vivo*	γ-Fe2O3-DMSA and Au-DMSA can be used as tracers for MSCs. Au-DMSA is not suitable for visualization and tracking. γ-Fe2O3-DMSA is a promising agent for MSC magnetic targeting
19	Moayeri *et al*[55], 2020	PLL hydrobromide coated SPIONs	Rat ADSC	Delivery and homing of transplanted MSCs in the target tissue	Transfection of ADSC by SPION/PLL is an appropriate protocol for cell therapy
20	Chung *et al*[57], 2018	Dex-IO NPs	hMSCs	Accelerate and optimize MSC therapeutics for Parkinson disease	NPs enhance the migration of hMSCs toward damaged DA-like cells, induce hMSCs to differentiate to DA-like neurons and promote the protection/regeneration effects of hMSCs
21	Li *et al*[84], 2020	Fe$_3$O$_4$@PDA NPs	Mouse bone marrow MSCs	Optimization of MSC-based therapeutic strategies for burn wound healing	NPs effectively incorporated into the MSCs without negative effects on cell properties and enhanced their migration ability
22	Dai *et al*[61], 2019	MIONs	mESCs	Induction of neural differentiation of stem cells	MIONs promoted the differentiation of the embryonic stem cells into nerve cells
23	Hachani *et al*[85], 2017	3,4-dihydroxyhydrocinnamic acid (DHCA) functionalized IONPs	hBM-MSCs	Imaging and contrast	It was significantly phagocytized by MSCs and produced significant contrast enhancement for proper tracking
24	Daquinag *et al*[66], 2013	Iron oxide (Fe$_2$O$_3$) and gold (Au) nanoparticles cross-linked with PLL	WAT ASC	WAT transplantation applications and WAT-based cell therapy	This NP-based 3D methodology potentially enhance WAT transplantation efficacy
25	Wang *et al*[67], 2016	Superparamagnetic Fe$_3$O$_4$ nanoparticles	hUCM-MSCs	Long-term banking of living cells	Magnetic induction heating in a magnetic field with Fe$_3$O$_4$ nanoparticles facilitates rewarming and cryopreservation outcome of hUCM-MSCs
26	Naseroleslami *et al*[68], 2021	SPIONs	hUCM-MSCs	Protection against myocardial injury	SPION-labeled MSCs in the presence of magnetic field reduces inflammation following myocardial injury
27	Zhang *et al*[69], 2020	Fe$_3$O$_4$@GO MNCs	Rat bone marrow mesenchymalstem cells	Bone tissue regeneration	Fe$_3$O$_4$@GO MNCs reduced cell damage caused by ROS, improved the activity of MSCs and promote osteogenic differentiation
28	Hamid *et al*[86], 2022	Combining Static Magnetic field with Samarium Cobalt (SmCO5)	hUC-MSCs	Proliferative properties o MSCs	Enhancement of MSCs proliferation without changing their stemless and immunophenotype
29	Van de Walle *et al*[72], 2019	Citrate coated iron oxide (maghemite) nanoparticles	hBM-MSCs	The long-term intracellular fate of MNP in MSCs and differentiation status	Intracellular *de novo* synthesis of magnetic nanoparticles was demonstrated due to the overexpression of H-subunit of ferritin. This

					process could prevent long-term cytotoxicity and enhance MSCs differentiation
30	Labusca *et al*[73], 2021	Fe$_3$O$_4$ MNP	(1) Human primary adipose derived MSCs; and (2) hWJMSCs	Cartilage engineering	Exposure to magnetic field increases ADSC-MNP chondrogenesis in ADSC, but not in WJMSC
31	Labusca *et al*[75], 2020	Fe$_3$O$_4$ magnetite MNP	Primary human ADSCs	Treatment of osteoporosis	Parameters of magnetic field and the exposure way interfere with ADSCs differentiation in terms of adipogenic and osteogenic conversion.
32	Ishmukhametov *et al*[87], 2022	Citrate-stabilized MNPs that are Functionalized with calf thymus DNA solution (50 µg/mL) and immobilized on glass surface	Human ADSCs	Differentiation of MSCs	Enhanced the Chondrogenesis and Osteogenesis in hTERT-transduced MSCs and the use of glass surface increased the chondrogenesis rate and reduced the need to high level of growth factors in the differentiation medium
33	Hao *et al*[88], 2021	Magnetic Scaffold made from Chitosan, Laponite and Fe$_3$O$_4$	hUC-MSCs	Proliferation and Osteogenesis	Enhanced the proliferation of hUC-MSCs and increased Osteogenesis markers; ALP, OCN and type I collagen
34	Zhang *et al*[89], 2022	3D magnetic scaffolds fabricated by incorporating MNPs into electrospun gelatin nanofibers coated with either citric acid or polyvinylpyrrolidone	BM-MSCs	Osteogenesis and Chondrogenesis	Chondrogenesis-related genes COL2A1 and ACAN were selectively enhanced by magnetic scaffolds with citric acid-coated MNPs (CAG). Osteogenesis-related genes (RUNX2 and SPARC were selectively upregulated by magnetic scaffolds with polyvinylpyrrolidone-coated MNPs
35	Ohki *et al*[90], 2020	SPIO and USPIO	hUC-MSCs	Labelling, Proliferation and differentiation	Remarkable increase in the signal intensity, proliferation and three-lineage differentiation (Osteogenesis, Adipogenesis, and Chondrogenesis)
36	Theruvath *et al*[91], 2021	Ferumoxytol and Ascorbic acid	BM-MSCs	Knee cartilage regeneration in minipigs	Hyaline-like cartilage regeneration in the knee joints of minipigs and improved Chondrogenesis were observed with significant upregulation in the amount of collagen type II
37	Xu *et al*[77], 2021	SPIOs	hUC-MSCs	Survival and Immunomodulation in Mouse Sepsis model	Enhanced the survival and immunomodulatory abilities of MSCs by increasing the levels of HO-1 and TRAF1 and promoted the polarization of macrophages to the M2 type. This was found to improve the liver- related injury in Sepsis
38	Liu *et al*[92], 2021	Fe$_3$O$_4$@PDA	hUC-MSCs	Homing and differentiation in rat model of Sciatic Nerve Chronic Compression Injury	Fe$_3$O$_4$@PDA-labeled MSCs showed better homing to the spinal cord under magnetic field guidance and decreases decreased spinal nerve demyelination and c-Fos expression

hBM-MSCs: Human bone marrow-derived mesenchymal stem cells; PDA: Polydopamine; SPIOs: Superparamagnetic iron oxide nanoparticles; AD-MSCs: Adipose tissue-derived mesenchymal stem cells; BM-MSCs: Bone marrow derived Mesenchymal stem cells; USPIO: Ultrasmall superparamagnetic iron oxide; MNPs: Magnetic nanoparticles; OCN: Osteocalcin; ROS: Reactive oxygen species; GO: Graphene oxide; WAT: White adipose tissue; ASC: Adipose stromal cells; MIONs: Magnetic iron oxide nanoparticles; Dex-IO NPs: Dextran-coated iron oxide nanoparticles; PLL: Poly-L-lysine; DMSA: 2,3-dimercaptosuccinic acid; WJ-MSCs: Wharton's Jelly of the human umbilical cord-derived MSCs; EVs: Extracellular vesicles; HPF: Heparin-protamine; MGIO: Microgel iron oxide nanoparticle; MAPK: Mitogen-activated protein kinases; PI3K: Phosphatidylinositol 3-kinase; mESCs: Mouse embryonic stem cells; IL: Interleukin; TNF-α: Tumor necrosis factor-α; MRI: Magnetic resonance imaging; USPIO-PAA-GlcN: Glucosamine-modified iron oxide nanoparticles; MNC: Magnetic nanocomposites; HO-1: Heme oxygenase-1.

used MNPs to improve the transplantation characteristics of MSCs. Combining Nanotechnology with MSCs opens new avenues to enhance their therapeutic outcomes and long-term regenerative abilities. The incorporation of MNPs with MSCs has been extensively investigated and it revealed great chances to increase their survival, promote their homing and retention at the site of injury, improve their tolerance to stress microenvironments and enhance their integration with host tissues and trigger their differentiation. The use of MNPs with MSCs still in need for further investigation to answer many concerns surrounding their combination. Some of these concerns are related to assessing the safety
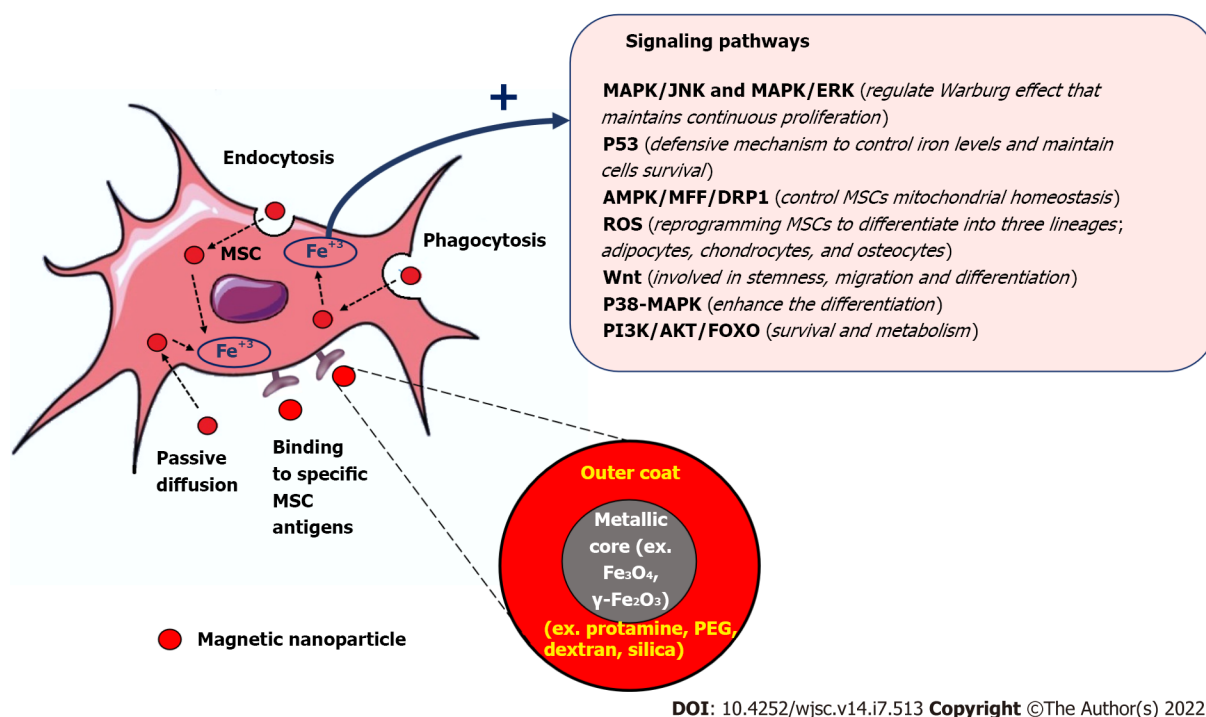
Figure 1 The prodigious therapeutic outcomes of combining mesenchymal stem cells with magnetic nanoparticles. MSC: Mesenchymal stem cell; MAPK: Mitogen-activated protein kinases; JNK: c-Jun NH2-terminal kinase; ERK: extracellular signal-regulated kinase; ROS: Reactive oxygen species; PI3K: Phosphatidylinositol 3-kinase; AKT: Protein kinase B; FOXO: Forkhead box O.

profile of MNPs on the long-run, determining the optimal non-toxic dose that can be added to MSCs based on the type of pathology and the ultimate target to be achieved, finding the best coating substrate to be used with MNPs without affecting their therapeutic functions, exploring the possibility of combining more than one MNPs for synergistic effects, finding the exact molecular mechanisms that are exerted by MNPs to alter the cellular pathways in MSCs, and studying the impact of the internal microenvironment which varies based on and the type of disease in influencing the uptake of MNPs by MSCs and their ultimate response. Future studies should also focus on addressing the role of MNPs in solving other MSCs therapy challenges including cellular heterogeneity which highly depends on the source of MSCs and the culturing procedures being used, the undesirable pre-transplantation differen-tiation, and the switch in their immunological characteristics under stress microenvironments. A Schematic summary depicted the role of MNPs in improving the transplantation and biological charac-teristics of MSCs can be found in Figure 1.

CONCLUSION

The regenerative abilities of MSCs have been thoroughly investigated and discussed. Despite the great improvement in understanding the curative mechanisms of MSCs, many challenges are still there which slow down the transferring of these cells in the treatment guidelines. Loss of tracking signal, poor migration and homing to the injury site, and undesirable differentiation are the most reported hurdles that thwart the therapeutic outcomes of MSCs in clinical trials. The new strategy of combining MSCs with MNPs has been proven to boost the success rate of MSCs transplantation. MNPs have been employed as an effective contrast agent for long term tracking and monitoring of injected MSCs. MNPs also increase the migration and homing tendency of MSCs and enhance the committed differentiation of these cells. Future studies should be designed to investigate the long term safety profile of these MNPs and determine the suitable formulation and doses based on the specificity of each disease model and the source of MSCs.

FOOTNOTES

errors; all authors have read and approved the final manuscript.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Open-Access: This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: https://creativecommons.org/Licenses/by-nc/4.0/

Country/Territory of origin: Jordan

ORCID number: Ejlal Abu-El-Rub 0000-0001-9217-2560; Ramada R Khasawneh 0000-0003-3873-925X; Fatimah Almahasneh 0000-0002-8799-2721.

S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

REFERENCES

1   **Sharma RR**, Pollock K, Hubel A, McKenna D. Mesenchymal stem or stromal cells: a review of clinical applications and manufacturing practices. *Transfusion* 2014; **54**: 1418-1437 [PMID: 24898458 DOI: 10.1111/trf.12421]

2   **Bianco P**, Robey PG, Simmons PJ. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* 2008; **2**: 313-319 [PMID: 18397751 DOI: 10.1016/j.stem.2008.03.002]

3   **Ullah I**, Subbarao RB, Rho GJ. Human mesenchymal stem cells - current trends and future prospective. *Biosci Rep* 2015; **35** [PMID: 25797907 DOI: 10.1042/BSR20150025]

4   **Burlacu A**, Grigorescu G, Rosca AM, Preda MB, Simionescu M. Factors secreted by mesenchymal stem cells and endothelial progenitor cells have complementary effects on angiogenesis in vitro. *Stem Cells Dev* 2013; **22**: 643-653 [PMID: 22947186 DOI: 10.1089/scd.2012.0273]

5   **Song N**, Scholtemeijer M, Shah K. Mesenchymal Stem Cell Immunomodulation: Mechanisms and Therapeutic Potential. *Trends Pharmacol Sci* 2020; **41**: 653-664 [PMID: 32709406 DOI: 10.1016/j.tips.2020.06.009]

6   **Marion NW**, Mao JJ. Mesenchymal stem cells and tissue engineering. *Methods Enzymol* 2006; **420**: 339-361 [PMID: 17161705 DOI: 10.1016/S0076-6879(06)20016-8]

7   **Joyce N**, Annett G, Wirthlin L, Olson S, Bauer G, Nolta JA. Mesenchymal stem cells for the treatment of neurodegenerative disease. *Regen Med* 2010; **5**: 933-946 [PMID: 21082892 DOI: 10.2217/rme.10.72]

8   **Kim N**, Cho SG. New strategies for overcoming limitations of mesenchymal stem cell-based immune modulation. *Int J Stem Cells* 2015; **8**: 54-68 [PMID: 26019755 DOI: 10.15283/ijsc.2015.8.1.54]

9   **Mollentze J**, Durandt C, Pepper MS. An *In Vitro* and *In Vivo* Comparison of Osteogenic Differentiation of Human Mesenchymal Stromal/Stem Cells. *Stem Cells Int* 2021; **2021**: 9919361 [PMID: 34539793 DOI: 10.1155/2021/9919361]

10   **Zhou T**, Yuan Z, Weng J, Pei D, Du X, He C, Lai P. Challenges and advances in clinical applications of mesenchymal stromal cells. *J Hematol Oncol* 2021; **14**: 24 [PMID: 33579329 DOI: 10.1186/s13045-021-01037-x]

11   **Galipeau J**, Sensébé L. Mesenchymal Stromal Cells: Clinical Challenges and Therapeutic Opportunities. *Cell Stem Cell* 2018; **22**: 824-833 [PMID: 29859173 DOI: 10.1016/j.stem.2018.05.004]

12   **Ali A**, Shah T, Ullah R, Zhou P, Guo M, Ovais M, Tan Z, Rui Y. Review on Recent Progress in Magnetic Nanoparticles: Synthesis, Characterization, and Diverse Applications. *Front Chem* 2021; **9**: 629054 [PMID: 34327190 DOI: 10.3389/fchem.2021.629054]

13   **Singh D**, McMillan JM, Liu XM, Vishwasrao HM, Kabanov AV, Sokolsky-Papkov M, Gendelman HE. Formulation design facilitates magnetic nanoparticle delivery to diseased cells and tissues. *Nanomedicine (Lond)* 2014; **9**: 469-485 [PMID: 24646020 DOI: 10.2217/nnm.14.4]

14   **Haun JB**, Yoon TJ, Lee H, Weissleder R. Magnetic nanoparticle biosensors. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 2010; **2**: 291-304 [PMID: 20336708 DOI: 10.1002/wnan.84]

15   **Yang HW**, Hua MY, Liu HL, Huang CY, Wei KC. Potential of magnetic nanoparticles for targeted drug delivery. *Nanotechnol Sci Appl* 2012; **5**: 73-86 [PMID: 24198498 DOI: 10.2147/NSA.S35506]

16   **Majidi S**, Zeinali Sehrig F, Samiei M, Milani M, Abbasi E, Dadashzadeh K, Akbarzadeh A. Magnetic nanoparticles: Applications in gene delivery and gene therapy. *Artif Cells Nanomed Biotechnol* 2016; **44**: 1186-1193 [PMID: 25727710 DOI: 10.3109/21691401.2015.1014093]

17   **Baldari S**, Di Rocco G, Piccoli M, Pozzobon M, Muraca M, Toietta G. Challenges and Strategies for Improving the Regenerative Effects of Mesenchymal Stromal Cell-Based Therapies. *Int J Mol Sci* 2017; **18** [PMID: 28974046 DOI: 10.3390/ijms18102087]

18   **Dasari A**, Xue J, Deb S. Magnetic Nanoparticles in Bone Tissue Engineering. *Nanomaterials (Basel)* 2022; **12** [PMID: 35269245 DOI: 10.3390/nano12050757]

19   **Akbarzadeh A**, Samiei M, Davaran S. Magnetic nanoparticles: preparation, physical properties, and applications in biomedicine. *Nanoscale Res Lett* 2012; **7**: 144 [PMID: 22348683 DOI: 10.1186/1556-276X-7-144]

20   **Ganapathe LS**, Mohamed MA, Mohamad Yunus R, Berhanuddin DD. Magnetite (Fe3O4) Nanoparticles in Biomedical Application: From Synthesis to Surface Functionalisation. *Magnetochemistry* 2020; **6** [DOI:

10.3390/magnetochemistry6040068]

21   **Mahmoudi M**, Sant S, Wang B, Laurent S, Sen T. Superparamagnetic iron oxide nanoparticles (SPIONs): development, surface modification and applications in chemotherapy. *Adv Drug Deliv Rev* 2011; **63**: 24-46 [PMID: 20685224 DOI: 10.1016/j.addr.2010.05.006]

22   **Suzuki M**, Bachelet-Violette L, Rouzet F, Beilvert A, Autret G, Maire M, Menager C, Louedec L, Choqueux C, Saboural P, Haddad O, Chauvierre C, Chaubet F, Michel JB, Serfaty JM, Letourneur D. Ultrasmall superparamagnetic iron oxide nanoparticles coated with fucoidan for molecular MRI of intraluminal thrombus. *Nanomedicine (Lond)* 2015; **10**: 73-87 [PMID: 24960075 DOI: 10.2217/nnm.14.51]

23   **Zhu N**, Ji H, Yu P, Niu J, Farooq MU, Akram MW, Udego IO, Li H, Niu X. Surface Modification of Magnetic Iron Oxide Nanoparticles. *Nanomaterials (Basel)* 2018; **8** [PMID: 30304823 DOI: 10.3390/nano8100810]

24   **Kurtz A**. Mesenchymal stem cell delivery routes and fate. *Int J Stem Cells* 2008; **1**: 1-7 [PMID: 24855503 DOI: 10.15283/ijsc.2008.1.1.1]

25   **Andreas K**, Georgieva R, Ladwig M, Mueller S, Notter M, Sittinger M, Ringe J. Highly efficient magnetic stem cell labeling with citrate-coated superparamagnetic iron oxide nanoparticles for MRI tracking. *Biomaterials* 2012; **33**: 4515-4525 [PMID: 22445482 DOI: 10.1016/j.biomaterials.2012.02.064]

26   **Cheng SH**, Yu D, Tsai HM, Morshed RA, Kanojia D, Lo LW, Leoni L, Govind Y, Zhang L, Aboody KS, Lesniak MS, Chen CT, Balyasnikova IV. Dynamic In Vivo SPECT Imaging of Neural Stem Cells Functionalized with Radiolabeled Nanoparticles for Tracking of Glioblastoma. *J Nucl Med* 2016; **57**: 279-284 [PMID: 26564318 DOI: 10.2967/jnumed.115.163006]

27   **Guo B**, Feng Z, Hu D, Xu S, Middha E, Pan Y, Liu C, Zheng H, Qian J, Sheng Z, Liu B. Precise Deciphering of Brain Vasculatures and Microscopic Tumors with Dual NIR-II Fluorescence and Photoacoustic Imaging. *Adv Mater* 2019; **31**: e1902504 [PMID: 31169334 DOI: 10.1002/adma.201902504]

28   **Patrick PS**, Kolluri KK, Zaw Thin M, Edwards A, Sage EK, Sanderson T, Weil BD, Dickson JC, Lythgoe MF, Lowdell M, Janes SM, Kalber TL. Lung delivery of MSCs expressing anti-cancer protein TRAIL visualised with ^{89}Zr-oxine PET-CT. *Stem Cell Res Ther* 2020; **11**: 256 [PMID: 32586403 DOI: 10.1186/s13287-020-01770-z]

29   **Estelrich J**, Sánchez-Martín MJ, Busquets MA. Nanoparticles in magnetic resonance imaging: from simple to dual contrast agents. *Int J Nanomedicine* 2015; **10**: 1727-1741 [PMID: 25834422 DOI: 10.2147/IJN.S76501]

30   **Andrzejewska A**, Jablonska A, Seta M, Dabrowska S, Walczak P, Janowski M, Lukomska B. Labeling of human mesenchymal stem cells with different classes of vital stains: robustness and toxicity. *Stem Cell Res Ther* 2019; **10**: 187 [PMID: 31238982 DOI: 10.1186/s13287-019-1296-8]

31   **Conner SD**, Schmid SL. Regulated portals of entry into the cell. *Nature* 2003; **422**: 37-44 [PMID: 12621426 DOI: 10.1038/nature01451]

32   **Alvarim LT**, Nucci LP, Mamani JB, Marti LC, Aguiar MF, Silva HR, Silva GS, Nucci-da-Silva MP, DelBel EA, Gamarra LF. Therapeutics with SPION-labeled stem cells for the main diseases related to brain aging: a systematic review. *Int J Nanomedicine* 2014; **9**: 3749-3770 [PMID: 25143726 DOI: 10.2147/IJN.S65616]

33   **Li L**, Jiang W, Luo K, Song H, Lan F, Wu Y, Gu Z. Superparamagnetic iron oxide nanoparticles as MRI contrast agents for non-invasive stem cell labeling and tracking. *Theranostics* 2013; **3**: 595-615 [PMID: 23946825 DOI: 10.7150/thno.5366]

34   **Frascione D**, Diwoky C, Almer G, Opriessnig P, Vonach C, Gradauer K, Leitinger G, Mangge H, Stollberger R, Prassl R. Ultrasmall superparamagnetic iron oxide (USPIO)-based liposomes as magnetic resonance imaging probes. *Int J Nanomedicine* 2012; **7**: 2349-2359 [PMID: 22661890 DOI: 10.2147/IJN.S30617]

35   **Guldris N**, Argibay B, Gallo J, Iglesias-Rey R, Carbó-Argibay E, Kolen'ko YV, Campos F, Sobrino T, Salonen LM, Bañobre-López M, Castillo J, Rivas J. Magnetite Nanoparticles for Stem Cell Labeling with High Efficiency and Long-Term in Vivo Tracking. *Bioconjug Chem* 2017; **28**: 362-370 [PMID: 27977143 DOI: 10.1021/acs.bioconjchem.6b00522]

36   **Lee ES**, Shuter B, Chan J, Chong MS, Ding J, Teoh SH, Beuf O, Briguet A, Tam KC, Choolani M, Wang SC. The use of microgel iron oxide nanoparticles in studies of magnetic resonance relaxation and endothelial progenitor cell labelling. *Biomaterials* 2010; **31**: 3296-3306 [PMID: 20116846 DOI: 10.1016/j.biomaterials.2010.01.037]

37   **Thu MS**, Bryant LH, Coppola T, Jordan EK, Budde MD, Lewis BK, Chaudhry A, Ren J, Varma NR, Arbab AS, Frank JA. Self-assembling nanocomplexes by combining ferumoxytol, heparin and protamine for cell tracking by magnetic resonance imaging. *Nat Med* 2012; **18**: 463-467 [PMID: 22366951 DOI: 10.1038/nm.2666]

38   **Unterweger H**, Dézsi L, Matuszak J, Janko C, Poettler M, Jordan J, Bäuerle T, Szebeni J, Fey T, Boccaccini AR, Alexiou C, Cicha I. Dextran-coated superparamagnetic iron oxide nanoparticles for magnetic resonance imaging: evaluation of size-dependent imaging properties, storage stability and safety. *Int J Nanomedicine* 2018; **13**: 1899-1915 [PMID: 29636608 DOI: 10.2147/IJN.S156528]

39   **Mesentier-Louro LA**, Zaverucha-do-Valle C, da Silva-Junior AJ, Nascimento-Dos-Santos G, Gubert F, de Figueirêdo AB, Torres AL, Paredes BD, Teixeira C, Tovar-Moll F, Mendez-Otero R, Santiago MF. Distribution of mesenchymal stem cells and effects on neuronal survival and axon regeneration after optic nerve crush and cell therapy. *PLoS One* 2014; **9**: e110722 [PMID: 25347773 DOI: 10.1371/journal.pone.0110722]

40   **Min KA**, Shin MC, Yu F, Yang M, David AE, Yang VC, Rosania GR. Pulsed magnetic field improves the transport of iron oxide nanoparticles through cell barriers. *ACS Nano* 2013; **7**: 2161-2171 [PMID: 23373613 DOI: 10.1021/nn3057565]

41   **Prijic S**, Scancar J, Romih R, Cemazar M, Bregar VB, Znidarsic A, Sersa G. Increased cellular uptake of biocompatible superparamagnetic iron oxide nanoparticles into malignant cells by an external magnetic field. *J Membr Biol* 2010; **236**: 167-179 [PMID: 20602230 DOI: 10.1007/s00232-010-9271-4]

42   **Ngen EJ**, Artemov D. Advances in Monitoring Cell-Based Therapies with Magnetic Resonance Imaging: Future Perspectives. *Int J Mol Sci* 2017; **18** [PMID: 28106829 DOI: 10.3390/ijms18010198]

43   **Lee ES**, Chan J, Shuter B, Tan LG, Chong MS, Ramachandra DL, Dawe GS, Ding J, Teoh SH, Beuf O, Briguet A, Tam KC, Choolani M, Wang SC. Microgel iron oxide nanoparticles for tracking human fetal mesenchymal stem cells through magnetic resonance imaging. *Stem Cells* 2009; **27**: 1921-1931 [PMID: 19544438 DOI: 10.1002/stem.112]

44   **Mailänder V**, Lorenz MR, Holzapfel V, Musyanovych A, Fuchs K, Wiesneth M, Walther P, Landfester K, Schrezenmeier H. Carboxylated superparamagnetic iron oxide particles label cells intracellularly without transfection agents. *Mol Imaging*

Biol 2008; **10**: 138-146 [PMID: 18297365 DOI: 10.1007/s11307-007-0130-3]

45   **Yáñez-Mó M**, Siljander PR, Andreu Z, Zavec AB, Borràs FE, Buzas EI, Buzas K, Casal E, Cappello F, Carvalho J, Colás E, Cordeiro-da Silva A, Fais S, Falcon-Perez JM, Ghobrial IM, Giebel B, Gimona M, Graner M, Gursel I, Gursel M, Heegaard NH, Hendrix A, Kierulf P, Kokubun K, Kosanovic M, Kralj-Iglic V, Krämer-Albers EM, Laitinen S, Lässer C, Lener T, Ligeti E, Linē A, Lipps G, Llorente A, Lötvall J, Manček-Keber M, Marcilla A, Mittelbrunn M, Nazarenko I, Nolte-'t Hoen EN, Nyman TA, O'Driscoll L, Olivan M, Oliveira C, Pállinger É, Del Portillo HA, Reventós J, Rigau M, Rohde E, Sammar M, Sánchez-Madrid F, Santarém N, Schallmoser K, Ostenfeld MS, Stoorvogel W, Stukelj R, Van der Grein SG, Vasconcelos MH, Wauben MH, De Wever O. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles* 2015; **4**: 27066 [PMID: 25979354 DOI: 10.3402/jev.v4.27066]

46   **Dabrowska S**, Del Fattore A, Karnas E, Frontczak-Baniewicz M, Kozlowska H, Muraca M, Janowski M, Lukomska B. Imaging of extracellular vesicles derived from human bone marrow mesenchymal stem cells using fluorescent and magnetic labels. *Int J Nanomedicine* 2018; **13**: 1653-1664 [PMID: 29593411 DOI: 10.2147/IJN.S159404]

47   **Li X**, Wei Z, Lv H, Wu L, Cui Y, Yao H, Li J, Zhang H, Yang B, Jiang J. Iron oxide nanoparticles promote the migration of mesenchymal stem cells to injury sites. *Int J Nanomedicine* 2019; **14**: 573-589 [PMID: 30666115 DOI: 10.2147/IJN.S184920]

48   **Yun WS**, Choi JS, Ju HM, Kim MH, Choi SJ, Oh ES, Seo YJ, Key J. Enhanced Homing Technique of Mesenchymal Stem Cells Using Iron Oxide Nanoparticles by Magnetic Attraction in Olfactory-Injured Mouse Models. *Int J Mol Sci* 2018; **19** [PMID: 29734748 DOI: 10.3390/ijms19051376]

49   **Ahn YJ**, Kong TH, Choi JS, Yun WS, Key J, Seo YJ. Strategies to enhance efficacy of SPION-labeled stem cell homing by magnetic attraction: a systemic review with meta-analysis. *Int J Nanomedicine* 2019; **14**: 4849-4866 [PMID: 31308662 DOI: 10.2147/IJN.S204910]

50   **Ahn YJ**, Yun WS, Choi JS, Kim WC, Lee SH, Park DJ, Park JE, Key J, Seo YJ. Biodistribution of poly clustered superparamagnetic iron oxide nanoparticle labeled mesenchymal stem cells in aminoglycoside induced ototoxic mouse model. *Biomed Eng Lett* 2021; **11**: 39-53 [PMID: 33747602 DOI: 10.1007/s13534-020-00181-6]

51   **Meng Y**, Shi C, Hu B, Gong J, Zhong X, Lin X, Zhang X, Liu J, Liu C, Xu H. External magnetic field promotes homing of magnetized stem cells following subcutaneous injection. *BMC Cell Biol* 2017; **18**: 24 [PMID: 28549413 DOI: 10.1186/s12860-017-0140-1]

52   **Braniste T**, Cobzac V, Ababii P, Plesco I, Raevschi S, Didencu A, Maniuc M, Nacu V, Ababii I, Tiginyanu I. Mesenchymal stem cells proliferation and remote manipulation upon exposure to magnetic semiconductor nanoparticles. *Biotechnol Rep (Amst)* 2020; **25**: e00435 [PMID: 32090026 DOI: 10.1016/j.btre.2020.e00435]

53   **Silva LH**, da Silva JR, Ferreira GA, Silva RC, Lima EC, Azevedo RB, Oliveira DM. Labeling mesenchymal cells with DMSA-coated gold and iron oxide nanoparticles: assessment of biocompatibility and potential applications. *J Nanobiotechnology* 2016; **14**: 59 [PMID: 27431051 DOI: 10.1186/s12951-016-0213-x]

54   **Panseri S**, Montesi M, Iafisco M, Adamiano A, Ghetti M, Cenacchi G, Tampieri A. Magnetic Labelling of Mesenchymal Stem Cells with Iron-Doped Hydroxyapatite Nanoparticles as Tool for Cell Therapy. *J Biomed Nanotechnol* 2016; **12**: 909-921 [PMID: 27305814 DOI: 10.1166/jbn.2016.2248]

55   **Moayeri A**, Darvishi M, Amraei M. Homing of Super Paramagnetic Iron Oxide Nanoparticles (SPIONs) Labeled Adipose-Derived Stem Cells by Magnetic Attraction in a Rat Model of Parkinson's Disease. *Int J Nanomedicine* 2020; **15**: 1297-1308 [PMID: 32161459 DOI: 10.2147/IJN.S238266]

56   **Labusca L**, Herea DD, Mashayekhi K. Stem cells as delivery vehicles for regenerative medicine-challenges and perspectives. *World J Stem Cells* 2018; **10**: 43-56 [PMID: 29849930 DOI: 10.4252/wjsc.v10.i5.43]

57   **Chung TH**, Hsu SC, Wu SH, Hsiao JK, Lin CP, Yao M, Huang DM. Dextran-coated iron oxide nanoparticle-improved therapeutic effects of human mesenchymal stem cells in a mouse model of Parkinson's disease. *Nanoscale* 2018; **10**: 2998-3007 [PMID: 29372743 DOI: 10.1039/c7nr06976f]

58   **Dong F**, Harvey J, Finan A, Weber K, Agarwal U, Penn MS. Myocardial CXCR4 expression is required for mesenchymal stem cell mediated repair following acute myocardial infarction. *Circulation* 2012; **126**: 314-324 [PMID: 22685115 DOI: 10.1161/CIRCULATIONAHA.111.082453]

59   **Li X**, Wei Z, Li B, Li J, Lv H, Wu L, Zhang H, Yang B, Zhu M, Jiang J. In vivo migration of Fe$_3$O$_4$@polydopamine nanoparticle-labeled mesenchymal stem cells to burn injury sites and their therapeutic effects in a rat model. *Biomater Sci* 2019; **7**: 2861-2872 [PMID: 31070196 DOI: 10.1039/c9bm00242a]

60   **Maulik G**, Shrikhande A, Kijima T, Ma PC, Morrison PT, Salgia R. Role of the hepatocyte growth factor receptor, c-Met, in oncogenesis and potential for therapeutic inhibition. *Cytokine Growth Factor Rev* 2002; **13**: 41-59 [PMID: 11750879 DOI: 10.1016/S1359-6101(01)00029-6]

61   **Dai R**, Hang Y, Liu Q, Zhang S, Wang L, Pan Y, Chen H. Improved neural differentiation of stem cells mediated by magnetic nanoparticle-based biophysical stimulation. *J Mater Chem B* 2019; **7**: 4161-4168

62   **Du V**, Luciani N, Richard S, Mary G, Gay C, Mazuel F, Reffay M, Menasché P, Agbulut O, Wilhelm C. A 3D magnetic tissue stretcher for remote mechanical control of embryonic stem cell differentiation. *Nat Commun* 2017; **8**: 400 [PMID: 28900152 DOI: 10.1038/s41467-017-00543-2]

63   **Jin HJ**, Bae YK, Kim M, Kwon SJ, Jeon HB, Choi SJ, Kim SW, Yang YS, Oh W, Chang JW. Comparative analysis of human mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord blood as sources of cell therapy. *Int J Mol Sci* 2013; **14**: 17986-18001 [PMID: 24005862 DOI: 10.3390/ijms140917986]

64   **Holzwarth JM**, Ma PX. Biomimetic nanofibrous scaffolds for bone tissue engineering. *Biomaterials* 2011; **32**: 9622-9629 [PMID: 21944829 DOI: 10.1016/j.biomaterials.2011.09.009]

65   **Pankhurst QA**, Connolly J, Jones SK, Dobson J. Applications of magnetic nanoparticles in biomedicine. *J Phys Appl Phys* 2003; **36**: R167-R181 [DOI: 10.1088/0022-3727/36/13/201]

66   **Daquinag AC**, Souza GR, Kolonin MG. Adipose tissue engineering in three-dimensional levitation tissue culture system based on magnetic nanoparticles. *Tissue Eng Part C Methods* 2013; **19**: 336-344 [PMID: 23017116 DOI: 10.1089/ten.TEC.2012.0198]

67   **Wang J**, Zhao G, Zhang Z, Xu X, He X. Magnetic induction heating of superparamagnetic nanoparticles during rewarming

augments the recovery of hUCM-MSCs cryopreserved by vitrification. *Acta Biomater* 2016; **33**: 264-274 [PMID: 26802443 DOI: 10.1016/j.actbio.2016.01.026]

68   **Naseroleslami M**, Aboutaleb N, Mokhtari B. Amniotic membrane mesenchymal stem cells labeled by iron oxide nanoparticles exert cardioprotective effects against isoproterenol (ISO)-induced myocardial damage by targeting inflammatory MAPK/NF-κB pathway. *Drug Deliv Transl Res* 2021; **11**: 242-254 [PMID: 32441012 DOI: 10.1007/s13346-020-00788-3]

69   **Zhang H**, Li S, Liu Y, Yu Y, Lin S, Wang Q, Miao L, Wei H, Sun W. Fe$_3$O$_4$@GO magnetic nanocomposites protect mesenchymal stem cells and promote osteogenic differentiation of rat bone marrow mesenchymal stem cells. *Biomater Sci* 2020; **8**: 5984-5993 [PMID: 32985626 DOI: 10.1039/d0bm00906g]

70   **Wang Q**, Tang Y, Ke Q, Yin W, Zhang C, Guo Y, Guan J. Magnetic lanthanum-doped hydroxyapatite/chitosan scaffolds with endogenous stem cell-recruiting and immunomodulatory properties for bone regeneration. *J Mater Chem B* 2020; **8**: 5280-5292 [PMID: 32441294 DOI: 10.1039/d0tb00342e]

71   **Zhang ZG**, Jiang Q, Zhang R, Zhang L, Wang L, Arniego P, Ho KL, Chopp M. Magnetic resonance imaging and neurosphere therapy of stroke in rat. *Ann Neurol* 2003; **53**: 259-263 [PMID: 12557295 DOI: 10.1002/ana.10467]

72   **Van de Walle A**, Plan Sangnier A, Abou-Hassan A, Curcio A, Hémadi M, Menguy N, Lalatonne Y, Luciani N, Wilhelm C. Biosynthesis of magnetic nanoparticles from nano-degradation products revealed in human stem cells. *Proc Natl Acad Sci U S A* 2019; **116**: 4044-4053 [PMID: 30760598 DOI: 10.1073/pnas.1816792116]

73   **Labusca L**, Herea DD, Emanuela Minuti A, Stavila C, Danceanu C, Plamadeala P, Chiriac H, Lupu N. Magnetic Nanoparticles and Magnetic Field Exposure Enhances Chondrogenesis of Human Adipose Derived Mesenchymal Stem Cells But Not of Wharton Jelly Mesenchymal Stem Cells. *Front Bioeng Biotechnol* 2021; **9**: 737132 [PMID: 34733830 DOI: 10.3389/fbioe.2021.737132]

74   **Fan J**, Tan Y, Jie L, Wu X, Yu R, Zhang M. Biological activity and magnetic resonance imaging of superparamagnetic iron oxide nanoparticles-labeled adipose-derived stem cells. *Stem Cell Res Ther* 2013; **4**: 44 [PMID: 23618360 DOI: 10.1186/scrt191]

75   **Labusca L**, Herea DD, Danceanu CM, Minuti AE, Stavila C, Grigoras M, Gherca D, Stoian G, Ababei G, Chiriac H, Lupu N. The effect of magnetic field exposure on differentiation of magnetite nanoparticle-loaded adipose-derived stem cells. *Mater Sci Eng C Mater Biol Appl* 2020; **109**: 110652 [PMID: 32228923 DOI: 10.1016/j.msec.2020.110652]

76   **Wang Y**, Zhou S, Yang R, Rahman M, Sequeira RC, Cao N, Zhang Y, Zhao W, Fu Q. Magnetic targeting of super-paramagnetic iron oxide nanoparticle labeled myogenic-induced adipose-derived stem cells in a rat model of stress urinary incontinence. *Nanomedicine* 2020; **30**: 102281 [PMID: 32763385 DOI: 10.1016/j.nano.2020.102281]

77   **Xu Y**, Liu X, Li Y, Dou H, Liang H, Hou Y. SPION-MSCs enhance therapeutic efficacy in sepsis by regulating MSC-expressed TRAF1-dependent macrophage polarization. *Stem Cell Res Ther* 2021; **12**: 531 [PMID: 34627385 DOI: 10.1186/s13287-021-02593-2]

78   **Maggio ND**, Martella E, Meikle S, Columbaro M, Lucarelli E, Santin M, Banfi A. Rapid and efficient magnetization of mesenchymal stem cells by dendrimer-functionalized magnetic nanoparticles. *Nanomedicine (Lond)* 2016; **11**: 1519-1534 [PMID: 27188451 DOI: 10.2217/nnm-2016-0085]

79   **Hu K**, Yu T, Tang S, Xu X, Guo Z, Qian J, Cheng Y, Zhao Y, Yan S, Zhang H, Wan M, Du C, Feng Y, Liu Q, Gu Z, Chen B, Zhang F, Gu N. Dual anisotropicity comprising 3D printed structures and magnetic nanoparticle assemblies: towards the promotion of mesenchymal stem cell osteogenic differentiation. *NPG Asia Mater* 2021; **13**: 19 [DOI: 10.1038/s41427-021-00288-x]

80   **Huang J**, Wang D, Chen J, Liu W, Duan L, You W, Zhu W, Xiong J. Osteogenic differentiation of bone marrow mesenchymal stem cells by magnetic nanoparticle composite scaffolds under a pulsed electromagnetic field. *Saudi Pharm J* 2017; **25**: 575-579 [PMID: 28579894 DOI: 10.1016/j.jsps.2017.04.026]

81   **Kono Y**, Takegaki J, Ohba T, Matsuda K, Negoro R, Fujita S, Fujita T. Magnetization of mesenchymal stem cells using magnetic liposomes enhances their retention and immunomodulatory efficacy in mouse inflamed skeletal muscle. *Int J Pharm* 2021; **596**: 120298 [PMID: 33529784 DOI: 10.1016/j.ijpharm.2021.120298]

82   **Unterweger H**, Janko C, Schwarz M, Dézsi L, Urbanics R, Matuszak J, Őrfi E, Fülöp T, Bäuerle T, Szebeni J, Journé C, Boccaccini AR, Alexiou C, Lyer S, Cicha I. Non-immunogenic dextran-coated superparamagnetic iron oxide nanoparticles: a biocompatible, size-tunable contrast agent for magnetic resonance imaging. *Int J Nanomedicine* 2017; **12**: 5223-5238 [PMID: 28769560 DOI: 10.2147/IJN.S138108]

83   **Han L**, Guo Y, Jia L, Zhang Q, Sun L, Yang Z, Dai Y, Lou Z, Xia Y. 3D magnetic nanocomposite scaffolds enhanced the osteogenic capacities of rat bone mesenchymal stem cells *in vitro* and in a rat calvarial bone defect model by promoting cell adhesion. *J Biomed Mater Res A* 2021; **109**: 1670-1680 [PMID: 33876884 DOI: 10.1002/jbm.a.37162]

84   **Li X**, Wang Y, Shi L, Li B, Li J, Wei Z, Lv H, Wu L, Zhang H, Yang B, Xu X, Jiang J. Magnetic targeting enhances the cutaneous wound healing effects of human mesenchymal stem cell-derived iron oxide exosomes. *J Nanobiotechnology* 2020; **18**: 113 [PMID: 32799868 DOI: 10.1186/s12951-020-00670-x]

85   **Hachani R**, Birchall MA, Lowdell MW, Kasparis G, Tung LD, Manshian BB, Soenen SJ, Gsell W, Himmelreich U, Gharagouzloo CA, Sridhar S, Thanh NTK. Assessing cell-nanoparticle interactions by high content imaging of biocompatible iron oxide nanoparticles as potential contrast agents for magnetic resonance imaging. *Sci Rep* 2017; **7**: 7850 [PMID: 28798327 DOI: 10.1038/s41598-017-08092-w]

86   **Hamid HA**, Ramasamy R, Mustafa MK, Hosseinpour Sarmadi V, Miskon A. Magnetic exposure using Samarium Cobalt (SmCo$_5$) increased proliferation and stemness of human Umbilical Cord Mesenchymal Stem Cells (hUC-MSCs). *Sci Rep* 2022; **12**: 8904 [PMID: 35618759 DOI: 10.1038/s41598-022-12653-z]

87   **Ishmukhametov I**, Batasheva S, Rozhina E, Akhatova F, Mingaleeva R, Rozhin A, Fakhrullin R. DNA/Magnetic Nanoparticles Composite to Attenuate Glass Surface Nanotopography for Enhanced Mesenchymal Stem Cell Differentiation. *Polymers (Basel)* 2022; **14** [PMID: 35054750 DOI: 10.3390/polym14020344]

88   **Hao M**, Xiong M, Liu Y, Tan W, Cai H. Magnetic-driven dynamic culture promotes osteogenesis of mesenchymal stem cell. *Bio Biopro* 2021; **8**: 15 [DOI: 10.1186/s40643-021-00368-4]

89   **Zhang J**, Zhang M, Lin R, Du Y, Wang L, Yao Q, Zannettino A, Zhang H. Chondrogenic preconditioning of mesenchymal

stem/stromal cells within a magnetic scaffold for osteochondral repair. *Biofabrication* 2022; **14** [PMID: 35226893 DOI: 10.1088/1758-5090/ac5935]

90   **Ohki A**, Saito S, Fukuchi K. Magnetic resonance imaging of umbilical cord stem cells labeled with superparamagnetic iron oxide nanoparticles: effects of labelling and transplantation parameters. *Sci Rep* 2020; **10**: 13684 [PMID: 32792506 DOI: 10.1038/s41598-020-70291-9]

91   **Theruvath AJ**, Mahmoud EE, Wu W, Nejadnik H, Kiru L, Liang T, Felt S, Daldrup-Link HE. Ascorbic Acid and Iron Supplement Treatment Improves Stem Cell-Mediated Cartilage Regeneration in a Minipig Model. *Am J Sports Med* 2021; **49**: 1861-1870 [PMID: 33872071 DOI: 10.1177/03635465211005754]

92   **Liu M**, Yu W, Zhang F, Liu T, Li K, Lin M, Wang Y, Zhao G, Jiang J. Fe$_3$O$_4$@Polydopamine-Labeled MSCs Targeting the Spinal Cord to Treat Neuropathic Pain Under the Guidance of a Magnetic Field. *Int J Nanomedicine* 2021; **16**: 3275-3292 [PMID: 34007177 DOI: 10.2147/IJN.S296398]

Basic Study

Application of extracellular vesicles from mesenchymal stem cells promotes hair growth by regulating human dermal cells and follicles

Ramya Lakshmi Rajendran, Prakash Gangadaran, Mi Hee Kwack, Ji Min Oh, Chae Moon Hong, Young Kwan Sung, Jaetae Lee, Byeong-Cheol Ahn

Ramya Lakshmi Rajendran, Prakash Gangadaran, Ji Min Oh, Chae Moon Hong, Jaetae Lee, Byeong-Cheol Ahn, Department of Nuclear Medicine, School of Medicine, Kyungpook National University, Daegu 41944, South Korea

Prakash Gangadaran, Mi Hee Kwack, Young Kwan Sung, Byeong-Cheol Ahn, BK21 FOUR KNU Convergence Educational Program of Biomedical Sciences for Creative Future Talents, Department of Biomedical Sciences, School of Medicine, Kyungpook National University, Daegu 41944, South Korea

Mi Hee Kwack, Young Kwan Sung, Department of Immunology, School of Medicine, Kyungpook National University, Daegu 41944, South Korea

Chae Moon Hong, Jaetae Lee, Byeong-Cheol Ahn, Department of Nuclear Medicine, Kyungpook National University Hospital, Daegu 41944, South Korea

Corresponding author: Byeong-Cheol Ahn, MD, PhD, Professor, Department of Nuclear Medicine, School of Medicine, Kyungpook National University, No. 680 Gukchaebosangro, Junggu, Daegu 41944, South Korea. abc2000@knu.ac.kr

Abstract

BACKGROUND
Dermal papillae (DP) and outer root sheath (ORS) cells play important roles in hair growth and regeneration by regulating the activity of hair follicle (HF) cells.

AIM
To investigate the effects of human mesenchymal stem cell-derived extracellular vesicles (hMSC-EVs) on DP and ORS cells as well as HFs. EVs are known to regulate various cellular functions. However, the effects of hMSC-EVs on hair growth, particularly on human-derived HF cells (DP and ORS cells), and the possible mechanisms underlying these effects are unknown.

METHODS
hMSC-EVs were isolated and characterized using transmission electron microscopy, nanoparticle tracking analysis, western blotting, and flow cytometry. The activation of DP and ORS cells was analyzed using cellular proliferation, migration, western blotting, and real-time polymerase chain reaction. HF growth was evaluated *ex vivo* using human HFs.

RESULTS
Wnt3a is present in a class of hMSC-EVs and associated with the EV membrane. hMSC-EVs promote the proliferation of DP and ORS cells. Moreover, they translocate β-catenin into the nucleus of DP cells by increasing the expression of β-catenin target transcription factors (Axin2, EP2 and LEF1) in DP cells. Treatment with hMSC-EVs also promoted the migration of ORS cells and enhanced the expression of keratin (K) differentiation markers (K6, K16, K17, and K75) in ORS cells. Furthermore, treatment with hMSC-EVs increases hair shaft elongation in cultured human HFs.

CONCLUSION
These findings suggest that hMSC-EVs are potential candidates for further preclinical and clinical studies on hair loss treatment.

Core Tip: Alopecia is a common medical problem affecting both males and females. This study found that Wnt3a is enriched in human mesenchymal stem cell-derived extracellular vesicles (hMSC-EVs) and associated with their EVs' surface. hMSC-EVs associated wnt3a can activate the Wnt/β-catenin signaling in recipient dermal papillae cells. hMSC-EVs activate keratin differentiation in recipient outer root sheath cells and increase hair shaft elongation. These findings open up for new hair growth treatment strategies to be developed for alopecia.

INTRODUCTION
Hair loss is a common and progressive condition affecting both men and women. Within hair follicles (HFs), cells and their secretory factors undergo complex and intricate interactions for the progression of the HF cycle from telogen to anagen[1,2]. Hair loss can be stopped and hair regrowth can be improved to a certain extent by minoxidil or finasteride treatment, but complete recovery is not possible. Hair transplant surgery is another option to avoid baldness. It is not a cure for male pattern baldness and is associated with complications such as edema, and rarely bleeding, folliculitis, numbness of the scalp, telogen effluvium, and infection[3,4]. The dermal papilla (DP) and outer root sheath (ORS) cells support the regulation of the hair cycle. However, they gradually lose their key hair-inducing properties under pathological conditions[5]. The restoration of DP and ORS cell functions is required to promote hair regrowth.

Extracellular vesicles (EVs) are spherical vesicles that are released by nearly all cells into the extracellular milieu, and are found in body fluids and culture media. EVs comprise functional lipids, proteins, and nucleic acids, and act as mediators of intercellular communication. EVs are classified as exosomes, small EVs, microvesicles, and apoptotic bodies. Exosomes are released by cellular multivesicular bodies, whereas microvesicles are formed by the outward budding of the plasma membrane; both are secreted under normal cellular conditions. In contrast, apoptotic bodies form during cell death[6,7].

In recent years, EVs have emerged as potential therapeutic candidates for various diseases, including ischemic diseases, wound healing, and hair regrowth, by delivering their cargo to target cells[8-12]. EVs or nanovesicles from DP cells[13-17], fibroblasts[18,19], stem cells[11,20], macrophages[21,22] and neural progenitor cells[23] have been shown to have potential therapeutic effects on hair growth in recent studies. Nearly half of these studies have reported enhanced hair regrowth using DP cells as the source cells, which showed potential as therapeutic candidates for hair regrowth. However, clinical translation of EVs derived from DP cells is limited because HFs are not readily available for isolating DP cells, and they gradually lose key hair-inducing properties upon *in vitro* culture[13,24]. Stem cells, which can be easily isolated from bone marrow (BM), adipose tissue, and the umbilical cord and generated using

induced pluripotent stem cells, have been used for regenerative therapies in the last few decades, including hair regeneration[25-28]. In our previous report, we studied the efficacy of mesenchymal stem cell (MSC)-derived EVs on hair regrowth in addition to the efficacy of mouse BM-MSC-EVs on human DP cells using a mouse model[11]. In another study, human MSC-EVs (hMSC-EVs) were used in a mouse model[29].

In this study, we investigated the effects of human BM-MSCs-EVs (hBM-MSCs-EVs) on hair growth. Additionally, we examined the possible molecular mechanisms responsible for hair regrowth. Finally, human DP cells, human ORS cells, and human HFs were treated with hMSC-EV and then examined for the activation of DP and ORS cells and their effects on hair shaft elongation in human HFs.

MATERIALS AND METHODS

Cell culture
BM-MSCs (normal, human; PCS-500-012™) were purchased from the American Type Culture Collection (Manassas, VA, United States). Cells were cultured in Dulbecco's Modified Eagle's (DMEM)-F12 medium (HyClone, Logan, UT, United States) supplemented with 10% EV-depleted fetal bovine serum (FBS; Hyclone; ultracentrifuged at 120000 × g for 18 h at 4 °C) and antibiotics (1% penicillin–streptomycin) (Gibco, Carlsbad, CA, United States) and maintained at 37 °C and 5% CO_2.

Isolation and culture of human DP and ORS cells
During hair transplantation of male patients with androgenic alopecia, biopsy specimens from the occipital scalps were obtained after receiving consent. The Medical Ethics committee of Kyungpook National University Hospital (Daegu, Korea) approved all the described studies (IRB No. KNU 2018-0155). The HFs were dissected to isolate DP cells from the bulbs, and the cells were transferred to tissue culture dishes coated with bovine type I collagen and cultured in low-glucose DMEM (HyClone, Logan, UT, United States) supplemented with 1% antibiotic–antimycotic and 20% heat-inactivated FBS at 37 °C. The cells were cultured for seven days with medium replacement every three days. The cells were then cultured in low-glucose DMEM supplemented with 10% heat-inactivated FBS in 100-mm culture dishes. Once the cells reached subconfluence, they were harvested using 0.25% trypsin and 10 mmol/L ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) (split at a 1:5 ratio). Cells from passage 2 were used for further experiments[30].

The same hair specimens were used to isolate ORS cells. The hair shaft and bulb regions of the HFs were removed (to avoid contamination by other cells). HFs were trimmed and immersed in DMEM supplemented with 20% FBS in tissue culture dishes coated with rat collagen type I (Corning, Kennebunk, ME, United States). Cells were cultured for three days, and the medium was changed to keratinocyte growth medium, EpiLife medium (Gibco BRL) with 1% antibiotic–antimycotic solution, and 1% EpiLife defined growth supplement medium. After reaching subconfluence, the cells were harvested using 0.25% trypsin and 10 mmol/L EDTA in PBS (split at a 1:5 ratio) and maintained in EpiLife medium. Cells from passage 2 were used for further experiments[18].

Isolation of hMSC-EVs
hMSC-EVs were isolated from the culture medium of human BM-MSCs (from passage 3 to 6) by ultracentrifugation as previously described[10]. The culture medium was centrifuged at 1500 × g for 10 min to remove the cells. Next, it was centrifuged at 4000 × g for 20 min to remove the cell debris. The collected culture media was filtered through a 0.45-µm syringe filter and ultracentrifuged at 100000 × g for 60 min. The collected hMSC-EV pellets were resuspended in PBS and ultracentrifuged at 100000 × g for 60 min. The hMSC-EVs were then reconstituted in 50–100 µL PBS and stored at −80 °C until use. All ultracentrifugation procedures were performed at 4 °C using an SW28 rotor (Beckman Coulter). A Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, MA, United States) was used to measure the amount of EVs.

Transmission electron microscopy
hMSC-EV pellets were resuspended in 100 µL of 2% paraformaldehyde. The samples were then added to Formvar or carbon transmission electron microscope (TEM) grids, and the membranes were air-dried for 20 min in a clean environment. The grids were washed with PBS (100 µL) and incubated in 50 µL of 1% glutaraldehyde for five minutes. The grids were then washed with distilled water for 7 × 2 min cycles and observed under an HT 7700 TEM (Hitachi, Tokyo, Japan) to view the morphology of the hMSC-EVs[9].

Nanoparticle tracking analysis
The measurement of hMSC-EVs was performed by nanoparticle tracking analysis (NTA) using NanoSight LM10 (Malvern). hMSC-EVs were diluted 1000-fold with Milli-Q water, and then a sterile syringe was used to inject the sample into the chamber while ensuring that no bubbles were present.

Measurements (n = 5) were performed and evaluated using the NanoSight NTA software. The NanoSight software found that the measured values were the same as the measured particle sizes.

Western blot analysis

Western blotting was performed as previously described[31]. To extract proteins, whole cells and EVs were treated with radio immunoprecipitation assay buffer (Thermo Fisher Scientific) containing a cocktail of protease inhibitors. Total protein concentration was measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Equal quantities of proteins (10 µg) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Burlington, MA, United States). Blots were probed with primary antibodies against Alix (dilution 1:4000; Abcam, Cambridge, MA, United States), cytochrome C (dilution 1:2500; Abcam), GM130 (dilution 1:5000; Abcam), Wnt3a (dilution 1:2500; Abcam), PCNA (dilution 1:5000; Cell Signaling Technology, Danvers, MA, United States), and anti-rabbit secondary antibodies (dilution 1:8000; Cell Signaling Technology, Danvers, MA, United States) conjugated to horseradish peroxidase. Signals were detected using enhanced chemiluminescence (GE Healthcare, Waukesha, WI, United States) according to the manufacturer's protocol. Blot images were cropped and prepared using MS PowerPoint (Microsoft, CA, United States).

Flow cytometry

Flow cytometry was performed as previously described[21]. hMSC-EVs were attached to 4 µm aldehyde or sulfate latex beads (Invitrogen, Carlsbad, CA, United States) by mixing 5 µg of the sample with 10 µL of beads for 15 min. The final volume was made up to 1 mL using PBS and mixed for 2 h in a rotary shaker. The sample reaction was stopped by adding 100 mmol/L glycine (1 mL) and 2% bovine serum albumin in PBS for 30 min in a rotary shaker. EVs were bound to beads and incubated overnight at 4 °C with Wnt3a. The beads were then incubated for 60 min at 37 °C with a fluorescein isothiocyanate (FITC)-labeled anti-rabbit antibody. They were resuspended in 1 mL PBS for flow cytometric analysis using a BD FACS Aria III instrument, as *per* the manufacturer's instructions (BD Biosciences, Franklin Lakes, NJ, United States).

The EV internalization assay

hMSC-EVs were labeled with DiD dye (hMSC-EVs/DiD) as described previously[11]. DP or ORS cells (1 × 10⁴) were cultured on eight-well chamber slides and incubated overnight. The DP was then incubated with unlabeled hMSC-EVs (10 µg/mL) and hMSC-EVs/DiD (5 and 10 µg/mL) for 2 h at 37 °C in 5% CO_2. The ORS cells were then incubated with unlabeled hMSC-EVs (5 µg/mL) and hMSC-EVs/DiD (2.5, 5 µg/mL) for 2 h at 37 °C in 5% CO_2. The cells were subsequently fixed in paraformaldehyde and mounted using mounting medium with 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, United States). A confocal laser scanning microscope (LSM 800 with AiryScan, Zeiss, Oberkochen, Germany) was used to observe and record the cellular internalization of hMSC-EVs into DP or ORS cells.

In vitro cell proliferation assay

DP or ORS cells were seeded (0.5 × 10⁴/well) in 96-well plates and maintained overnight at 37 °C and 5% CO_2. Cells treated with hMSC-EVs (DP cells: 2, 4, 6, 8, and 10 µg/mL) and ORS cells (1–5 µg/mL) were maintained for 24 h at 37 °C and 5% CO_2. CCK8 (10 µL) (CCK8 assay kit, Dojindo Molecular Technologies, Kyushu, Japan) solution was added to each well. Two hours later, according to the manufacturer's instructions, a spectrophotometer was used to measure the optical density at 450 nm to observe the cell proliferation rate.

β-catenin localization in DP cells by immunofluorescence assay

DP cells (1 × 10⁴) were seeded on an eight-well chamber slide and incubated overnight. hMSC-EVs (10 µg/mL) were added and incubated for an additional 24 h. The cells in the chamber were then fixed with 4% paraformaldehyde, probed with a primary anti-β-catenin antibody (dilution 1:200; Cell Signaling Technology) overnight and washed with PBS. The fixed cells were then incubated with Alexa Fluor FITC-conjugated anti-rabbit antibody for 60 min at room temperature for 45 min. Slides were washed three times with PBS and mounted using mounting medium with DAPI (Vector Laboratories). Images were analyzed using a confocal microscope (LSM 5 exciter, Zeiss, Oberkochen, Germany).

β-catenin trans-localization in DP cells by western blotting

DP cells (1 × 10⁶) were seeded on a 6-well plate and incubated overnight. Next, hMSC-EVs (5 and 10 µg/mL) were added and incubated for an additional 24 h. The nuclear fraction was isolated using an NE-PER™ Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Real-time polymerase chain reaction

Cells were lysed using TRIzol solution (Invitrogen) and total RNA was extracted according to the manufacturer's instructions. A real-time polymerase chain reaction (RT-PCR) was performed as described previously[21] using the SsoAdvancedTM Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, United States) in a CFX96 touch-RT-PCR system (Bio-Rad). The PCR primer sequences used in this study are listed in the Supplementary Table 1.

Cell migration assay

Migration assays were performed in 24-well cell culture inserts containing trans-parent PET membranes with 8.0-mm pores (BD Biosciences). Human ORS cells were seeded on the upper chamber insert at 5×10^3/well in 0.5 mL serum-free medium containing 0, 2.5, or 5 μg/mL hMSC-EVs and cultured for 24 h. The medium was supplemented with 10% FBS in the lower chamber as a chemoattractant. After 24 h, the cells on the lower surface were fixed with 2% paraformaldehyde, stained with crystal violet, viewed under phase-contrast microscopy, and enumerated.

Hair shaft elongation of human HFs

Human HFs were isolated and cultured as described previously[32]. HFs were treated with varying concentrations of hMSC-EVs (0, 0.1, 0.5, and 1 μg/mL) and hair shaft elongation was measured on day 6.

Statistical analysis

The mean ± SD is used to express all data. Two-group comparisons were performed using Student's t-test in Microsoft Excel (Microsoft, Redmond, WA, United States) or GraphPad Prism 9 software version 9.0.0 (121) (GraphPad Software, San Diego, Inc., CA, United States). Statistical significance was set at $P < 0.05$.

RESULTS

Characterization of hMSC-EVs and detection of Wnt3a associated with EV-membrane

The morphology of the isolated hMSC-EVs was analyzed using TEM. TEM imaging of hMSC-EVs showed that most hMSC-EVs were spherical, which is the classical morphology of EVs. Moreover, hMSC-EVs were intact and undamaged after the isolation procedure (Figure 1A). The results of NTA of hMSC-EVs showed that their average diameter was 168.4 ± 78.4 nm (Mode: 144.3 nm) (Figure 1B). Western blotting analysis of EV biomarkers revealed that Alix was present in hMSC-EVs. Cytochrome C (a mitochondrial protein) and GM130 (a Golgi apparatus protein), which are negative EV markers, were absent in hMSC-EVs, confirming that hMSC-EVs were not contaminated with other cells or organelles. Moreover, the presence and enrichment of Wnt3a were greater in hMSC-EVs than in hMSCs (Figure 1C). Flow cytometry was used to confirm the location of Wnt3a in hMSC-EVs, which showed that 34.22% of hMSC-EVs had Wnt3a on their membranes (Figure 1D and E).

hMSC-EVs promote the proliferation and activation of DP cells

To examine the interaction and integration of hMSC-EVs with recipient DP cells, hMSC-EVs were labeled with DiD dye, and the labeled hMSC-EVs/DiD cells were incubated with DP cells for 4 h. Confocal microscopy showed that hMSC-EVs interacted and integrated inside the cells (Figure 2A). The effects of hMSC-EVs on the proliferation of DP cells were examined, and the results showed that hMSC-EV treatment significantly increased the proliferation of DP cells ($P < 0.001$) with 2–6 μg/mL of hMSC-EVs and ($P < 0.01$) with 8–10 μg/mL of hMSC-EVs (Figure 2B). Since hMSC-EVs showed the presence of Wnt3a, we examined the translocation of β-catenin into the nucleus of DP cells after treatment with hMSC-EVs (10 μg/mL), which revealed a strong signal in the nucleus of DP cells (Figure 2C). In addition, we observed a dose-dependent increase in β-catenin levels in the nuclear fraction of hMSC-EV-treated cells compared with that of control-treated cells (Figure 2D). Furthermore, we examined the expression of Wnt/β-catenin target transcription factors (Axin2, EP2 and LEF1). RT-PCR results showed that there was a significant ($P < 0.001$ or $P < 0.01$) upregulation of Axin2, EP2 and LEF1 expression in DP cells in a dose-dependent manner compared to the control (Figure 2E).

hMSC-EVs promote the proliferation and migration of human ORS cells

Confocal microscopy revealed the interaction and integration of hMSC-EVs into ORS cells (Figure 3A). The effect of hMSC-EVs on the proliferation of ORS cells was investigated. The results showed that hMSC-EV treatment significantly increased the proliferation of ORS cells ($P < 0.001$) at 1–5 μg/mL (Figure 3B). As the migration of ORS cells is a hallmark of hair elongation, we examined the migration of ORS cells using hMSC-EVs. After treatment with hMSC-EVs (2.5 and 5 μg/mL), ORS cells showed significantly increased migration in a dose-dependent manner at both concentrations ($P < 0.01$ at 2.5 μg/mL and $P < 0.001$ at 5 μg/mL) (Figure 3C and D). Furthermore, we examined the expression of
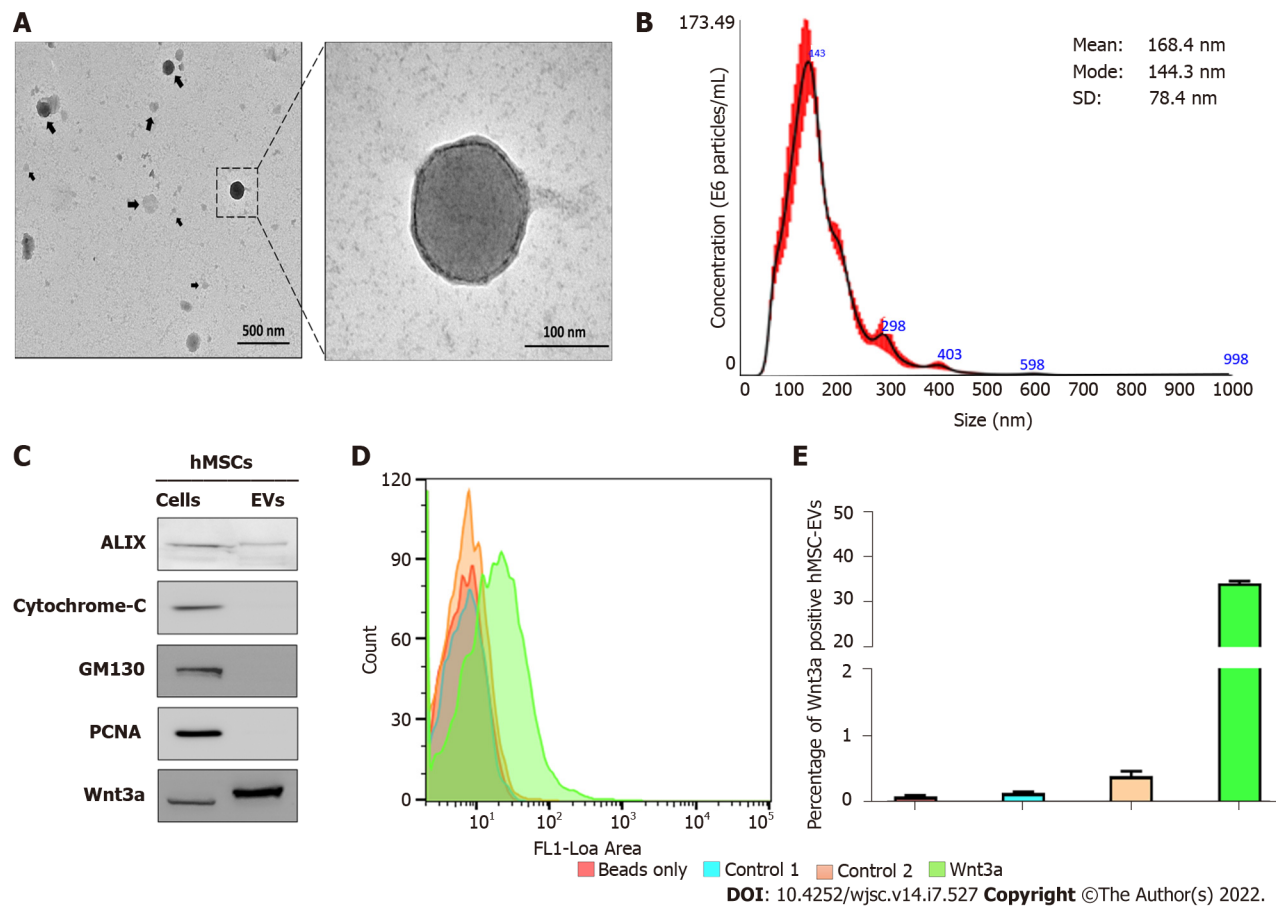
Figure 1 Isolation and characterization of human mesenchymal stem cell-derived extracellular vesicles. A: The morphology of human mesenchymal stem cell-derived extracellular vesicles (hMSC-EVs) was confirmed using transmission electron microscopy (scale bars: 500 and 100 nm); B: hMSC-EV size was determined using nanoparticle tracking analysis (n = 5); C: Western blot analysis using Alix, cytochrome C, GM130, PCNA, and Wnt3a antibodies on hMSCs and hMSC-EVs; D and E: Flow cytometry count graphs of only beads, control 1 (beads + hMSC-EVs), control 2 [beads + hMSC-EVs + Secondary fluorescein isothiocyanate (FITC) antibody], and Wnt3a (beads + hMSC-EVs + Wnt3a antibody + Secondary FITC antibody) (n = 3). The values obtained from experiments are shown mean ± SD. FITC: Fluorescein isothiocyanate; hMSC-EVs: Human mesenchymal stem cell-derived extracellular vesicles, NTA: Nanoparticle tracking analysis.

keratin (K) differentiation markers (K6, K16, K17, and K75) in ORS cells after treatment with hMSC-EVs (2.5 and 5 µg/mL). RT-PCR results showed a significant upregulation of all K mRNAs in a dose-dependent manner compared to the control. K75 showed the highest expression ($P < 0.001$), followed by K16 ($P < 0.001$) and K6 ($P < 0.001$) at both concentrations; K17 showed significant upregulation at 2.5 µg/mL ($P < 0.05$); and hMSC-EV treatment at 5 µg/mL showed no significant difference ($P > 0.05$) compared to the control (Figure 3E).

hMSC-EVs elongate human HFs

To examine the elongation of hair shafts, mini-organ cultures were performed using human scalp HFs. The HFs were treated with hMSC-EV (0, 0.05, and 0.01 µg/mL) and Wnt inhibitor-XAV939 (5 µM) treatments for six days; the results showed that hMSC-EVs increased hair shaft length significantly ($P < 0.01$) at 0.05 µg/mL and ($P < 0.001$) at 0.1 µg/mL compared to control (vehicle). The XAV939 treatment significantly ($P < 0.001$) reduced the hair shaft elongation compared to control (vehicle). Combination treatment with hMSC-EV (0.05 and 0.01 µg/mL) and Wnt inhibitor-XAV939 (5 µM) significantly ($P < 0.001$) abolished hMSC-EVs-induced hair shaft elongation (Figure 4).

DISCUSSION

EVs were isolated from the hMSC culture medium by serial centrifugation, filtration, and ultracentrifugation. The isolated hMSC-EVs displayed intact EV morphology (round) and size distribution. Moreover, hMSC-EVs were enriched in Alix (a typical biomarker of EVs) and lacked cytochrome C (a mitochondrial marker), GM130 (a golgi marker), and PCNA (a nuclear marker), which confirmed that our hMSC-EVs were not contaminated with cell organelles, consistent with previous reports[9-11]. The Wnt/β-catenin signaling cascade is crucial for the development and maintenance of HFs[13,33]. The
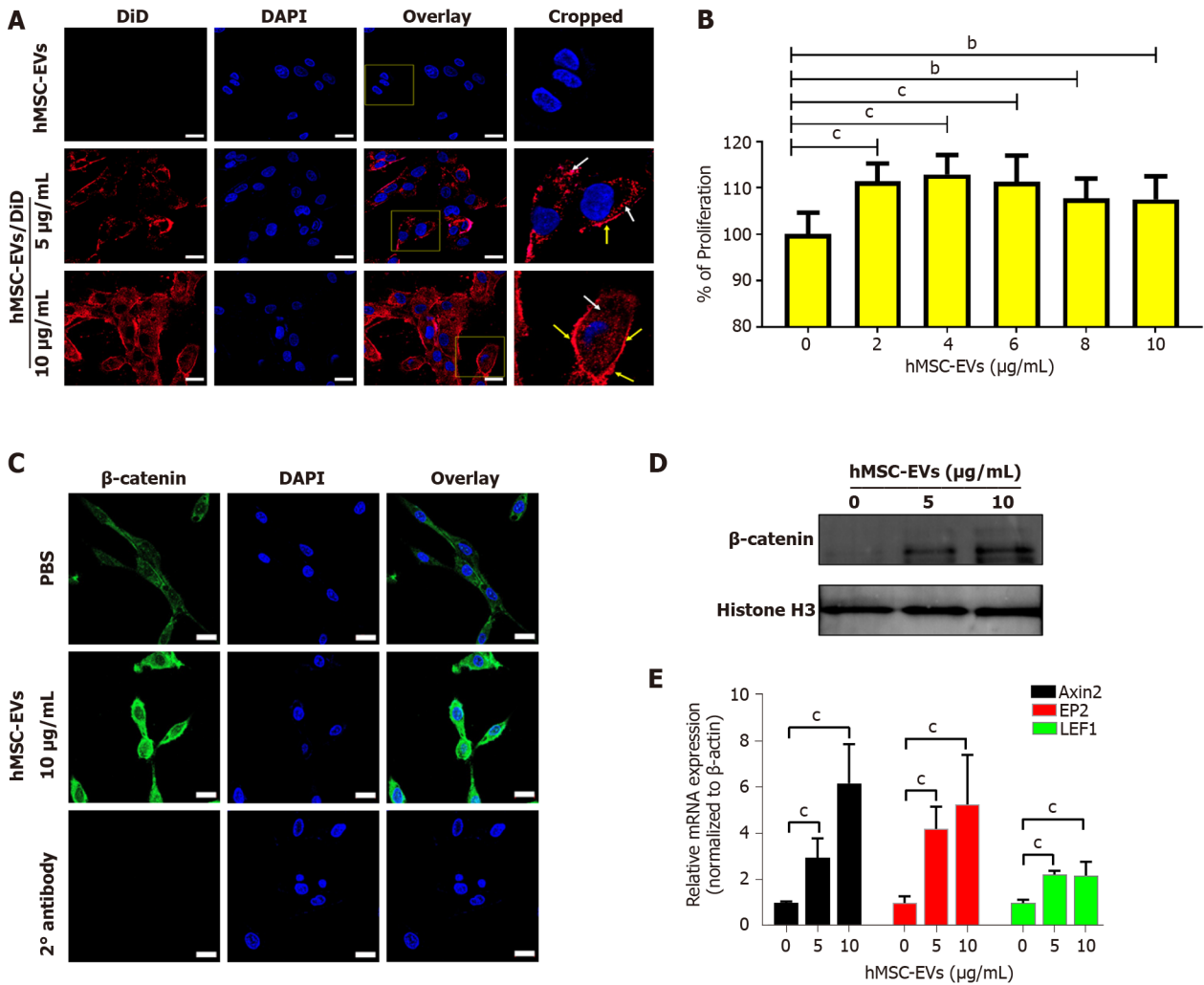
Figure 2 Interaction of human mesenchymal stem cell-derived extracellular vesicles with dermal papillae cells leads to cell proliferation and activation of Wnt/β-catenin signaling. A: Dermal papillae (DP) cells incubated for 2 h with non-labeled human mesenchymal stem cell-derived extracellular vesicles (hMSC-EVs) (10 μg/mL) and DiD-labeled hMSC-EVs (5 and 10 μg/mL; hMSC-EVs/DiD) (scale bar: 20 μm); B: DP cell proliferation was determined using a CCK8 assay 24 h after treatment with 0–10 μg hMSC-EVs (n = 5); C: β-catenin immunofluorescence assay in DP cells after 24 h of treatment with hMSC-EVs (10 μg/mL) (scale bar: 20 μm); D: The levels of β-catenin in the nuclear fraction of DP cells treated with hMSC-EVs (5 and 10 μg/mL) with histone H3 used as a loading control for nuclear fraction; E: Quantitative real-time polymerase chain reaction results of mRNA expression of Axin2, EP2 and LEF1 in DP cells treated with hMSC-EVs (5 and 10 μg/mL) for 24 h (n = 3). The values obtained from experiments are shown mean ± SD (^bP < 0.01; ^cP < 0.001. Student's t-test was used for comparison). hMSC-EVs: Human mesenchymal stem cell-derived extracellular vesicles; DP: Dermal papillae.

presence of Wnt3a in hMSC-EVs was confirmed, and Wnt3a was more enriched in EVs than in cells. Several previous studies[34-37] have well documented the enrichment of Wnt proteins on EVs. Furthermore, a significant proportion of Wnt3a (34.22%) was associated with the EV membranes. Our previous study with macrophage-and fibroblast-derived EVs also showed that they have > 90% (macrophage-derived EVs) or > 70% (fibroblast-derived EVs) associated with the EV membrane[18,21] and A recent study showed that Wnt3a, Wnt5a, and Wnt7a were present on the surface of small EVs isolated from a mouse hippocampal cell line (HT-22), which is in agreement with our current study[37].

To exert the therapeutic effects of any EV, an interaction with target/recipient cells or internalization into target/recipient cells is needed[6,7,13]. Our results revealed that hMSC-EVs actively interacted and integrated into DP cells. In the hair growth process, activation and maintenance of the Wnt/β-catenin signaling cascade in DP cells are crucial[1,11]. In this study, we observed increased proliferation of DP cells *in vitro* on treatment with hMSC-EVs. Most studies on various EVs have shown an increase in DP cell proliferation upon treatment[11,13,15,17]. Furthermore, our results revealed that treatment of DP cells with hMSC-EV translocated β-catenin into the nucleus, which is a requirement for the activation of hair-inducing transcription factors[38,39]. Additionally, hMSC-EVs increased the expression of hair-inducing transcription factors in DP cells (Axin2, EP2 and LEF1). Similar results were observed in other studies that used EVs for treatment[15,18,21].

ORS cells are a putative source of stem cells with therapeutic capacity. Survival, migration, and differentiation are important for HF maintenance[40,41]. Our results show that the interaction and integration

DOI: 10.4252/wjsc.v14.i7.527 **Copyright** ©The Author(s) 2022.
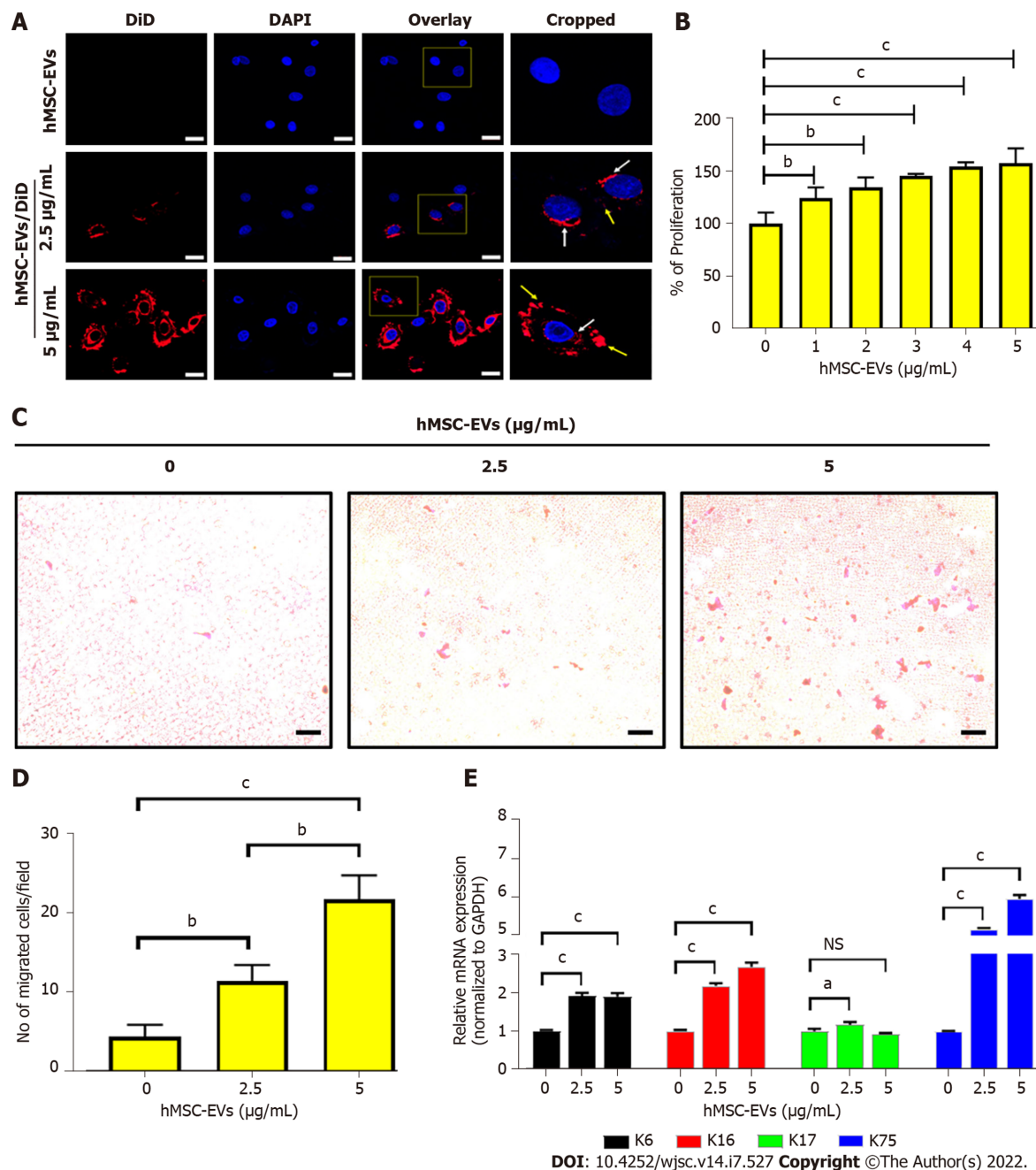
Figure 3 Interaction of human mesenchymal stem cell-derived extracellular vesicles with outer root sheath cells leads to cell proliferation, migration, and differentiation. A: Outer root sheath (ORS) cells incubated for 2 h with non-labeled human mesenchymal stem cell-derived extracellular vesicles (hMSC-EVs) (5 μg/mL) and DiD-labeled hMSC-EVs (2.5 and 5 μg/mL; hMSC-EVs/DiD) (scale bar: 20 μm); B: ORS cell proliferation was determined using a CCK8 assay 24 h after treatment with 0–5 μg/mL hMSC-EVs (*n* = 4); C and D: Phase-contrast microscopy images of migrated ORS cells 24 h after treatment with hMSC-EVs (2.5 and 5 μg/mL; scale bar: 50 μm); the quantified data of migrated cells are shown in (A) (*n* = 3); E: Quantitative real-time polymerase chain reaction results of mRNA expressions of keratin (K) 6, K16, K17, and K75 in ORS cells treated with hMSC-EVs (5 and 10 μg/mL) for 24 h (*n* = 3). The values obtained from experiments are shown mean ± SD ($^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. Student's t-test was used for comparison). NS: Not significant; hMSC-EVs: Human mesenchymal stem cell-derived extracellular vesicles; ORS: Outer root sheath.

of hMSC-EVs into ORS cells increased cellular proliferation and migration, which are necessary for hair growth. Furthermore, hMSC-EV treatment increased the expression of differentiation markers (K6, K16, K17, and K75), indicating the differentiation of cultured ORS cells into follicular lineages[42]. Finally, we investigated the hair-inducing properties of hMSC-EVs on human HFs. We observed that hMSC-EVs increased hair shaft length, which was abolished by the Wnt inhibitor. These findings suggest a potential therapeutic effect of hMSC-EVs in human HFs through Wnt/β-catenin signaling. Several other studies using EVs in HFs have reported an increase in hair shaft elongation[15,16,18,21].

In the present study, we showed the enrichment of Wnt3a in hMSC-EVs and some association of Wnt3a with the EV membrane. However, compared to macrophage-and fibroblast-derived EVs, hMSC-EVs showed a lower association between Wnt3a and the membrane[18,21,37]. We have not ruled out
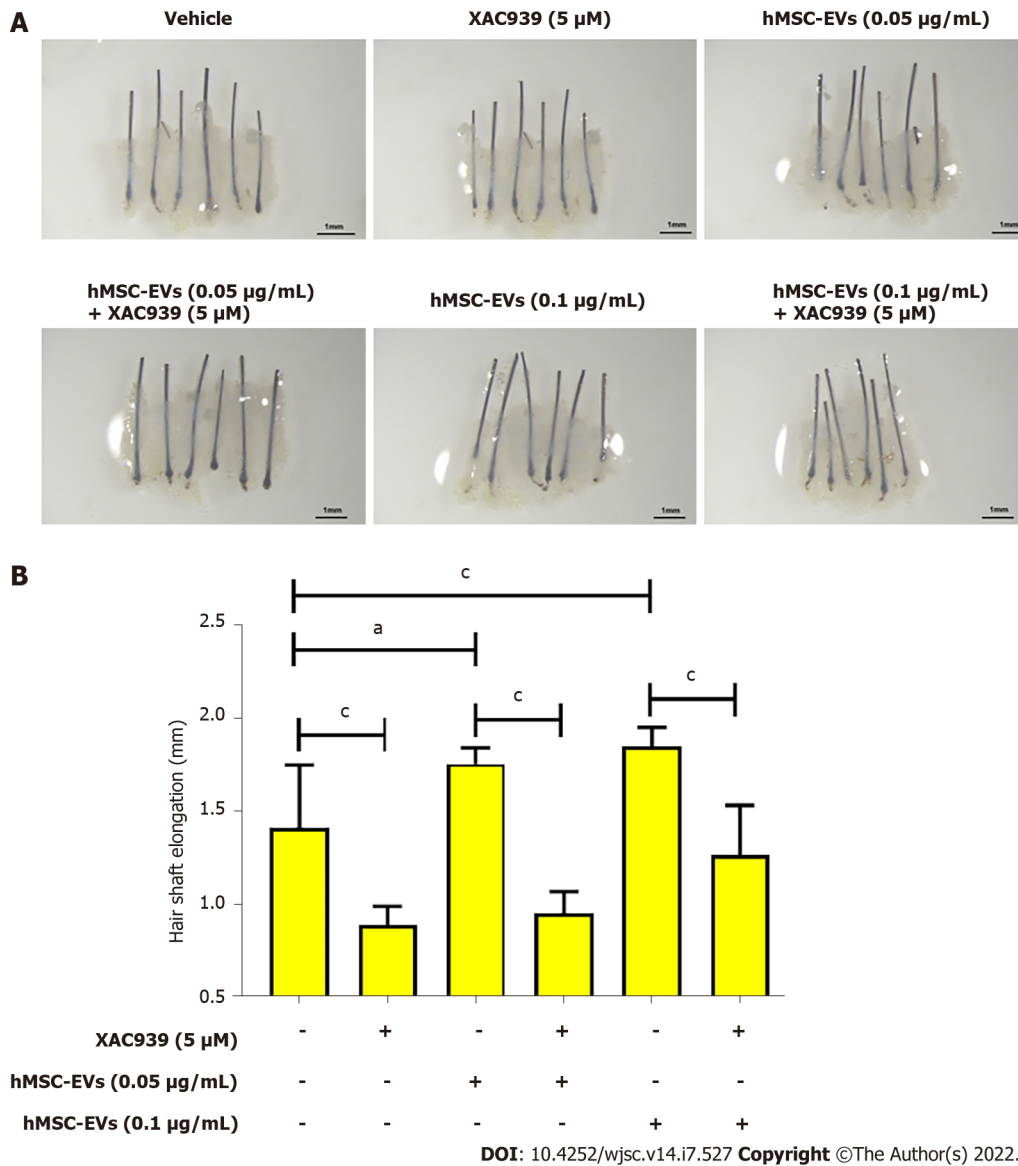
Figure 4 Human mesenchymal stem cell-derived extracellular vesicles treatment promoted human hair follicle shaft elongation. A: Representative images of human hair follicles of an individual after human mesenchymal stem cell-derived extracellular vesicles (0, 0.05, and 0.01 µg/mL) and XAV939 (5 µM) treatments; B: Quantified data of hair shaft elongation on day 6 (n = 6). ([a]P < 0.05; [c]P < 0.001. Student's t-test was used for comparison). hMSC-EVs: Human mesenchymal stem cell-derived extracellular vesicles.

that other proteins and miRNAs may play a role in hair regrowth because a few recent studies have shown that miRNA-100, miR-NA-140-5p, and miRNA-218-5p play certain roles in hair regrowth[13,16, 23]. Further, a complete proteomic and miRNA analysis is needed to reveal a more complete understanding of hair growth promoted by hMSC-EV treatment.

CONCLUSION

The present study demonstrated that hMSC-EVs enhance hair growth by activating HF cells and HFs. Thus, hMSC-EVs could be therapeutic candidates for hair loss treatment.

ARTICLE HIGHLIGHTS

Research background

Hair loss is one of the most common disorders in both sexes. Despite the availability of several treatment options, no definitive treatment method is currently available. Application of extracellular vesicles (EVs) has been suggested as a possible new treatment modality for hair loss.

Research motivation

Although cell-derived EV treatments have shown reasonable efficacy in hair loss studies, the molecular mechanisms and therapeutic effects are still relatively unknown.

Research objectives

We examined the effects of human mesenchymal stem cell-derived EVs (hMSC-EVs) on human dermal papillae (DP), outer root sheath (ORS) cells, and hair follicles (HF).

Research methods

Human DP cells, ORS cells, and HFs were treated with various amounts of hMSC-EVs to investigate the effect of hMSC-EVs on human cells *in vitro* and *ex vivo*.

Research results

The Wnt3a-containing hMSC-EVs treatment increased the proliferation of DP cells and the Wnt/β-catenin signaling cascade and activated transcription related to hair growth. Similarly, hMSC-EV treatment increased the proliferation, migration, and keratin differentiation of ORS cells. The *ex vivo* treatment with hMSC-EVs increased human HF shaft elongation.

Research conclusions

Application of hMSC-EVs may be a new potential strategy for hair loss treatment.

Research perspectives

Our findings demonstrate the effects of hMSC-EVs on hair cells and HFs.

FOOTNOTES

ORCID number: Ramya Lakshmi Rajendran 0000-0001-6987-0854; Prakash Gangadaran 0000-0002-0658-4604; Mi Hee Kwack 0000-0002-7381-6532; Ji Min Oh 0000-0001-8474-7221; Chae Moon Hong 0000-0002-5519-6982; Young Kwan Sung 0000-0002-5122-4616; Jaetae Lee 0000-0001-5676-4059; Byeong-Cheol Ahn 0000-0001-7700-3929.

L-Editor: A
P-Editor: Yu HG

REFERENCES

1 **Kishimoto J**, Burgeson RE, Morgan BA. Wnt signaling maintains the hair-inducing activity of the dermal papilla. *Genes Dev* 2000; **14**: 1181-1185 [PMID: 10817753]

2 **Oh JW**, Kloepper J, Langan EA, Kim Y, Yeo J, Kim MJ, Hsi TC, Rose C, Yoon GS, Lee SJ, Seykora J, Kim JC, Sung YK, Kim M, Paus R, Plikus MV. A Guide to Studying Human Hair Follicle Cycling In Vivo. *J Invest Dermatol* 2016; **136**: 34-44 [PMID: 26763421 DOI: 10.1038/JID.2015.354]

3 **Kerure AS**, Patwardhan N. Complications in Hair Transplantation. *J Cutan Aesthet Surg* 2018; **11**: 182-189 [PMID: 30886471 DOI: 10.4103/JCAS.JCAS_125_18]

4 **Zito PM**, Raggio BS.   Hair Transplantation. In: StatPearls. Treasure Island (FL): StatPearls Publishing, 2022

5 **Phillips TG**, Slomiany WP, Allison R. Hair Loss: Common Causes and Treatment. *Am Fam Physician* 2017; **96**: 371-378 [PMID: 28925637]

6 **Doyle LM**, Wang MZ. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. *Cells* 2019; **8** [PMID: 31311206 DOI: 10.3390/cells8070727]

7 **Simons M**, Raposo G. Exosomes--vesicular carriers for intercellular communication. *Curr Opin Cell Biol* 2009; **21**: 575-581 [PMID: 19442504 DOI: 10.1016/j.ceb.2009.03.007]

8 **De Jong OG**, Van Balkom BW, Schiffelers RM, Bouten CV, Verhaar MC. Extracellular vesicles: potential roles in regenerative medicine. *Front Immunol* 2014; **5**: 608 [PMID: 25520717 DOI: 10.3389/fimmu.2014.00608]

9 **Théry C**, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* 2006; **Chapter** 3: Unit 3.22 [PMID: 18228490 DOI: 10.1002/0471143030.cb0322s30]

10 **Vu NB**, Nguyen HT, Palumbo R, Pellicano R, Fagoonee S, Pham PV. Stem cell-derived exosomes for wound healing: current status and promising directions. *Minerva Med* 2021; **112**: 384-400 [PMID: 33263376 DOI: 10.23736/S0026-4806.20.07205-5]

11 **Rajendran RL**, Gangadaran P, Bak SS, Oh JM, Kalimuthu S, Lee HW, Baek SH, Zhu L, Sung YK, Jeong SY, Lee SW, Lee J, Ahn BC. Extracellular vesicles derived from MSCs activates dermal papilla cell in vitro and promotes hair follicle conversion from telogen to anagen in mice. *Sci Rep* 2017; **7**: 15560 [PMID: 29138430 DOI: 10.1038/s41598-017-15505-3]

12 **Öztürk S**, Elçin AE, Koca A, Elçin YM. Therapeutic Applications of Stem Cells and Extracellular Vesicles in Emergency Care: Futuristic Perspectives. *Stem Cell Rev Rep* 2021; **17**: 390-410 [PMID: 32839921 DOI: 10.1007/s12015-020-10029-2]

13 **Hu S**, Li Z, Lutz H, Huang K, Su T, Cores J, Dinh PC, Cheng K. Dermal exosomes containing miR-218-5p promote hair regeneration by regulating β-catenin signaling. *Sci Adv* 2020; **6**: eaba1685 [PMID: 32832660 DOI: 10.1126/sciadv.aba1685]

14 **Yan H**, Gao Y, Ding Q, Liu J, Li Y, Jin M, Xu H, Ma S, Wang X, Zeng W, Chen Y. Exosomal Micro RNAs Derived from Dermal Papilla Cells Mediate Hair Follicle Stem Cell Proliferation and Differentiation. *Int J Biol Sci* 2019; **15**: 1368-1382 [PMID: 31337968 DOI: 10.7150/ijbs.33233]

15 **Aberdam E**, Le Riche A, Bordes S, Closs B, Park BS, Aberdam D. Extracellular Vesicles Including Exosomes for Hair Follicle Regeneration. In: Jimenez F, Higgins C. (eds) Hair Follicle Regeneration. Humana, Cham. *Stem Cell Biol Regen Med* 2022; **72**: 205-218 [DOI: 10.1007/978-3-030-98331-4_9]

16 **Chen Y**, Huang J, Liu Z, Chen R, Fu D, Yang L, Wang J, Du L, Wen L, Miao Y, Hu Z. miR-140-5p in Small Extracellular Vesicles From Human Papilla Cells Stimulates Hair Growth by Promoting Proliferation of Outer Root Sheath and Hair Matrix Cells. *Front Cell Dev Biol* 2020; **8**: 593638 [PMID: 33425897 DOI: 10.3389/fcell.2020.593638]

17 **Zhou L**, Wang H, Jing J, Yu L, Wu X, Lu Z. Regulation of hair follicle development by exosomes derived from dermal papilla cells. *Biochem Biophys Res Commun* 2018; **500**: 325-332 [PMID: 29654758 DOI: 10.1016/j.bbrc.2018.04.067]

18 **Rajendran RL**, Gangadaran P, Kwack MH, Oh JM, Hong CM, Sung YK, Lee J, Ahn BC. Human fibroblast-derived extracellular vesicles promote hair growth in cultured human hair follicles. *FEBS Lett* 2021; **595**: 942-953 [PMID: 33523480 DOI: 10.1002/1873-3468.14050]

19 **le Riche A**, Aberdam E, Marchand L, Frank E, Jahoda C, Petit I, Bordes S, Closs B, Aberdam D. Extracellular Vesicles from Activated Dermal Fibroblasts Stimulate Hair Follicle Growth Through Dermal Papilla-Secreted Norrin. *Stem Cells* 2019; **37**: 1166-1175 [PMID: 31237401 DOI: 10.1002/stem.3043]

20 **Li Y**, Wang G, Wang Q, Zhang Y, Cui L, Huang X. Exosomes Secreted from Adipose-Derived Stem Cells Are a Potential Treatment Agent for Immune-Mediated Alopecia. *J Immunol Res* 2022; **2022**: 7471246 [PMID: 35155688 DOI: 10.1155/2022/7471246]

21 **Rajendran RL**, Gangadaran P, Seo CH, Kwack MH, Oh JM, Lee HW, Gopal A, Sung YK, Jeong SY, Lee SW, Lee J, Ahn BC. Macrophage-Derived Extracellular Vesicle Promotes Hair Growth. *Cells* 2020; **9** [PMID: 32244824 DOI: 10.3390/cells9040856]

22 **Rajendran RL**, Gangadaran P, Kwack MH, Oh JM, Hong CM, Gopal A, Sung YK, Lee J, Ahn BC. Engineered extracellular vesicle mimetics from macrophage promotes hair growth in mice and promotes human hair follicle growth. *Exp Cell Res* 2021; **409**: 112887 [PMID: 34678305 DOI: 10.1016/j.yexcr.2021.112887]

23 **Cao L**, Tian T, Huang Y, Tao S, Zhu X, Yang M, Gu J, Feng G, Ma Y, Xia R, Xu W, Wang L. Neural progenitor cell-derived nanovesicles promote hair follicle growth *via* miR-100. *J Nanobiotechnology* 2021; **19**: 20 [PMID: 33430889 DOI: 10.1186/s12951-020-00757-5]

24 **Taghiabadi E**, Nilforoushzadeh MA, Aghdami N. Maintaining Hair Inductivity in Human Dermal Papilla Cells: A Review of Effective Methods. *Skin Pharmacol Physiol* 2020; **33**: 280-292 [PMID: 33053562 DOI: 10.1159/000510152]

25   **Rajabzadeh N**, Fathi E, Farahzadi R. Stem cell-based regenerative medicine. *Stem Cell Investig* 2019; **6**: 19 [PMID: 31463312 DOI: 10.21037/sci.2019.06.04]

26   **Owczarczyk-Saczonek A**, Krajewska-Włodarczyk M, Kruszewska A, Banasiak Ł, Placek W, Maksymowicz W, Wojtkiewicz J. Therapeutic Potential of Stem Cells in Follicle Regeneration. *Stem Cells Int* 2018; **2018**: 1049641 [PMID: 30154860 DOI: 10.1155/2018/1049641]

27   **Bak DH**, Choi MJ, Kim SR, Lee BC, Kim JM, Jeon ES, Oh W, Lim ES, Park BC, Kim MJ, Na J, Kim BJ. Human umbilical cord blood mesenchymal stem cells engineered to overexpress growth factors accelerate outcomes in hair growth. *Korean J Physiol Pharmacol* 2018; **22**: 555-566 [PMID: 30181702 DOI: 10.4196/kjpp.2018.22.5.555]

28   **Zomer HD**, Vidane AS, Gonçalves NN, Ambrósio CE. Mesenchymal and induced pluripotent stem cells: general insights and clinical perspectives. *Stem Cells Cloning* 2015; **8**: 125-134 [PMID: 26451119 DOI: 10.2147/SCCAA.S88036]

29   **Yang G**, Chen Q, Wen D, Chen Z, Wang J, Chen G, Wang Z, Zhang X, Zhang Y, Hu Q, Zhang L, Gu Z. A Therapeutic Microneedle Patch Made from Hair-Derived Keratin for Promoting Hair Regrowth. *ACS Nano* 2019; **13**: 4354-4360 [PMID: 30942567 DOI: 10.1021/acsnano.8b09573]

30   **Kwack MH**, Kang BM, Kim MK, Kim JC, Sung YK. Minoxidil activates β-catenin pathway in human dermal papilla cells: a possible explanation for its anagen prolongation effect. *J Dermatol Sci* 2011; **62**: 154-159 [PMID: 21524889 DOI: 10.1016/j.jdermsci.2011.01.013]

31   **Ju MS**, Ahn HM, Han SG, Ko S, Na JH, Jo M, Lim CS, Ko BJ, Yu YG, Lee WK, Kim YJ, Jung ST. A human antibody against human endothelin receptor type A that exhibits antitumor potency. *Exp Mol Med* 2021; **53**: 1437-1448 [PMID: 34588605 DOI: 10.1038/s12276-021-00678-9]

32   **Bak SS**, Sung YK, Kim SK. 7-Phloroeckol promotes hair growth on human follicles in vitro. *Naunyn Schmiedebergs Arch Pharmacol* 2014; **387**: 789-793 [PMID: 24833298 DOI: 10.1007/s00210-014-0986-0]

33   **Clevers H**. Wnt/beta-catenin signaling in development and disease. *Cell* 2006; **127**: 469-480 [PMID: 17081971 DOI: 10.1016/j.cell.2006.10.018]

34   **Gross JC**, Chaudhary V, Bartscherer K, Boutros M. Active Wnt proteins are secreted on exosomes. *Nat Cell Biol* 2012; **14**: 1036-1045 [PMID: 22983114 DOI: 10.1038/ncb2574]

35   **Działo E**, Rudnik M, Koning RI, Czepiel M, Tkacz K, Baj-Krzyworzeka M, Distler O, Siedlar M, Kania G, Błyszczuk P. WNT3a and WNT5a Transported by Exosomes Activate WNT Signaling Pathways in Human Cardiac Fibroblasts. *Int J Mol Sci* 2019; **20** [PMID: 30901906 DOI: 10.3390/ijms20061436]

36   **Zhang L**, Wrana JL. The emerging role of exosomes in Wnt secretion and transport. *Curr Opin Genet Dev* 2014; **27**: 14-19 [PMID: 24791688 DOI: 10.1016/j.gde.2014.03.006]

37   **Torres VI**, Barrera DP, Varas-Godoy M, Arancibia D, Inestrosa NC. Selective Surface and Intraluminal Localization of Wnt Ligands on Small Extracellular Vesicles Released by HT-22 Hippocampal Neurons. *Front Cell Dev Biol* 2021; **9**: 735888 [PMID: 34722516 DOI: 10.3389/fcell.2021.735888]

38   **Kwack MH**, Kim MK, Kim JC, Sung YK. Wnt5a attenuates Wnt/β-catenin signalling in human dermal papilla cells. *Exp Dermatol* 2013; **22**: 229-231 [PMID: 23489428 DOI: 10.1111/exd.12101]

39   **Shin H**, Kwack MH, Shin SH, Oh JW, Kang BM, Kim AA, Kim J, Kim MK, Kim JC, Sung YK. Identification of transcriptional targets of Wnt/beta-catenin signaling in dermal papilla cells of human scalp hair follicles: EP2 is a novel transcriptional target of Wnt3a. *J Dermatol Sci* 2010; **58**: 91-96 [PMID: 20347274 DOI: 10.1016/j.jdermsci.2010.02.011]

40   **Pena JC**, Kelekar A, Fuchs EV, Thompson CB. Manipulation of outer root sheath cell survival perturbs the hair-growth cycle. *EMBO J* 1999; **18**: 3596-3603 [PMID: 10393176 DOI: 10.1093/emboj/18.13.3596]

41   **Li H**, Masieri FF, Schneider M, Bartella A, Gaus S, Hahnel S, Zimmerer R, Sack U, Maksimovic-Ivanic D, Mijatovic S, Simon JC, Lethaus B, Savkovic V. The Middle Part of the Plucked Hair Follicle Outer Root Sheath Is Identified as an Area Rich in Lineage-Specific Stem Cell Markers. *Biomolecules* 2021; **11** [PMID: 33503918 DOI: 10.3390/biom11020154]

42   **Chan CC**, Fan SM, Wang WH, Mu YF, Lin SJ. A Two-Stepped Culture Method for Efficient Production of Trichogenic Keratinocytes. *Tissue Eng Part C Methods* 2015; **21**: 1070-1079 [PMID: 25951188 DOI: 10.1089/ten.TEC.2015.0033]

ORIGINAL ARTICLE

Basic Study

miR-3682-3p directly targets FOXO3 and stimulates tumor stemness in hepatocellular carcinoma *via* a positive feedback loop involving FOXO3/PI3K/AKT/c-Myc

Qian Chen, Si-Bo Yang, Ye-Wei Zhang, Si-Yuan Han, Lei Jia, Bo Li, Yi Zhang, Shi Zuo

Qian Chen, Ye-Wei Zhang, Bo Li, Shi Zuo, Department of Hepatobiliary Surgery, Affiliated Hospital of Guizhou Medical University, Guiyang 550000, Guizhou Province, China

Si-Bo Yang, Department of Clinical Medicine, Guizhou Medical University, Guiyang 550000, Guizhou Province, China

Si-Yuan Han, Department of Infectious Diseases, SSL Central Hospital of Dongguan, Dongguan 523000, Guangdong Province, China

Lei Jia, Department of Organ Transplantation, Affiliated Hospital of Guizhou Medical University, Guiyang 550000, Guizhou Province, China

Yi Zhang, Department of Hepatobiliary Surgery, Guizhou Provincial People's Hospital, Guiyang 550000, Guizhou Province, China

Corresponding author: Shi Zuo, PhD, Chief Doctor, Department of Hepatobiliary Surgery, Affiliated Hospital of Guizhou Medical University, No. 28 Gui Medical Street, Beijing Road, Yunyan District, Guiyang 550000, Guizhou Province, China. 1056659393@qq.com

Abstract

BACKGROUND
Cancer stem cells (CSCs) have been implicated in tumorigenesis and tumor recurrence and metastasis are key therapeutic targets in cancer treatment. MicroRNAs display therapeutic potential by controlling the properties of CSCs; however, whether an association exists between miR-3682-3p and CSCs is unknown.

AIM
To investigate the mechanism by which miR-3682-3p promotes stemness maintenance in hepatocellular carcinoma (HCC).

METHODS
MiR-3682-3p expression in HCC cell lines and 34 pairs of normal and HCC specimens was assayed by quantitative polymerase chain reaction. The functional role of miR-3682-3p was investigated *in vitro* and *in vivo*. Dual-luciferase reporter and chromatin immunoprecipitation assays were performed for target asse-

ssment, and western blotting was utilized to confirm miR-3682-3p/target relationships.

RESULTS
We found that miR-3682-3p plays a key role in HCC pathogenesis by promoting HCC cell stemness. The upregulation of miR-3682-3p enhanced CSC spheroid-forming ability, side population cell fractions, and the expression of CSC factors in HCC cells *in vitro* and the tumorigenicity of transplanted HCC cells *in vivo*. Furthermore, silencing miR-3682-3p prolonged the survival of HCC-bearing mice. Mechanistically, we found that miR-3682-3p targets FOXO3 and enables FOXO3/β-catenin interaction, which promotes c-Myc expression through PI3K/AKT; c-Myc, in turn, activates miR-3682-3p, forming a positive feedback loop. Intriguingly, miR-3682-3p expression was induced by hepatitis B virus X protein (HBx) and was involved in HBx-induced tumor stemness-related pathogenesis.

CONCLUSION
Our findings reveal a novel mechanism by which miR-3682-3p promotes stemness in HCC stem cells. Silencing miR-3682-3p may represent a novel therapeutic strategy for HCC.

Core Tip: In this work, we identified miR-3682-3p as a key inducer of cancer stem cell properties, thereby promoting the pathogenesis of hepatocellular carcinoma (HCC). In brief, we found that the upregulation of miR-3682-3p enhanced the spheroid forming ability, the fraction of side population cells, and the expression of cancer stem cell factors in HCC cells in vitro as well as the tumorigenicity of transplanted HCC cells *in vivo*; furthermore, silencing miR-3682-3p significantly prolonged the survival time of HCC-bearing mice. Mechanistically, we found that miR-3682-3p targets FOXO3 and enables FOXO3/β-catenin interaction, which promotes c-Myc expression through PI3K/AKT; c-Myc, in turn, activates miR-3682-3p, resulting in the formation of a positive feedback loop. Taken together, we identified a novel positive feedback regulatory loop involving HBx, miR-3682-3p, FOXO3, β-catenin, and c-Myc that plays a pivotal role in the stemness of HCC. Our findings revealed a novel mechanism by which miR-3682-3p promotes stem cell maintenance in HCC, and silencing miR-3682-3p may represent a novel therapeutic strategy for the treatment of this cancer.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a commonly diagnosed malignant tumor in China and is associated with very high morbidity and mortality[1,2]. Despite notable advances in multimodal treatment strategies over recent decades, such as radiotherapy, immunotherapy, and noninvasive surgical resection, the prognosis of patients with HCC, with a 5-year relative overall survival rate of approximately only 12%, remains unsatisfactory[3,4]. This highlights the urgent need to elucidate the mechanisms underlying HCC tumorigenicity and metastasis and identify reliable biomarkers to guide early HCC diagnosis and therapeutic intervention[5,6]. Increasing evidence has indicated that cancer stem cells (CSCs) underlie the insensitivity of HCC to radiotherapy and chemotherapy and play a key role in HCC metastasis and recurrence[7]. Like most stem cells, CSCs have the potential for self-renewal and the ability to differentiate into multiple cell types, resulting in tumor heterogeneity, and ultimately leading to tumor recurrence and metastasis[8]. Accordingly, targeting CSCs may represent a promising therapeutic target for cancer treatment[9,10].

The hepatitis B virus X protein (HBx) can reportedly disrupt several pathways and associated functions by regulating the expression and activity of numerous genes, epigenetics-related molecules [such as microRNAs (miRNAs)], and long non-coding RNAs (lncRNAs)[11]. MiRNAs are endogenous non-coding RNA molecules that can bind to the 3'-untranslated regions (3'-UTRs) of target mRNAs and regulate their expression at the post-transcriptional level[12]. More than 2500 miRNAs have been

identified in humans to date, and many likely remain to be identified[13-15]. It is well documented that miRNAs play a significant role in regulating CSC function and cancer progression at multiple levels[16, 17]. MiR-3682-3p exerts both oncogenic and tumor-suppressive functions, depending on the cancer type. For instance, high miR-3682-3p expression is correlated with esophageal cancer[18]. Exosomal miR-3682-3p targets angiopoietin 1 by altering Ras-MEK1/2-ERK signaling, suggesting that exosomal-derived miR-3682-3p may act as a tumor suppressor in HCC[19]. However, a different study reported that miR-3682-3p induces the proliferation of HCC cells and inhibits their apoptosis through the FAS pathway, indicating that miR-3682-3p may instead exert oncogenic effects in HCC[20].

Given these conflicting reports on the role of miR-3682-3p in HCC, we performed early validation of miR-3682-3p expression using in situ hybridization, and found that the expression level of miR-3682-3p was significantly elevated in HCC tissue compared with that in controls, and was positively correlated with both malignancy and worse prognosis in HCC. Our data further indicated that high miR-3682-3p expression levels were positively correlated with hepatitis B virus (HBV) infection. However, whether miR-3682-3p also affects stemness in HCC remains unknown. In this study, we demonstrated that miR-3682-3p directly targets and inhibits the expression of FOXO3. The downregulation of FOXO3 Levels subsequently leads to the activation of a PI3K/AKT/β-catenin/c-Myc/miR-3682-3p positive feedback loop that promotes the stemness of HCC cells. In summary, our findings revealed a role for miR-3682-3p in promoting the stemness of HCC cells and suggested that this miRNA may represent a potential therapeutic target for the treatment of HCC.

MATERIALS AND METHODS

Clinical tissue specimens
A total of 34 patients with HCC who underwent surgical resection in the Department of Hepatobiliary Surgery of the Affiliated Hospital of Guizhou Medical University from June 2017 to June 2019 were selected for this study. All patients provided informed consent. The study was approved by the Ethics Committee of the Affiliated Hospital of Guizhou Medical University. HCC and paired paracancerous tissue specimens (at least 3 cm from the edge of the tumor) were collected after resection and were preserved in liquid nitrogen. HCC was pathologically confirmed in all cases. All the selected patients were undergoing first-time surgical resection of the primary lesion, and none had received radiotherapy, chemotherapy, or hormone therapy before surgery.

The HCC tissue microarray was purchased from the Shanghai Molecular Medicine Engineering Center (Shanghai, China) with approval from its Ethics Committee.

Cell culture
The cell lines used in this study (LO2, LM3, Huh7, Hep3B, HepG2, and MHCC97H) were provided by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone Laboratories, Inc., Logan, UT, United States) supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2.

RNA isolation and quantitative polymerase chain reaction
Total RNA was extracted from cultured HCC cells using Trizol reagent and reverse transcribed into cDNA, which served as a template for quantitative polymerase chain reaction (qPCR). Gene expression levels were detected separately using a standard SYBR Green RT-PCR kit (Thermo Scientific, Waltham, MA, United States) according to the manufacturer's instructions. The sequences of the primers used are detailed in Supplementary Table 1. Relative gene expression levels were calculated using the 2-△ △ Ct method and are reported as fold changes.

Lentivirus production and infection
Purchased miR-3682-3p overexpressing lentivirus with green fluorescence constructed and synthesized by Ji kai (Shanghai, China) and the corresponding control lentivirus in vacuo. The virus dosage was calculated at a cell fusion rate of 60%. Lentivirus was transfected into HCC cells at an MOI of 80 using a transfection reagent. The medium was changed after 8 h of transfection. Green fluorescence could be detected after 72 h of transfection under a fluorescence microscope, at which point the cells were collected for PCR-based detection of the miR-3682-3p level to determine the transfection efficiency (Supplementary Table 2).

Sphere formation assay
A total of 5 × 10³ HCC cells were inoculated into six-well ultra-low adsorption plates (Corning, Painted Post, NY, United States) containing 2% B27 (BD PharMingen, Carlsbad, CA, United States), 20 ng/mL epidermal growth factor, 20 ng/mL fibroblast growth factor in DMEM/F12 serum-free medium (Invitrogen). The cells were cultured for 14 d and then imaged and counted under a light microscope.

CSC sorting

Cells in the logarithmic growth phase were digested into single-cell suspensions and washed twice with PBS. A total of 1×10^6 cells/mL were resuspended in each of two tubes containing medium supplemented with 2% FBS. Verapamil (20 µL) was added to one tube (to inhibit dye efflux) and incubated for 30 min at 37 °C protected from light. Vybrant DyeCycle Violet stain (Sigma-Aldrich) at a final concentration of 5 µg/mL was then added to both tubes followed by incubation with shaking for 90 min at 37 °C in the dark, and with mixing every 10 min. The samples were subsequently centrifuged at $1500 \times g$, the supernatant was discarded, and the cells were resuspended in 1 mL of PBS. Finally, 5 µL of 7-ADD (BD PharMingen) was added to each tube followed by flow cytometric detection. Each set of experiments was repeated three times.

Dual-luciferase reporter assay

HCC cells were inoculated into 24-well plates, and the cells were fused to 70% with Lipofectamine 2000 (Invitrogen, Guangzhou, China). Plasmids containing the wild-type or mutated (mut) FOXO3 3′-UTR were co-transfected with miR-3682-3p mimic/inhibitor and the cells were assayed for luciferase activity after 48 h. To investigate the effect of c-Myc on miR-3682-3p transcription, vectors containing mutated c-Myc binding sites were constructed and co-transfected with c-Myc expression plasmids into HCC cells, following which the cells were assayed for luciferase activity.

Western blot analysis

Total protein was extracted from each group of HCC cells. Protein concentrations were determined by BCA. Equal amounts of protein were separated by 10% SDS-PAGE, transferred to PVDF membranes (Millipore, Bedford, MA, United States), incubated overnight at 4 °C with primary antibodies, and then labeled with secondary antibodies at room temperature for 1 h. Bands were developed using luminol-enhanced chemiluminescence (Thermo Scientific). The primary antibodies used for western blotting are described in Supplementary Table 3.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed using a ChIP kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, genomic DNA was extracted from HCC cells, the DNA was fragmented, and a c-Myc antibody was added to the reaction system for immunoprecipitation. IgG served as a negative control. The purified DNA was PCR-amplified and the products were separated on a 2% agarose gel. The primers used for PCR amplification are shown in Supplementary Table 1.

Co-immunoprecipitation

Total cellular protein was extracted using the Pierce Co-immunoprecipitation (Co-IP) Kit (Thermo Scientific). A total of 2 mg of protein was incubated with 5 µg of specific antibody or IgG (used as a negative control) overnight at 4 °C. After washing, the protein samples were subjected to western blotting.

Nude mouse tumor xenograft model

All animal experiments were performed according to the requirements of the Guizhou Medical University Animal Experiment Ethics Committee (2100555). Cyclophosphamide (200 µL) was injected daily for the first three days of the experiment to disrupt the immune system of BALB/c nude mice (SPF, Beijing, China). Then, the mice were divided into two groups of 20 mice each and administered HCC cells (1×10^6, 2×10^6, 4×10^6, and 8×10^6 cells, n = 5 mice per concentration) overexpressing or not miR-3682-3p by subcutaneous injection. The mice were euthanized after 21 d and the tumors were excised. Equal amounts of HCC cells (8×10^6) were subcutaneously injected into four-week-old BALB/c nude mice (n = 10 per group) to establish a HCC xenograft model. After one week, the mice received either saline or 10 nmol of a miR-3682-3p antagomir (RiboBio, Guangzhou, China) intraperitoneally twice a week for three weeks. The Kaplan-Meier method was used to evaluate the survival of the mice.

Immunohistochemistry and evaluation of immunohistochemical staining

FOXO3, OCT4, and SOX2 protein expression levels were assessed in tissue microarrays (Proteintech, Wuhan, China) according to the manufacturer's protocol as well as in animal tissues. The indirect streptavidin peroxidase method was used. Immunohistochemistry was scored by two registered pathologists from the Affiliated Hospital of Guizhou Medical University based on the intensity of coloration and area of the sections. The stained area was scored from 0 to 4 (0: < 5% staining; 1: 5%-25% staining; 2: 26%-50% staining; 3: 50%-75% staining; and 4: > 75% staining). The product of the two sets of scores was counted, and final staining scores of < 6 and ≥ 6 were considered to reflect low and high expression, respectively.

Statistical analysis

SPSS v.25.0 (SPSS Inc., Chicago, IL, United States) was used for statistical analysis. Data were expressed as means ± standard deviation. Differences between two groups were compared using *t*-tests. Patient clinicopathological parameters were analyzed using the χ^2 test, patient survival was analyzed using Kaplan-Meier survival curves, and comparisons of survival and recurrence rates between groups were performed using the log-rank test. *P* values < 0.05 were considered significant. One-way Cox regression was used to analyze all clinicopathological indicators and factors with *P* values < 0.05 were used for multivariate regression analysis.

RESULTS

High expression of miR-3682-3p reflected poor prognosis of HCC patients

Data from The Cancer Genome Atlas (TCGA) were accessed to identify miRNAs related to the survival of HCC patients. We identified miR-3682-3p as being distinctly highly expressed in HCC specimens from TCGA datasets (*P* = 0.001) (Figure 1A). The results of our previous in situ hybridization analysis in primary HCC tissues from a cohort of 90 HCC patients had suggested that a significant correlation exists between miR-3682-3p expression and tumor size and stage, intrahepatic GGT levels, and HBV surface antigen status. Additionally, high miR-3682-3p expression levels were associated with a shorter survival time in HCC patients[21]. To verify the above results, we obtained HCC and adjacent paracancerous tissue from a different cohort of 34 patients. RT-qPCR analysis showed that miR-3682-3p expression was significantly higher in HCC tissue than in adjacent liver tissue (*P* = 0.001) (Figure 1B) and was also significantly higher in HCC cell lines than in an immortalized hepatocyte line (LO2). Intriguingly, the expression of miR-3682-3p was upregulated in HBV-positive HCC cells (Hep3B, MHCC-97H, and HCCLM3) compared with that in HBV-negative (Huh7 and HepG2) HCC cells (Figure 1C). Furthermore, survival analysis of TCGA dataset suggested that HCC patients with low miR-3682-3p expression survived longer than those with high miR-3682-3p expression (*P* < 0.001) (Figure 1D). In summary, our results suggested that miR-3682-3p expression is upregulated in HCC tissues and is positively correlated with poor prognosis in HCC patients.

miR-3682-3p promotes stem cell properties in HCC cells via the PI3K/AKT/c-Myc signaling pathway

To investigate the effect of miR-3682-3p on CSCs, miR-3682-3p mimics, miR-3682-3p inhibitors, or miR-3682-3p-expressing lentiviral vectors were transfected into HCC cells. We first examined the transfection efficiency of miR-3682-3p by RT-qPCR (Supplementary Figure 1A and B). Overexpression of miR-3682-3p increased the sphere-forming ability of HCC cells (Figure 2A and Supplementary Figure 1C) as well as the percentage of side population cells (Figure 2B and Supplementary Figure 1D). Immunofluorescence staining and RT-qPCR further confirmed that miR-3682-3p increased the stemness of HCC cells (Figure 2C-E). In contrast, miR-3682-3p downregulation in LM3 and MHCC-97H cells following treatment with the miR-3682-3p inhibitor elicited the opposite result (Supplementary Figure 1E-H).

To investigate whether miR-3682-3p modulated the signaling pathway involved in the promotion of tumor stemness, we performed gene set enrichment analysis (GSEA), and found that miR-3682-3p expression was closely associated with PI3K/AKT activation in HCC (Figure 2F). Western blot was further performed to confirm this possibility, with the results showing that miR-3682-3p overexpression led to the upregulation of the expression of PI3K/AKT pathway-related proteins, including c-Myc, as well as that of stemness-related molecules such as CD44, CD133, SOX2, and OCT4. The simultaneous application of a PI3K inhibitor (LY294002) and the miR-3682-3p inhibitor in HCC cells overexpressing miR-3682-3p reversed the above-mentioned changes in protein levels (Figure 2G). Together, these data demonstrated that miR-3682-3p promotes a CSC-like phenotype in HCC cells through the PI3K/AKT/c-Myc signaling axis, thereby further contributing to the progression of HCC.

MiR-3682-3p promotes the tumorigenicity of HCC cells in vivo

Because we found that MiR-3682-3p could promote a CSC-like phenotype in vitro, we next assessed whether miR-3682-3p exerted a similar effect *in vivo*. For this, different concentrations (1×10^6, 2×10^6, 4×10^6, and 8×10^6) of HCC cells stably transfected with miR-3682-3p were subcutaneously injected into BALB/C nude mice. As shown in Figure 3A, the associated tumorigenicity rates were 20% (1/5), 60% (3/5), 100% (5/5), and 100% (5/5), respectively. For equal concentrations of control cells, the tumorigenicity rates were 0% (0/5), 20% (1/5), 60% (3/5), and 100% (5/5), respectively. Similarly, the tumor weight was greater in the miR-3682-3p overexpression group than in the control group (Figure 3B). RT-qPCR results confirmed that the miR-3682-3p expression levels were higher in xenografts derived from cells stably overexpressing miR-3682-3p than in negative control cell-derived xenografts (Figure 3C). Additionally, Kaplan-Meier curve analysis indicated that miR-3682-3p antagomir administration prolonged the survival time of mice compared with that of control animals (Figure 3D). The expression of SOX2 and OCT4 in nude mouse xenograft tumors was then detected by immunohistochemistry, and the same results were obtained (Figure 3E). Overall, these results provided further evidence that miR-
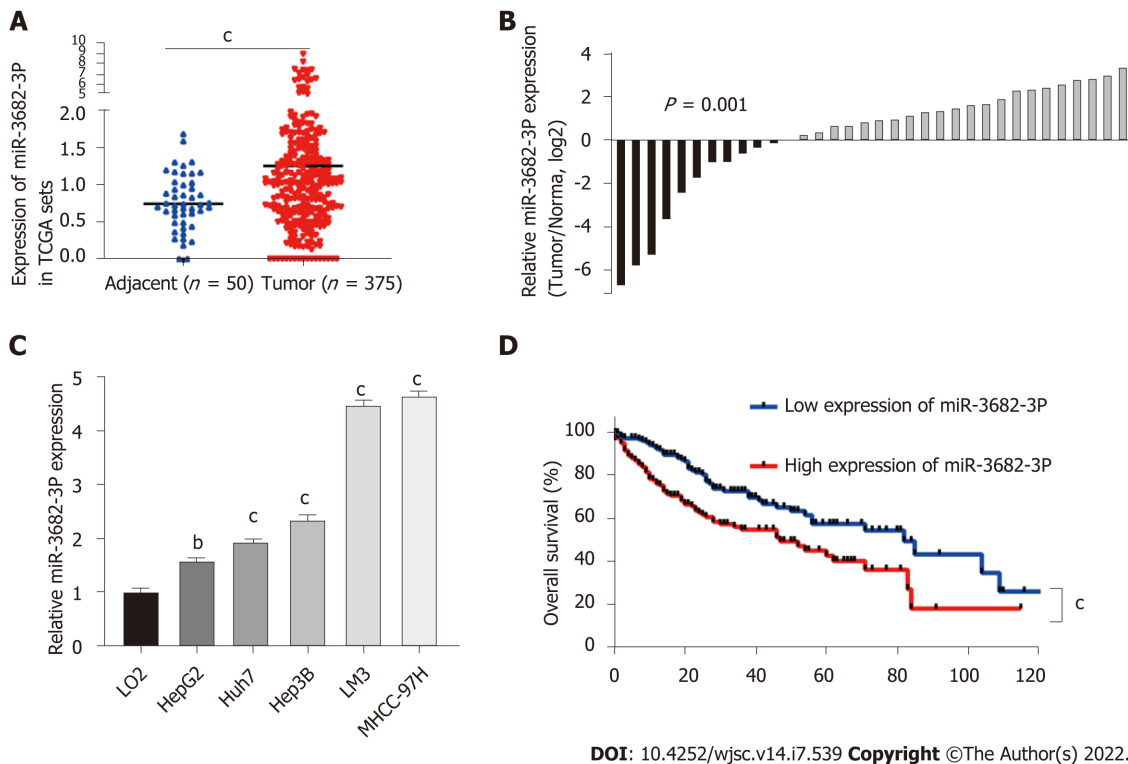
Figure 1 High expression of miR-3682-3p reflected poor prognosis of hepatocellular carcinoma. A: The expression levels of miR-3682-3p in normal and hepatocellular carcinoma (HCC) tissues in The Cancer Genome Atlas (TCGA) dataset; B: The expression of miR-3682-3p in 34 HCC and paired paracancerous tissue samples as determined by RT-qPCR; C: miR-3682-3p expression in HCC cells (HepG2, Huh7, Hep3B, LM3, and MHCC-97H) and normal liver epithelial (LO2) cells as determined by RT-qPCR; D: Analysis of Kaplan-Meier overall survival curves for HCC patients based on miR-3682-3p expression in TCGA dataset. [b]$P < 0.01$, [c]$P < 0.001$. Experiments were repeated three times.

3682-3p promotes the tumorigenicity of HCC cells *in vivo*.

FOXO3 is a direct target of miR-3682-3p

To understand how miR-3682-3p affects the stemness of HCC cells, miRNA walk 2.0 was used for the prediction of miR-382-3p targets, resulting in the identification of FOXO3 as a potential target gene of this miRNA (Figure 4A). MiR-3682-3p overexpression led to the suppression of FOXO3 protein levels, but did not affect FOXO3 transcript levels; meanwhile, the knockdown of miR-3682-3p elicited the opposite effect (Figure 4B-D). The interaction between miR-3682-3p and FOXO3 was further validated using a dual-luciferase reporter assay (Figure 4E). Additionally, immunohistochemical analysis of xenografts derived from miR-3682-3p-overexpressing HCC cells demonstrated that FOXO3 expression was downregulated in miR-3682-3p-overexpressing tumors (Figure 4F). To further demonstrate that FOXO3 mediates the oncogenic activity of miR-3682-3p, we evaluated the effect of FOXO3 on miR-3682-3p-modulated signals in HCC cells. We found that the overexpression of FOXO3 eliminated the promotive effects of miR-3682-3p on the expression of phosphorylated (p)-PI3K, p-AKT, β-catenin, SOX2, OCT4, CD133, CD44, and c-Myc (Figure 4G). Moreover, FOXO3 inhibited miR-3682-3p-induced stimulation of CSC-like phenotypes in HCC cells (Supplementary Figure 2A-D). Taken together, these results indicated that FOXO3 inhibits the miR-3682-3p-induced stemness of HCC cells.

It has been documented that FOXO3 interacts with β-catenin and plays a pivotal role in a variety of tumors[22,23]. Here, we identified a possible interaction between FOXO3 and β-catenin by CoIP (Figure 4H). Interestingly, we further found that FOXO3 co-localized with β-catenin in the cytoplasm (Figure 4I), while further immunofluorescence staining results showed that FOXO3 inhibited β-catenin nuclear translocation (Figure 4J).

In brief, the above findings indicated that miR-3682-3p directly binds to the 3′-UTR of FOXO3, thereby activating the PI3K/AKT/β-catenin/c-MYC signaling axis and promoting the stemness of HCC cells.

c-Myc binds to the miR-3682-3p promoter region to promote its transcriptional activation

Transcription factors can promote the transcription of downstream miRNAs, which then bind to and regulate the expression of their target genes. To determine the mechanism underlying the upregulation of miR-3682-3p expression in HCC, we performed GSEA, and found that miR-3682-3p was positively correlated with c-Myc (Figure 5A). Transcription factor binding site prediction using the UCSC and JASPAR databases indicated that c-Myc can bind three regions in the miR-3682-3p promoter (Figure 5B).
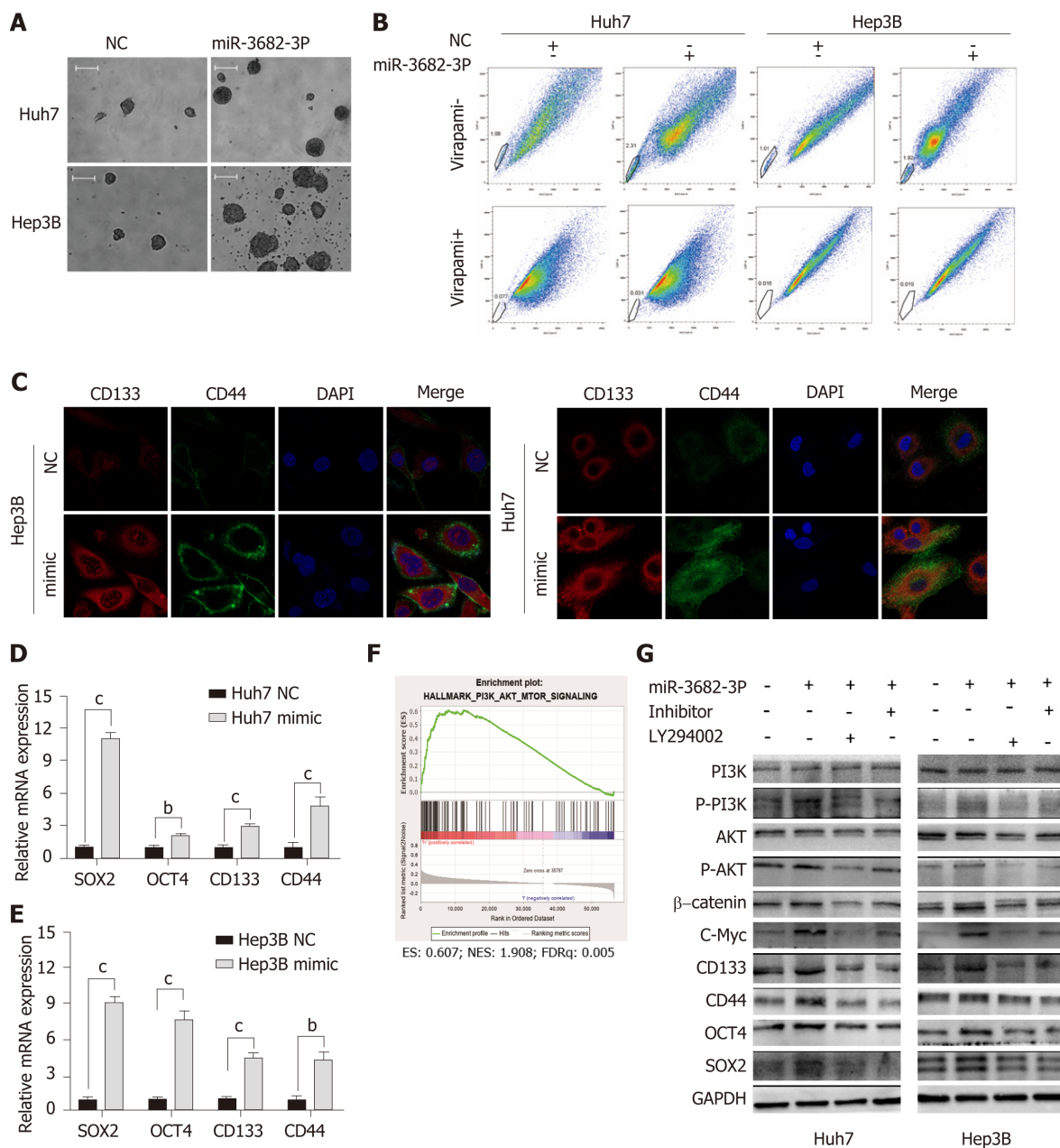
DOI: 10.4252/wjsc.v14.i7.539 **Copyright** ©The Author(s) 2022.

Figure 2 miR-3682-3p promotes the stem cell properties of hepatocellular carcinoma cells *via* the PI3K/AKT/c-Myc signaling pathway. A: Representative images of formed hepatospheres (× 100 magnification); scale bar: 200 μm; B: Flow cytometric analysis of the proportion of side population cells; C: Immunofluorescence staining was performed to assess the expression levels of CD44 and CD133 in hepatocellular carcinoma cells (scale bar: 5 μm); D and E: RT-qPCR analysis of SOX2, OCT4, CD133, and CD44 expression; F: Gene set enrichment analysis showing that miR-3682-3p regulates the PI3K/AKT signaling axis; G: Western blotting analysis of PI3K/AKT/c-Myc signaling- and stemness-related protein expression levels. [b]$P < 0.01$; [c]$P < 0.001$. Experiments were repeated three times. NC: Negative control.

RT-qPCR analysis showed that upregulation of c-Myc increased the expression levels of miR-3682-3p, whereas c-Myc downregulation elicited the opposite result (Figure 5C and D). ChIP and luciferase reporter assays further confirmed that c-Myc can bind to the three sites on the miR-3682-3p promoter (Figure 5E-G). In summary, these data suggested that in HCC, c-Myc regulates miR-3682-3p transcription through binding to its promoter region.

HBx induces miR-3682-3p expression, thereby enhancing the cancer stem cell-like properties of HCC cells

HBx is an HBV-encoded protein that displays oncogenic properties and is known to play a critical role in the development of many tumors. We have previously shown that miR-3682-3p expression is positively correlated with HBV infection. Here, we sought to determine whether there is a correlation between HBx and the miR-3682-3p/FOXO3/PI3K/AKT/β-catenin/c-Myc feedback loop. Our results demonstrated that the overexpression or knockdown of the HBx-encoding gene respectively increased

	1×10⁶	2×10⁶	4×10⁶	8×10⁶
NC	0/5	1/5	3/5	5/5
miR-3682-3p	1/5	3/5	5/5	5/5
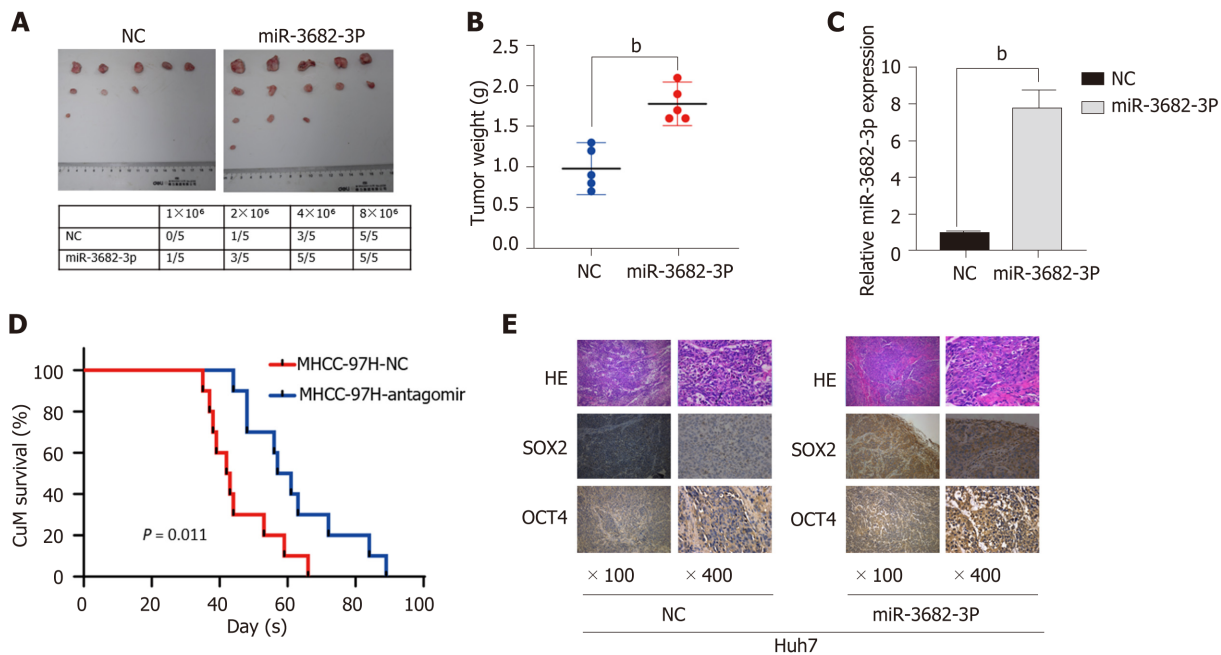
DOI: 10.4252/wjsc.v14.i7.539 **Copyright** ©The Author(s) 2022.

Figure 3 miR-3682-3p promotes the tumorigenicity of hepatocellular carcinoma cells *in vivo*. A: The tumorigenic rate of different concentrations of hepatocellular carcinoma cells (*n* = 5); B: Tumors in the miR-3682-3p overexpression group weighed significantly more than those of the control group (*n* = 5); C: Detection of miR-3682-3p expression in tumor tissues by RT-qPCR; D: Survival analysis revealed that miR-3682-3p antagomir prolongs the survival time of mice (*n* = 10, log-rank test); E: Immunohistochemical staining for stem cell-associated markers (SOX2 and OCT4) in xenograft tumors (*n* = 5) (× 100 magnification scale bar: 200 μm; × 400 magnification scale bar: 50 μm). ^bP < 0.01. NC: Negative control; HE: Hematoxylin and eosin.

and decreased the expression level of miR-3682-3p (Figure 6A and B). We further showed that miR-3682-3p mediated the oncogenic effects resulting from the HBx-mediated induction of PI3K/AKT/c-Myc signaling (Figure 6C and D), *i.e.*, HBx promoted stemness by upregulating miR-3682-3p expression.

Pathoclinical features of FOXO3 expression
Finally, to obtain additional evidence to support our conclusions, tissue microarrays containing 90 HCC and paired adjacent non-tumor tissue samples were subjected to FOXO3 immunohistochemical analysis and scored for cell staining (Figure 7A and B). Survival analysis indicated that low FOXO3 expression correlated with reduced overall survival time in HCC patients (Figure 7C and D), while high FOXO3 expression elicited the opposite effect (Figure 7C and D). Subsequently, FOXO3 expression was evaluated in relation to the clinicopathological characteristics of HCC (Table 1). A one-step univariate Cox regression analysis indicated that AJCC staging (*P* = 0.022), relapse (*P* = 0.002), GGT (*P* = 0.035), Edmondson-Steiner grading (*P* < 0.001), and FOXO3 expression (*P* = 0.006). However, no differences were identified using multivariate Cox regression analysis (Table 2). Combined with our early fluorescence in situ hybridization analysis scores, our immunohistochemical analysis revealed that FOXO3 expression was negatively correlated with miR-3682-3p expression (Figure 7E).

DISCUSSION

HCC is one of the most commonly diagnosed cancers worldwide[24]. Despite improvements in the treatment of HCC, this disease continues to be associated with high morbidity and mortality[5,25]. CSCs play key roles in tumorigenesis and tumor metastasis, and targeting CSCs has the potential to treat a variety of cancers[26]. Multiple hepatic CSC biomarkers have already been identified. In this study, we have added a genetic signature of these liver CSCs and revealed the signaling pathways that regulate HCC[27].

Epigenetic modification is a key regulatory mechanism in tumor pathogenesis and involves the participationof numerous miRNAs[28]. Recent studies have shown that specific miRNAs exhibit good therapeutic potential associated with the control of cancer cell stemness[29]. In addition, strategies to upregulate or inhibit miRNA levels in tumors, such as the design of miRNA mimics or antagomiRs, have shown promising preliminary clinical results in tumor treatment, especially those targeting CSCs. Studies have revealed that miR-3682-3p plays a major role in a variety of cancers, including HCC. In addition, miR-3682-3p has been implicated as a key player in bladder cancer drug resistance by inhibiting its activation by BML1 and thereby affecting[30]. However, no study to date has investigated

Table 1 Correlations between FOXO3 expression and the clinicopathological features of hepatocellular carcinoma patients, *n* (%)

Characteristics	*n*	FOXO3 expression		*P* value
		Low	High	
Age (yr)				
> 50	50	30 (60.00)	20 (40.00)	0.633
≤ 50	40	22 (55.00)	18 (45.00)	
Gender				
Male	80	46 (57.50)	34 (42.50)	1.000
Female	10	6 (60.00)	4 (40.00)	
AJCC stage				
I	52	25 (48.08)	27 (51.92)	0.029
II-III	38	27 (71.05)	11 (28.95)	
HBsAg				
Negative	19	8 (42.11)	11 (57.89)	0.119
Positive	71	44 (61.97)	27 (38.03)	
Recurrence				
No	41	19 (46.34)	22 (53.76)	0.032
Yes	49	33 (67.35)	16 (32.65)	
AFP (μg/L)				
> 400	33	19 (57.6)	14 (42.4)	0.976
≤ 400	57	33 (57.9)	24 (42.1)	
Total bilirubin (μmol/L)				
>20	15	5 (33.33)	10 (66.67)	0.036
≤ 20	75	47 (62.67)	28 (37.33)	
ALT (U/L)				
> 45	31	20 (64.52)	11 (35.48)	0.348
≤ 45	59	32 (54.24)	27 (45.76)	
GGT				
> 40	59	37 (62.7)	22 (37.3)	0.191
≤ 40	31	15 (48.4)	16 (51.6)	
Edmondson-Steiner grade				
I-II	61	28 (45.90)	33 (54.10)	0.001
III-IV	29	24 (82.76)	5 (17.24)	
Tumor number				
Single	79	45 (56.97)	34 (45.03)	0.675
Multiple	11	7 (63.64)	4 (36.36)	
Tumor size (cm)				
> 5	28	24 (85.71)	4 (14.29)	0.001
≤ 5	62	28 (45.16)	34 (54.84)	

stemness in HCC or whether miR-3682-3p has a role in this process. To address this, we investigated the role of miR-3682-3p in regulating the properties of HCC stem cells as well as the underlying mechanism. We found that the inhibition of miR-3682-3p suppressed the development of HCC, demonstrating that miR-3682-3p may serve as a prognostic marker for this cancer.

Table 2 Univariate and multivariate survival analysis of clinicpathological variables of hepatocellular carcinoma patients

Characteristics	Overall survival					
	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
FOXO3 expression Low *vs* high	0.311	(0.134-0.721)	**0.006**	0.504	(0.164-1.553)	0.233
Age (yr) ≤ 50 *vs* > 50	0.721	(0.352-1.476)	0.371			
Gender Male *vs* female	0.520	(0.124-2.176)	0.371			
AJCC stage I *vs* II-III	2.277	(1.123-4.616)	0.022	1.203	(0.555-2.604)	0.640
HBsAg Negative *vs* positive	0.667	(0.257-1.736)	0.407			
Recurrence No *vs* yes	87.223	(4.860-1565.443)	0.002			
AFP (ng/mL) ≤ 400 *vs* > 400	0.888	(0.434-1.818)	0.746			
Total bilirubin (μmol/L) ≤ 20 *vs* > 20	1.113	(0.428-2.893)	0.826			
ALT (U/L) ≤ 45 *vs* > 45	1.046	(0.504-2.171)	0.903			
GGT (U/L) ≤ 40 *vs* > 40	0.386	(0.159-0.937)	0.035	0.594	(0.233-1.516)	0.276
Edmondson-Steiner grade I-II *vs* III-IV	3.664	(1.791-7.497)	< 0.001	1.839	(0.560-3.445)	0.478
Tumor number Singlevs *vs* multiple	1.810	(0.742-4.417)	0.192			
Tumor size (cm) ≤ 5 *vs* > 5	0.504	(0.250-1.018)	0.056			

FOXO3 is a key member of the large FOX family of transcription factors[31]. In mammals, FOXO3 is known to have a wide range of biological functions[32]. Studies have confirmed that FOXO3 exerts an oncogenic role in a variety of cancers, including breast cancer[33], through the promotion of tumor cell proliferation, metastasis, stemness, and drug resistance, among other biological behaviors. Several studies have illustrated the interactive relationship between FOXO3 and β-catenin and the functional role of FOXO3 in the PI3K/AKT signaling axis[22]. In the present study, we found that miR-3682-3p directly targets FOXO3 and suppresses its expression, thereby enhancing P3K/AKT/c-Myc and downstream signaling, including upregulating the expression of known stemness markers, and, consequently, promoting the stemness of HCC cells.

c-Myc is an integral member of the MYC family of transcription factors. It is known to be involved in the progression of various cancers[34,35], including through the regulation of the expression of numerous miRNAs, thereby promoting tumor stemness, metastasis, proliferation, and chemoresistance [36,38]. We have previously shown the existence of three binding sites for c-Myc in the miR-3682-3p promoter using online bioinformatics tools. The results of the current study suggested that miR-3682-3p targets FOXO3 and enables FOXO3/β-catenin interaction, which promotes c-Myc expression; c-Myc, in turn, activates miR-3682-3p, thereby forming a positive feedback loop.

Despite the consensus that HBx is closely related to HBV-associated HCC[9,39], the role of miRNAs in HBx-associated HCC is poorly understood[40]. HBx is known to influence the development of HCC by regulating β-catenin entry into the nucleus, which, in turn, affects downstream signaling[39-41]. In

A

FOXO3 wt 3'UTR: 5'… GAGTTCCAT TGGTATTGTCAC…TTTGCCTCTACCTAGTGA…3'UTR

hsa-miR-3682-3p:3'…GAUGGAGGUGGACAUAGUAGU…5' 3'…GAUGGAGGUGGACAUAGU…5'

mut1 3'UTR:5'…GGGT CTTGTT ACATTGTAGC…TTTGCCTCTACCTAGTGA….3'

mut2 3'UTR:5'…GAGATCCATT TGGTATTGTCAC….TTTGCCTGAUGGAGTGA…3'

mut1+2 3'UTR:5'…GGGTCTTGTT ACATTGTAGc…TTTGCCTGAUGGAGTGA…3'

B

C

D

E

F

G

H

I

DOI: 10.4252/wjsc.v14.i7.539 Copyright ©The Author(s) 2022.
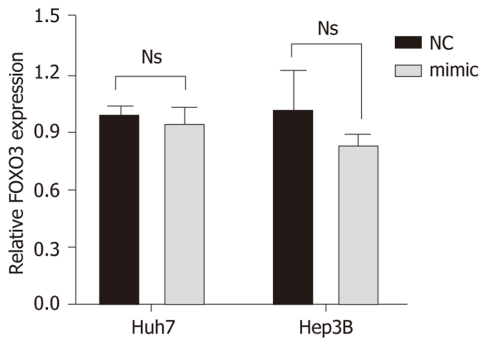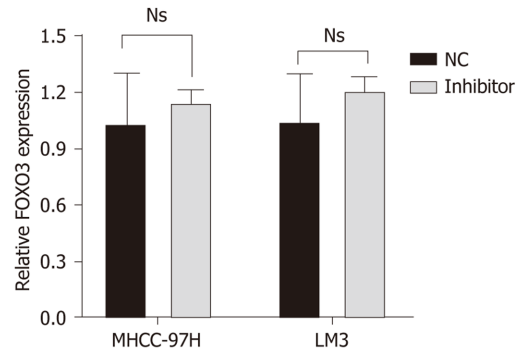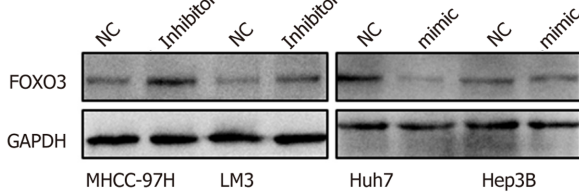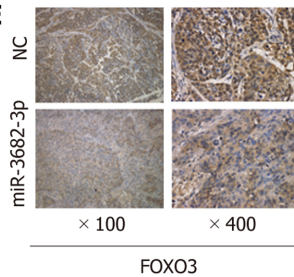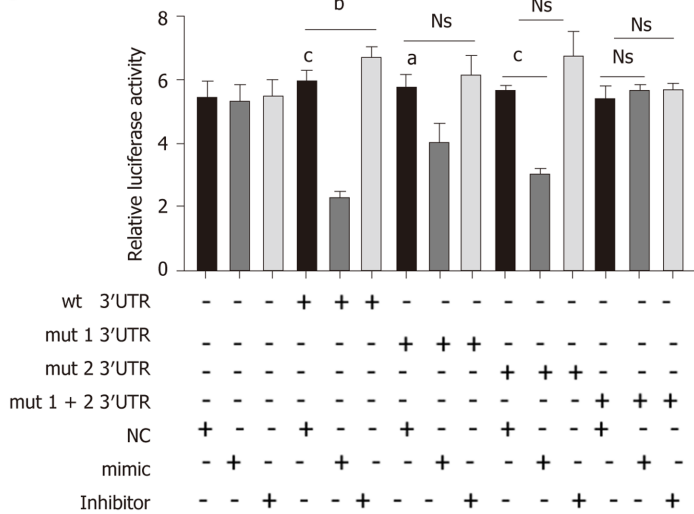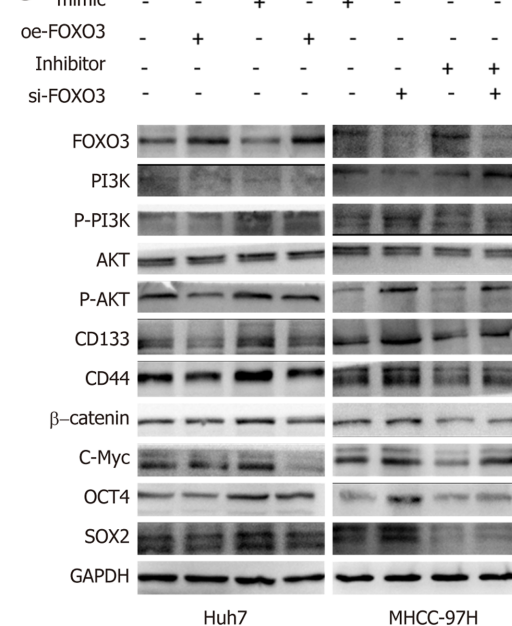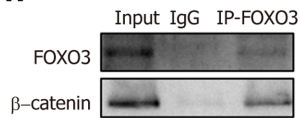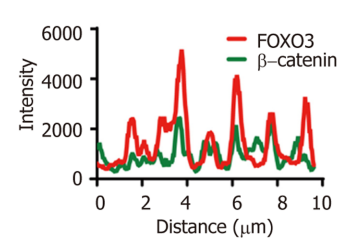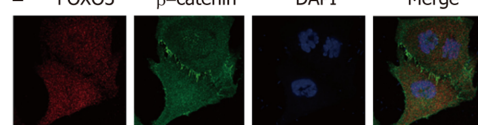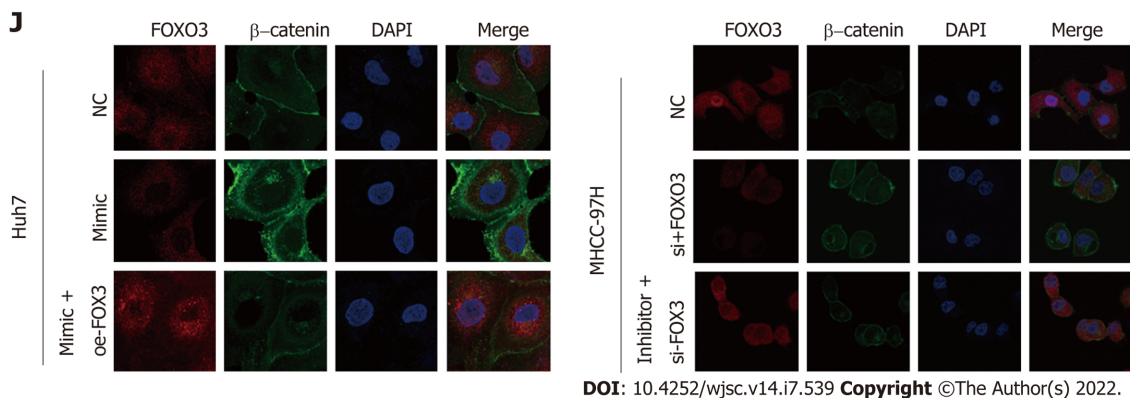
Figure 4 FOXO3 is a direct target of miR-3682-3p. A: Binding sites for miR-3682-3p in the 3'-UTR of FOXO3 were predicted using bioinformatics tools; B-D: The effect of miR-3682-3p on FOXO3 mRNA and protein levels as determined by RT-qPCR and western blot, respectively; E: Dual-luciferase reporter assay was used to detect the interaction between miR-3682-3p and the 3'-UTR of FOXO3; F: Immunohistochemical analysis of FOXO3 expression in xenograft tumors (*n* = 5) (× 100 magnification scale bar: 200 μm, × 400 magnification scale bar: 50 μm); G: Western blot-based analysis of the effects of FOXO3 on stemness- and PI3K/AKT/c-Myc pathway-related proteins; H: Immunoprecipitation assays for the interaction between endogenous FOXO3 and β-catenin; I: Immunofluorescence staining-based assessment of FOXO3/β-catenin co-localization; J: Immunofluorescence staining of FOXO3 and β-catenin in hepatocellular carcinoma cells (scale bar: 5 μm). $^aP <$ 0.05, $^cP < 0.001$. Experiments were repeated three times. NC: Negative control; wt: Wild-type; mut: Mutant.

DOI: 10.4252/wjsc.v14.i7.539 Copyright ©The Author(s) 2022.
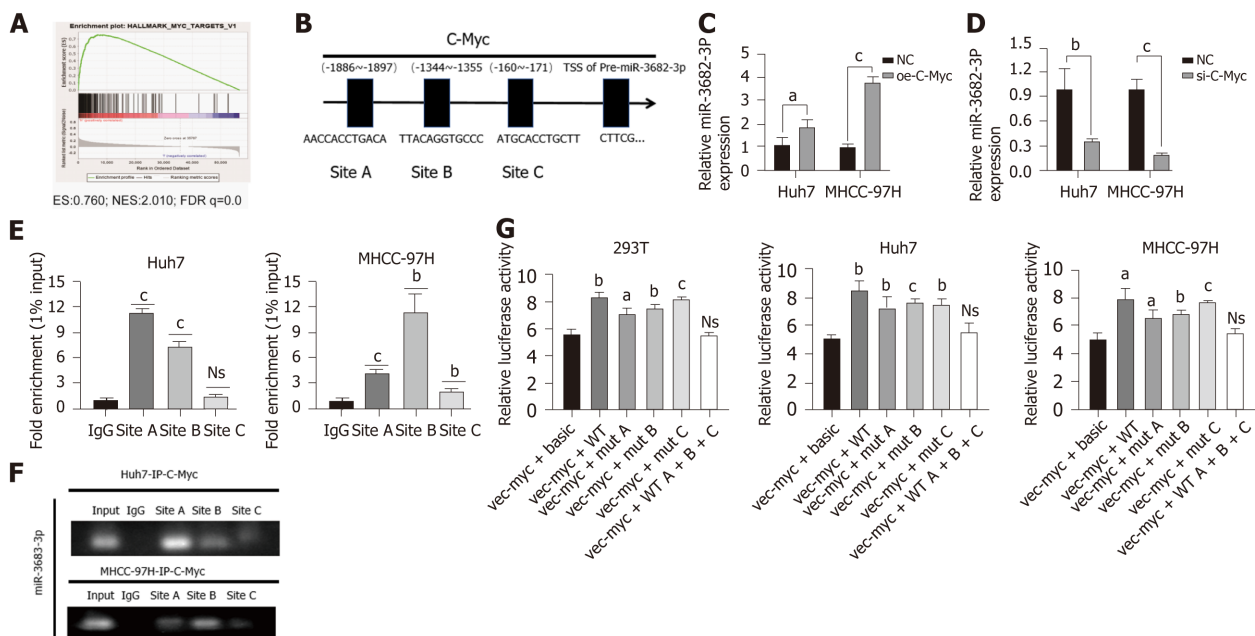
Figure 5 c-Myc binds to the miR-3682-3p promoter region and promotes its transcriptional activation. A: Gene set enrichment analysis revealed that miR-3682-3p is positively associated with c-Myc; B: Bioinformatic-based prediction of c-Myc binding sites in the miR-3682-3p promoter region; C and D: RT-qPCR analysis of miR-3682-3p levels after c-Myc silencing and overexpression; E and F: Chromatin immunoprecipitation validated the binding of c-Myc to the miR-3682-3p promoter; G: Dual-luciferase reporter assay confirmed the binding of c-Myc to the miR-3682-3p promoter. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$. Experiments were repeated three times. NC: Negative control.

the present study, we found that miR-3682-3p expression was positively correlated with HBV infection. We further found that HBx can promote miR-3682-3p expression, thereby downregulating that of FOXO3 and promoting stemness in HCC.

CONCLUSION

We identified a novel positive feedback regulatory loop involving miR-3682-3p, FOXO3, β-catenin, and c-Myc that plays a pivotal role in the stemness of HCC (Figure 7F). Our findings unveiled a novel mechanism by which miR-3682-3p promotes stem cell maintenance in HCC progression and elucidated for the first time the regulatory relationship between HBV and miR-3682-3p.

DOI: 10.4252/wjsc.v14.i7.539 **Copyright** ©The Author(s) 2022.
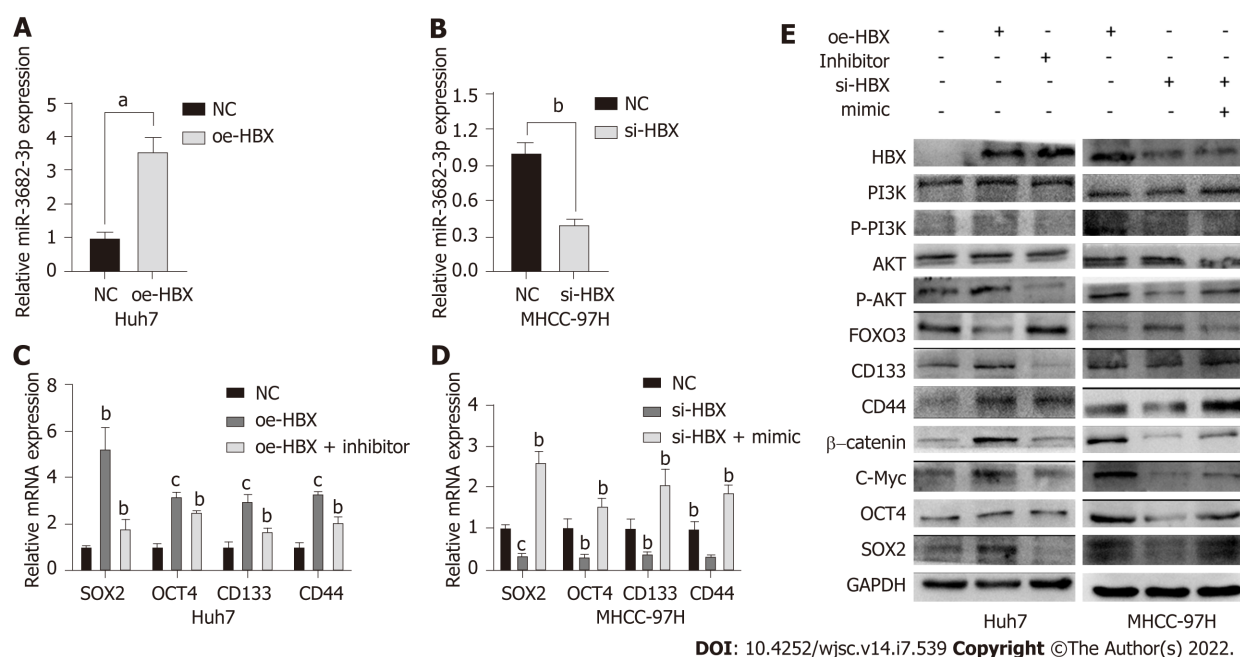
Figure 6 HBx induces miR-3682-3p expression, thereby enhancing the cancer stem cell-like properties of hepatocellular carcinoma cells.
A and B: The levels of miR-3682-3p after the overexpression and silencing of HBx were detected by RT-qPCR; C and D: RT-qPCR and western blot analysis of the effect of HBx on stemness markers. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$. Experiments were repeated three times. NC: Negative control.

DOI: 10.4252/wjsc.v14.i7.539 **Copyright** ©The Author(s) 2022.
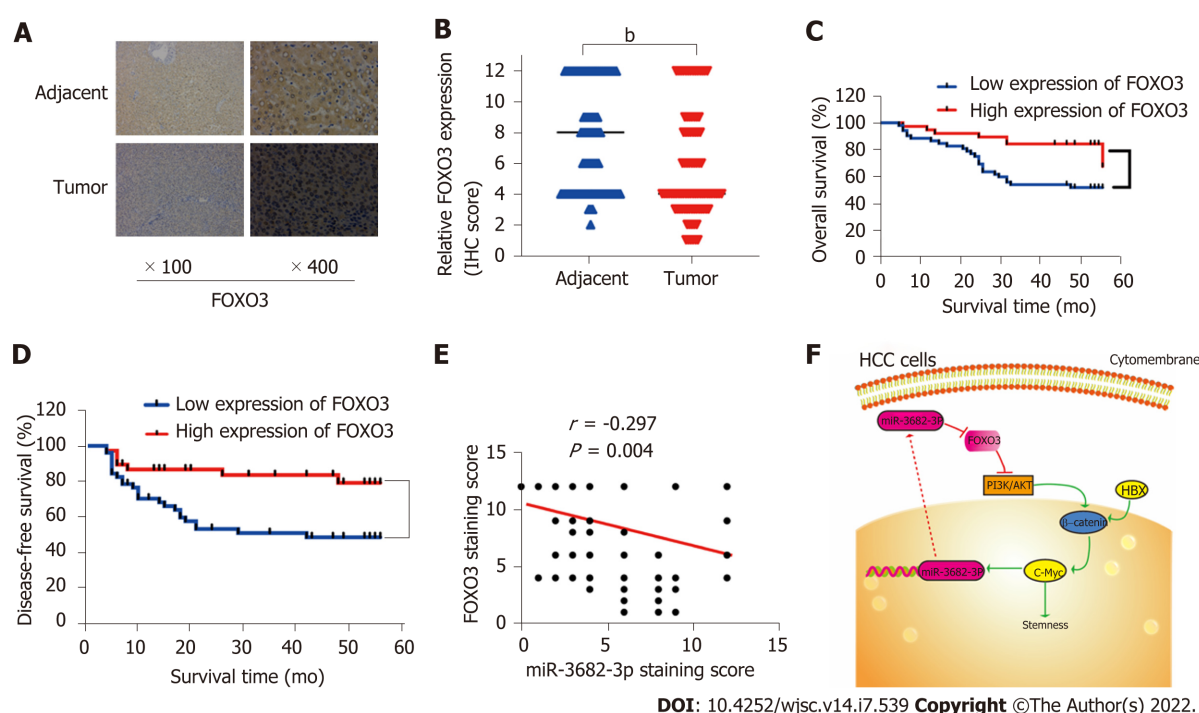
Figure 7 Pathoclinical features of FOXO3 expression. A: FOXO3 expression in hepatocellular carcinoma (HCC) tissue microarray (× 100 magnification scale bar: 200 μm; × 400 magnification scale bar: 50 μm); B: Immunohistochemical scoring of FOXO3 staining; C: Kaplan-Meier survival analysis showing the FOXO3 expression level-related overall survival in HCC; D: Kaplan-Meier survival analysis showing the FOXO3 expression level-related disease-free survival in HCC; E: Correlation between FOXO1 and miR-3682-3p expression (Spearman's rank correlation test); F: Schematic representation of the HBx-induced miR-3682-3p/FOXO3/PI3K/AKT/c-Myc feedback loop that promotes HCC stemness. [b]$P < 0.01$.

ARTICLE HIGHLIGHTS

Research background
Cancer stem cells (CSCs) have been implicated in tumorigenesis and tumor recurrence and metastasis are key therapeutic targets in cancer treatment. MicroRNAs display therapeutic potential by controlling the properties of CSCs; however, whether an association exists between miR-3682-3p and CSCs is unknown.

Research motivation
However, whether an association exists between miR-3682-3p and CSCs is unknown. Here, we investigated whether miR-3682-3p has a role in hepatocellular carcinoma (HCC).

Research objectives
To investigate the mechanism by which miR-3682-3p promotes stemness maintenance in HCC.

Research methods
MiR-3682-3p expression in HCC cell lines and 34 pairs of normal and HCC specimens was assayed by qPCR. The functional role of miR-3682-3p was investigated *in vitro* and *in vivo*. Dual-luciferase reporter and chromatin immunoprecipitation assays were performed for target assessment, and western blotting was utilized to confirm miR-3682-3p/target relationships.

Research results
We found that miR-3682-3p plays a key role in HCC pathogenesis by promoting HCC cell stemness. The upregulation of miR-3682-3p enhanced CSC spheroid-forming ability, side population cell fractions, and the expression of CSC factors in HCC cells *in vitro* and the tumorigenicity of transplanted HCC cells *in vivo*. Furthermore, silencing miR-3682-3p prolonged the survival of HCC-bearing mice. Mechanistically, we found that miR-3682-3p targets FOXO3 and enables FOXO3/β-catenin interaction, which promotes c-Myc expression through PI3K/AKT; c-Myc, in turn, activates miR-3682-3p, forming a positive feedback loop. Intriguingly, miR-3682-3p expression was induced by hepatitis B virus X protein (HBx) and was involved in HBx-induced tumor stemness-related pathogenesis.

Research conclusions
Our findings reveal a novel mechanism by which miR-3682-3p promotes stemness in HCC stem cells. Silencing miR-3682-3p may represent a novel therapeutic strategy for HCC.

Research perspectives
This study has shed some light on the mechanism of action of miR-3682-3p in promoting stemness maintenance in HCC and provides a potential target for the treatment of HCC.

FOOTNOTES

revised according to the ARRIVE Guidelines.

ORCID number: Shi Zuo 0000-0002-8595-5062.

REFERENCES

1 **Siegel RL**, Miller KD, Fuchs HE, Jemal A. Cancer Statistics, 2021. *CA Cancer J Clin* 2021; **71**: 7-33 [PMID: 33433946 DOI: 10.3322/caac.21654]

2 **Ferlay J**, Colombet M, Soerjomataram I, Parkin DM, Piñeros M, Znaor A, Bray F. Cancer statistics for the year 2020: An overview. *Int J Cancer* 2021 [PMID: 33818764 DOI: 10.1002/ijc.33588]

3 **Lim H**, Ramjeesingh R, Liu D, Tam VC, Knox JJ, Card PB, Meyers BM. Optimizing Survival and the Changing Landscape of Targeted Therapy for Intermediate and Advanced Hepatocellular Carcinoma: A Systematic Review. *J Natl Cancer Inst* 2021; **113**: 123-136 [PMID: 32898239 DOI: 10.1093/jnci/djaa119]

4 **Finn RS**, Zhu AX. Evolution of Systemic Therapy for Hepatocellular Carcinoma. *Hepatology* 2021; **73** Suppl 1: 150-157 [PMID: 32380571 DOI: 10.1002/hep.31306]

5 **Harding-Theobald E**, Louissaint J, Maraj B, Cuaresma E, Townsend W, Mendiratta-Lala M, Singal AG, Su GL, Lok AS, Parikh ND. Systematic review: radiomics for the diagnosis and prognosis of hepatocellular carcinoma. *Aliment Pharmacol Ther* 2021; **54**: 890-901 [PMID: 34390014 DOI: 10.1111/apt.16563]

6 **Lin YL**, Li Y. Study on the hepatocellular carcinoma model with metastasis. *Genes Dis* 2020; **7**: 336-350 [PMID: 32884988 DOI: 10.1016/j.gendis.2019.12.008]

7 **Zeng Z**, Lu Q, Liu Y, Zhao J, Zhang Q, Hu L, Shi Z, Tu Y, Xiao Z, Xu Q, Huang D. Effect of the Hypoxia Inducible Factor on Sorafenib Resistance of Hepatocellular Carcinoma. *Front Oncol* 2021; **11**: 641522 [PMID: 34307125 DOI: 10.3389/fonc.2021.641522]

8 **Ma XL**, Hu B, Tang WG, Xie SH, Ren N, Guo L, Lu RQ. CD73 sustained cancer-stem-cell traits by promoting SOX9 expression and stability in hepatocellular carcinoma. *J Hematol Oncol* 2020; **13**: 11 [PMID: 32024555 DOI: 10.1186/s13045-020-0845-z]

9 **Lin X**, Li AM, Li YH, Luo RC, Zou YJ, Liu YY, Liu C, Xie YY, Zuo S, Liu Z, Fang WY. Silencing MYH9 blocks HBx-induced GSK3β ubiquitination and degradation to inhibit tumor stemness in hepatocellular carcinoma. *Signal Transduct Target Ther* 2020; **5**: 13 [PMID: 32296025 DOI: 10.1038/s41392-020-0111-4]

10 **Xiao Y**, Sun Y, Liu G, Zhao J, Gao Y, Yeh S, Gong L, Chang C. Androgen receptor (AR)/miR-520f-3p/SOX9 signaling is involved in altering hepatocellular carcinoma (HCC) cell sensitivity to the Sorafenib therapy under hypoxia *via* increasing cancer stem cells phenotype. *Cancer Lett* 2019; **444**: 175-187 [PMID: 30448543 DOI: 10.1016/j.canlet.2018.11.004]

11 **Gao Y**, Gu J, Wang Y, Fu D, Zhang W, Zheng G, Wang X. Hepatitis B virus X protein boosts hepatocellular carcinoma progression by downregulating microRNA-137. *Pathol Res Pract* 2020; **216**: 152981 [PMID: 32527447 DOI: 10.1016/j.prp.2020.152981]

12 **Wang X**, He Y, Mackowiak B, Gao B. MicroRNAs as regulators, biomarkers and therapeutic targets in liver diseases. *Gut* 2021; **70**: 784-795 [PMID: 33127832 DOI: 10.1136/gutjnl-2020-322526]

13 **Li J**, Guan X, Fan Z, Ching LM, Li Y, Wang X, Cao WM, Liu DX. Non-Invasive Biomarkers for Early Detection of Breast Cancer. *Cancers (Basel)* 2020; **12** [PMID: 32992445 DOI: 10.3390/cancers12102767]

14 **Cao L**, Huang C, Cui Zhou D, Hu Y, Lih TM, Savage SR, Krug K, Clark DJ, Schnaubelt M, Chen L, da Veiga Leprevost F, Eguez RV, Yang W, Pan J, Wen B, Dou Y, Jiang W, Liao Y, Shi Z, Terekhanova NV, Cao S, Lu RJ, Li Y, Liu R, Zhu H, Ronning P, Wu Y, Wyczalkowski MA, Easwaran H, Danilova L, Mer AS, Yoo S, Wang JM, Liu W, Haibe-Kains B, Thiagarajan M, Jewell SD, Hostetter G, Newton CJ, Li QK, Roehrl MH, Fenyö D, Wang P, Nesvizhskii AI, Mani DR, Omenn GS, Boja ES, Mesri M, Robles AI, Rodriguez H, Bathe OF, Chan DW, Hruban RH, Ding L, Zhang B, Zhang H; Clinical Proteomic Tumor Analysis Consortium. Proteogenomic characterization of pancreatic ductal adenocarcinoma. *Cell* 2021; **184**: 5031-5052.e26 [PMID: 34534465 DOI: 10.1016/j.cell.2021.08.023]

15 **Zhao Q**, Liu Y, Wang T, Yang Y, Ni H, Liu H, Guo Q, Xi T, Zheng L. MiR-375 inhibits the stemness of breast cancer cells by blocking the JAK2/STAT3 signaling. *Eur J Pharmacol* 2020; **884**: 173359 [PMID: 32738343 DOI: 10.1016/j.ejphar.2020.173359]

16 **Wang L**, Lv Y, Li C, Yang G, Fu B, Peng Q, Jian L, Hou D, Wang J, Zhao C, Yang P, Zhang K, Wang L, Wang Z, Wang H, Xu W. Transformable Dual-Inhibition System Effectively Suppresses Renal Cancer Metastasis through Blocking Endothelial Cells and Cancer Stem Cells. *Small* 2020; **16**: e2004548 [PMID: 32881381 DOI: 10.1002/smll.202004548]

17 **Liu Y**, Lu LL, Wen D, Liu DL, Dong LL, Gao DM, Bian XY, Zhou J, Fan J, Wu WZ. MiR-612 regulates invadopodia of hepatocellular carcinoma by HADHA-mediated lipid reprogramming. *J Hematol Oncol* 2020; **13**: 12 [PMID: 32033570

DOI: 10.1186/s13045-019-0841-3]

18   **Zhao Y**, Xu L, Wang X, Niu S, Chen H, Li C. A novel prognostic mRNA/miRNA signature for esophageal cancer and its immune landscape in cancer progression. *Mol Oncol* 2021; **15**: 1088-1109 [PMID: 33463006 DOI: 10.1002/1878-0261.12902]

19   **Dong SS**, Dong DD, Yang ZF, Zhu GQ, Gao DM, Chen J, Zhao Y, Liu BB. Exosomal miR-3682-3p Suppresses Angiogenesis by Targeting ANGPT1 *via* the RAS-MEK1/2-ERK1/2 Pathway in Hepatocellular Carcinoma. *Front Cell Dev Biol* 2021; **9**: 633358 [PMID: 33869178 DOI: 10.3389/fcell.2021.633358]

20   **Yao B**, Niu Y, Li Y, Chen T, Wei X, Liu Q. High-matrix-stiffness induces promotion of hepatocellular carcinoma proliferation and suppression of apoptosis *via* miR-3682-3p-PHLDA1-FAS pathway. *J Cancer* 2020; **11**: 6188-6203 [PMID: 33033502 DOI: 10.7150/jca.45998]

21   **Liu S**, Wen Y, Quan B, Lin J, Zhu Z, Tang J, Han S. High expression of mir-3682-3p is an unfavorable prognostic factor of hepatocellular carcinoma. *Nanfang Yike Daxue Xuebao* 2021; **41**: 1885-1891 [DOI: 10.12122/j.issn.1673-4254.2021.12.19]

22   **Cao MQ**, You AB, Zhu XD, Zhang W, Zhang YY, Zhang SZ, Zhang KW, Cai H, Shi WK, Li XL, Li KS, Gao DM, Ma DN, Ye BG, Wang CH, Qin CD, Sun HC, Zhang T, Tang ZY. miR-182-5p promotes hepatocellular carcinoma progression by repressing FOXO3a. *J Hematol Oncol* 2018; **11**: 12 [PMID: 29361949 DOI: 10.1186/s13045-018-0555-y]

23   **Salem M**, Shan Y, Bernaudo S, Peng C. miR-590-3p Targets Cyclin G2 and FOXO3 to Promote Ovarian Cancer Cell Proliferation, Invasion, and Spheroid Formation. *Int J Mol Sci* 2019; **20** [PMID: 31013711 DOI: 10.3390/ijms20081810]

24   **Semmler G**, Meyer EL, Kozbial K, Schwabl P, Hametner-Schreil S, Zanetto A, Bauer D, Chromy D, Simbrunner B, Scheiner B, Stättermayer AF, Pinter M, Schöfl R, Russo FP, Greenfield H, Schwarz M, Schwarz C, Gschwantler M, Alonso López S, Manzano ML, Ahumada A, Bañares R, Pons M, Rodríguez-Tajes S, Genescà J, Lens S, Trauner M, Ferenci P, Reiberger T, Mandorfer M. HCC risk stratification after cure of hepatitis C in patients with compensated advanced chronic liver disease. *J Hepatol* 2022; **76**: 812-821 [PMID: 34871626 DOI: 10.1016/j.jhep.2021.11.025]

25   **Sanduzzi-Zamparelli M**, Mariño Z, Lens S, Sapena V, Iserte G, Pla A, Granel N, Bartres C, Llarch N, Vilana R, Nuñez I, Darnell A, Belmonte E, García-Criado A, Díaz A, Muñoz-Martinez S, Ayuso C, Bianchi L, Fuster-Anglada C, Rimola J, Forner A, Torres F, Bruix J, Forns X, Reig M. Liver cancer risk after HCV cure in patients with advanced liver disease without non-characterized nodules. *J Hepatol* 2022; **76**: 874-882 [PMID: 34856322 DOI: 10.1016/j.jhep.2021.11.023]

26   **Zou Y**, Lin X, Bu J, Lin Z, Chen Y, Qiu Y, Mo H, Tang Y, Fang W, Wu Z. Timeless-Stimulated miR-5188-FOXO1/β-Catenin-c-Jun Feedback Loop Promotes Stemness *via* Ubiquitination of β-Catenin in Breast Cancer. *Mol Ther* 2020; **28**: 313-327 [PMID: 31604679 DOI: 10.1016/j.ymthe.2019.08.015]

27   **Gu Y**, Ji F, Liu N, Zhao Y, Wei X, Hu S, Jia W, Wang XW, Budhu A, Ji J, Zhao B, Roessler S, Zheng X. Loss of miR-192-5p initiates a hyperglycolysis and stemness positive feedback in hepatocellular carcinoma. *J Exp Clin Cancer Res* 2020; **39**: 268 [PMID: 33256802 DOI: 10.1186/s13046-020-01785-7]

28   **Chen Y**, Peng C, Chen J, Chen D, Yang B, He B, Hu W, Zhang Y, Liu H, Dai L, Xie H, Zhou L, Wu J, Zheng S. WTAP facilitates progression of hepatocellular carcinoma *via* m6A-HuR-dependent epigenetic silencing of ETS1. *Mol Cancer* 2019; **18**: 127 [PMID: 31438961 DOI: 10.1186/s12943-019-1053-8]

29   **Jiang N**, Zou C, Zhu Y, Luo Y, Chen L, Lei Y, Tang K, Sun Y, Zhang W, Li S, He Q, Zhou J, Chen Y, Luo J, Jiang W, Ke Z. HIF-1α-regulated miR-1275 maintains stem cell-like phenotypes and promotes the progression of LUAD by simultaneously activating Wnt/β-catenin and Notch signaling. *Theranostics* 2020; **10**: 2553-2570 [PMID: 32194819 DOI: 10.7150/thno.41120]

30   **Chen MK**, Zhou JH, Wang P, Ye YL, Liu Y, Zhou JW, Chen ZJ, Yang JK, Liao DY, Liang ZJ, Xie X, Zhou QZ, Xue KY, Guo WB, Xia M, Bao JM, Yang C, Duan HF, Wang HY, Huang ZP, Qin ZK, Liu CD. BMI1 activates P-glycoprotein *via* transcription repression of *miR-3682-3p* and enhances chemoresistance of bladder cancer cell. *Aging (Albany NY)* 2021; **13**: 18310-18330 [PMID: 34270461 DOI: 10.18632/aging.203277]

31   **Lin Z**, Niu Y, Wan A, Chen D, Liang H, Chen X, Sun L, Zhan S, Chen L, Cheng C, Zhang X, Bu X, He W, Wan G. RNA m6 A methylation regulates sorafenib resistance in liver cancer through FOXO3-mediated autophagy. *EMBO J* 2020; **39**: e103181 [PMID: 32368828 DOI: 10.15252/embj.2019103181]

32   **Yao S**, Fan LY, Lam EW. The FOXO3-FOXM1 axis: A key cancer drug target and a modulator of cancer drug resistance. *Semin Cancer Biol* 2018; **50**: 77-89 [PMID: 29180117 DOI: 10.1016/j.semcancer.2017.11.018]

33   **Liu H**, Song Y, Qiu H, Liu Y, Luo K, Yi Y, Jiang G, Lu M, Zhang Z, Yin J, Zeng S, Chen X, Deng M, Jia X, Gu Y, Chen D, Zheng G, He Z. Downregulation of FOXO3a by DNMT1 promotes breast cancer stem cell properties and tumorigenesis. *Cell Death Differ* 2020; **27**: 966-983 [PMID: 31296961 DOI: 10.1038/s41418-019-0389-3]

34   **Fu Q**, Song X, Liu Z, Deng X, Luo R, Ge C, Li R, Li Z, Zhao M, Chen Y, Lin X, Zhang Q, Fang W. miRomics and Proteomics Reveal a miR-296-3p/PRKCA/FAK/Ras/c-Myc Feedback Loop Modulated by HDGF/DDX5/β-catenin Complex in Lung Adenocarcinoma. *Clin Cancer Res* 2017; **23**: 6336-6350 [PMID: 28751441 DOI: 10.1158/1078-0432.CCR-16-2813]

35   **Sun W**, Li J, Zhou L, Han J, Liu R, Zhang H, Ning T, Gao Z, Liu B, Chen X, Ba Y. The c-Myc/miR-27b-3p/ATG10 regulatory axis regulates chemoresistance in colorectal cancer. *Theranostics* 2020; **10**: 1981-1996 [PMID: 32104496 DOI: 10.7150/thno.37621]

36   **Deng X**, Liu Z, Liu X, Fu Q, Deng T, Lu J, Liu Y, Liang Z, Jiang Q, Cheng C, Fang W. miR-296-3p Negatively Regulated by Nicotine Stimulates Cytoplasmic Translocation of c-Myc *via* MK2 to Suppress Chemotherapy Resistance. *Mol Ther* 2018; **26**: 1066-1081 [PMID: 29525743 DOI: 10.1016/j.ymthe.2018.01.023]

37   **Komoll RM**, Hu Q, Olarewaju O, von Döhlen L, Yuan Q, Xie Y, Tsay HC, Daon J, Qin R, Manns MP, Sharma AD, Goga A, Ott M, Balakrishnan A. MicroRNA-342-3p is a potent tumour suppressor in hepatocellular carcinoma. *J Hepatol* 2021; **74**: 122-134 [PMID: 32738449 DOI: 10.1016/j.jhep.2020.07.039]

38   **Li J**, Zhang S, Zou Y, Wu L, Pei M, Jiang Y. miR-145 promotes miR-133b expression through c-myc and DNMT3A-mediated methylation in ovarian cancer cells. *J Cell Physiol* 2020; **235**: 4291-4301 [PMID: 31612498 DOI: 10.1002/jcp.29306]

39   **Huang P**, Xu Q, Yan Y, Lu Y, Hu Z, Ou B, Zhang H, Mao K, Zhang J, Wang J, Xiao Z. HBx/ERα complex-mediated LINC01352 downregulation promotes HBV-related hepatocellular carcinoma *via* the miR-135b-APC axis. *Oncogene* 2020;

39: 3774-3789 [PMID: 32157216 DOI: 10.1038/s41388-020-1254-z]

40   **Lin X**, Zuo S, Luo R, Li Y, Yu G, Zou Y, Zhou Y, Liu Z, Liu Y, Hu Y, Xie Y, Fang W. HBX-induced miR-5188 impairs FOXO1 to stimulate β-catenin nuclear translocation and promotes tumor stemness in hepatocellular carcinoma. *Theranostics* 2019; **9**: 7583-7598 [PMID: 31695788 DOI: 10.7150/thno.37717]

41   **Salerno D**, Chiodo L, Alfano V, Floriot O, Cottone G, Paturel A, Pallocca M, Plissonnier ML, Jeddari S, Belloni L, Zeisel M, Levrero M, Guerrieri F. Hepatitis B protein HBx binds the DLEU2 lncRNA to sustain cccDNA and host cancer-related gene transcription. *Gut* 2020; **69**: 2016-2024 [PMID: 32114505 DOI: 10.1136/gutjnl-2019-319637]

ORIGINAL ARTICLE

Basic Study

Intratracheal administration of umbilical cord-derived mesenchymal stem cells attenuates hyperoxia-induced multi-organ injury *via* heme oxygenase-1 and JAK/STAT pathways

Na Dong, Pan-Pan Zhou, Dong Li, Hua-Su Zhu, Ling-Hong Liu, Hui-Xian Ma, Qing Shi, Xiu-Li Ju

Na Dong, Hua-Su Zhu, Department of Pediatrics, Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan 250012, Shandong Province, China

Pan-Pan Zhou, Xiu-Li Ju, Department of Pediatrics, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China

Dong Li, Ling-Hong Liu, Hui-Xian Ma, Qing Shi, Xiu-Li Ju, Stem Cell and Regenerative Medicine Research Center, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China

Corresponding author: Xiu-Li Ju, PhD, Chief Doctor, Department of Pediatrics, Qilu Hospital of Shandong University, No. 107 Wenhua West Road, Jinan 250012, Shandong Province, China. jxlqlyy@163.com

Abstract

BACKGROUND

Bronchopulmonary dysplasia (BPD) is not merely a chronic lung disease, but a systemic condition with multiple organs implications predominantly associated with hyperoxia exposure. Despite advances in current management strategies, limited progress has been made in reducing the BPD-related systemic damage. Meanwhile, although the protective effects of human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) or their exosomes on hyperoxia-induced lung injury have been explored by many researchers, the underlying mechanism has not been addressed in detail, and few studies have focused on the therapeutic effect on systemic multiple organ injury.

AIM

To investigate whether hUC-MSC intratracheal administration could attenuate hyperoxia-induced lung, heart, and kidney injuries and the underlying regulatory mechanisms.

METHODS

Neonatal rats were exposed to hyperoxia (80% O_2), treated with hUC-MSCs intratracheal (iT) or intraperitoneal (iP) on postnatal day 7, and harvested on postnatal day 21. The tissue sections of the lung, heart, and kidney were analyzed morphometrically. Protein contents of the bronchoalveolar lavage fluid (BALF), myeloper-

oxidase (MPO) expression, and malondialdehyde (MDA) levels were examined. Pulmonary inflammatory cytokines were measured *via* enzyme-linked immunosorbent assay. A comparative transcriptomic analysis of differentially expressed genes (DEGs) in lung tissue was conducted *via* RNA-sequencing. Subsequently, we performed reverse transcription-quantitative polymerase chain reaction and western blot analysis to explore the expression of target mRNA and proteins related to inflammatory and oxidative responses.

RESULTS
iT hUC-MSCs administration improved pulmonary alveolarization and angiogenesis ($P < 0.01$, $P < 0.01$, $P < 0.001$, and $P < 0.05$ for mean linear intercept, septal counts, vascular medial thickness index, and microvessel density respectively). Meanwhile, treatment with hUC-MSCs iT ameliorated right ventricular hypertrophy (for Fulton's index, $P < 0.01$), and relieved reduced nephrogenic zone width ($P < 0.01$) and glomerular diameter ($P < 0.001$) in kidneys. Among the beneficial effects, a reduction of BALF protein, MPO, and MDA was observed in hUC-MSCs groups ($P < 0.01$, $P < 0.001$, and $P < 0.05$ respectively). Increased pro-inflammatory cytokines tumor necrosis factor-alpha, interleukin (IL)-1β, and IL-6 expression observed in the hyperoxia group were significantly attenuated by hUC-MSCs administration ($P < 0.01$, $P < 0.001$, and $P < 0.05$ respectively). In addition, we observed an increase in anti-inflammatory cytokine IL-10 expression in rats that received hUC-MSCs iT compared with rats reared in hyperoxia ($P < 0.05$). Transcriptomic analysis showed that the DEGs in lung tissues induced by hyperoxia were enriched in pathways related to inflammatory responses, epithelial cell proliferation, and vasculature development. hUC-MSCs administration blunted these hyperoxia-induced dysregulated genes and resulted in a shift in the gene expression pattern toward the normoxia group. hUC-MSCs increased heme oxygenase-1 (HO-1), JAK2, and STAT3 expression, and their phosphorylation in the lung, heart, and kidney ($P < 0.05$). Remarkably, no significant difference was observed between the iT and iP administration.

CONCLUSION
iT hUC-MSCs administration ameliorates hyperoxia-induced lung, heart, and kidney injuries by activating HO-1 expression and JAK/STAT signaling. The therapeutic benefits of local iT and iP administration are equivalent.

Core Tip: In the present study, we used a newborn rat model of postnatal hyperoxia exposure to simulate clinical bronchopulmonary dysplasia (BPD) and the associated heart and kidney injuries in preterm infants. Improved lung, heart, and kidney development, as well as reduced inflammatory and oxidative responses, were observed with human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) administration. We demonstrated that hUC-MSCs ameliorate hyperoxia-induced systemic organ injuries by activating heme oxygenase-1 expression and JAK/STAT pathway. Overall, our study shows that intratracheal administration is a more attractive route of MSCs administration in preterm infants for the prevention and treatment of BPD and hyperoxia-induced systemic damage.

INTRODUCTION

Bronchopulmonary dysplasia (BPD) is the most common chronic lung disease in premature infants and is a heterogeneous disease predominantly associated with oxygen supplementation clinically[1]. Data from major cohort studies demonstrate an increasing BPD prevalence of 11%–50%, most likely due to the increased survival of newborns at an extremely low gestational age[2,3]. Current management strategies include the use of volume-targeted and non-invasive ventilation, as well as targeted use of

steroids along with adjunct medical therapies including surfactant, caffeine, and vitamin A[2,4]. Despite advances in these interventions resulting in improved survival and decreased morbidity, limited progress has been made in reducing the risk of BPD development.

BPD is characterized by varying magnitudes of impairment in alveolar septation, lung fibrosis, and abnormal vascular development and remodeling. Moreover, increasing evidence has suggested that BPD is not merely a lung disease but a systemic condition with short-term and long-term multiple organ implications[3,5]. Clinical and experimental findings demonstrated that chronic exposure to hyperoxia causes oxidative stress and leads to certain implications, including neurodevelopmental impairments [6], retinopathy of prematurity[7,8], renal vascular and tubular development impairments[9,10], and associated cardiac disease[11,12]. Notably, hyperoxia exposure in neonates results in long-term cardiac defects and renal abnormalities in later adult life. More specifically, recent studies on experimental models have revealed that hyperoxia-treated mice exhibited significantly reduced stroke volume and ejection fractions, the appearance of left ventricular (LV) dysfunction, right ventricular (RV) dysfunction, and pulmonary hypertension (PH)[13,14]. In addition, hyperoxia exposure reduces kidney size, glomerular density, and glomerular filtration rate, along with increases in the renal tubular necrosis, dilation, regeneration, and interstitial inflammation[10,15]. Hence, new effective therapeutic interventions are urgently needed to attenuate BPD-associated multiple organ damage, rather than focusing on single organ injuries.

Mesenchymal stem cells (MSCs) are multipotent stromal cells that have immunomodulatory and anti-inflammatory properties, as well as low immunogenicity, and have shown great potential for the management of a range of different neonatal conditions, including BPD, in both preclinical models and clinical trials, by systemic or local intratracheal (iT) administration[16-20]. The therapeutic mechanism of MSCs may predominantly include paracrine or indirect pathways, which are responsible for the beneficial effects of local MSCs administration on systemic damage[21,22]. Neonates with very low birth weight are often intubated at birth, making local iT instillation an attractive route of administration[17]. Notably, higher isolation efficiency and proliferation capacity, lower immunogenicity, and greater overall immunomodulatory and anti-inflammatory effects were observed for human umbilical cord-derived MSCs (hUC-MSCs) over stem cells harvested from other sources[23]. Moreover, recent studies confirmed that MSCs are poorly immunogenic, and both allogeneic and xenogeneic MSCs showed equal efficacy without side effects[24,25]. Xenogeneic hUC-MSCs transplantation has been extensively investigated over the past decades on various animal models with promising effects[16,18,19]. However, beyond the pulmo-protective properties of systemic or local iT hUC-MSCs administration, its potential therapeutic effects on hyperoxia-induced heart and kidney injuries have not yet been evaluated.

Extensive evidence has demonstrated that heme oxygenase-1 (HO-1) is an inducible enzyme with potent anti-oxidant, anti-inflammatory, and anti-apoptotic attributes[26,27]. The protective response regulated by HO-1 has been reported in multiple animal models of hyperoxia-induced injury[28-30]. Furthermore, it is generally considered that the JAK2 and STAT3 signaling pathway are involved in the various patho-physiological responses[31] and also play a key role in the development of pulmonary [32], cardiovascular[33], and renal[34] diseases. Interestingly, the evidence demonstrated that cross-talk may exist between HO-1 and JAK/STAT pathways[35-37]. However, whether HO-1 and JAK/STAT signaling pathways are involved in the protective effects of MSCs remains unclear.

In the present study, we used a newborn rat model of postnatal hyperoxia exposure to simulate clinical BPD and the associated heart and kidney injuries in preterm infants. This study aimed to investigate whether iT administration of hUC-MSCs could simultaneously attenuate hyperoxia-induced lung, heart, and kidney injuries in experimental neonatal rat models *via* mechanisms that involve activation of the HO-1 and JAK2/STAT3 signaling pathways.

MATERIALS AND METHODS

hUC-MSCs culture and identification

hUC-MSCs were provided by the Cell and tissue bank of Shandong province (Jinan, China) and cultured in alpha-minimal essential medium (Gibco, Carlsbad, CA, United States) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco) at 37 ℃ with 5% CO_2. Cells between passage three and five were used in this study.

Phenotypic analysis was conducted by flow cytometry[38]. Briefly, hUC-MSCs were washed with phosphate-buffered saline (PBS) and resuspended to a concentration of 1×10^6 cells/mL. Then, the cells were stained with antibodies against the following cell surface epitopes: Phycoerythrin (PE)-anti CD29 (1:20; 303004, BioLegend, San Diego, CA, United States), PE-anti CD31 (303106, BioLegend), PE-anti CD44 (12-0441-82, eBioscience, San Diego, CA, United States), PE-anti CD45 (368510, BioLegend), PE-anti CD73 (344004, BioLegend), PE-anti CD90 (12-0909-42, eBioscience), PE-anti CD105 (12-1057-42, eBioscience), and PE-anti CD271 (12-9400-42, eBioscience). Flow cytometry was performed with the Guava easyCyte 6HT (EMD Millipore, Billerica, MA, United States), and the data were examined using the Flowjo™ software (V10, BD Life Sciences, Franklin Lakes, NJ, United States). To demonstrate the multilineage differentiation potential, hUC-MSCs were seeded in 6-well plates and cultured in

adipogenic (HUXUC-90031, Cyagen, Santa Clara, CA, United States), and osteogenic (HUXUC-90021, Cyagen) differentiation medium, or in a loosely capped tube (430790, Corning, NY, United States) in chondrogenic differentiation (HUXUC-90042, Cyagen) medium. The medium was changed every 3 d in accordance with the manufacturer's instructions. At the end of 21 d, all cells were fixed with 4% paraformaldehyde (PFA) and processed with oil red O, alizarin red, and alcian blue.

Animal model and experimental groups

Timed pregnant Sprague Dawley rats were obtained from SPF (Beijing) Biotechnology Co., Ltd. (Beijing, China) and kept under a 12/12 h light-dark cycle, controlled temperature of 25 ± 2 °C, and relative humidity of 55% (± 10%) with free access to food and water. Pregnant rats were housed individually and allowed to deliver vaginally at term. Newborn rats were then randomly assigned to four experimental groups as follows: Normoxia group (n = 12, exposed to room air), hyperoxia group [n = 12, exposed to 80% O_2, treated with PBS 40 µL on postnatal day 7 (P7)], hyperoxia + iT-MSC group (n = 12, exposed to 80% O_2, treated with 4 × 10^5 hUC-MSCs, 40 µL, iT, on P7), and hyperoxia + intraperitoneal (iP)-MSC group(n = 12, exposed to 80% O_2, treated with 4 × 10^5 hUC-MSCs, 40 µL, iP, on P7). The nursing mothers were alternated between the 80% O_2 and the room air groups every 24 h to minimize oxygen toxicity in the mothers.

On postnatal day 21 (P21), rats were euthanized *via* iP injection of pentobarbital sodium. Bronchoalveolar lavage fluid (BALF) was obtained after ligation of the right main bronchus by back-flushing 1 mL of cold PBS three times *via* endotracheal intubation. The lungs, heart, and kidneys were excised and fixed overnight in 4% PFA for histological analysis, or frozen at -80 °C for reverse transcription-quantitative polymerase chain reaction (RT-qPCR), western blotting, and cytokine analyses. All animal procedures and protocols complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee on Animal Experiments of Shandong University Qilu Hospital (DWLL-2021-035, Jinan, China).

iT administration

For iT administration, the neonatal rats on P7 were euthanized *via* iP injection of pentobarbital sodium and restrained on a board with the neck in hyperflexion. The 29-gauge needle syringe (320310, Becton, Dickinson and Company, NJ, United States) with the tip smoothened and wrapped with a 0.3 mm flexible capillary pipe was prepared as endotracheal intubation. The light source was placed close to the neck of the rat. The rat's tongue was wrapped and pulled outside the mouth with gauze in the right hand, the root of the tongue was gently pressed with a small tweezer to expose the glottis with the left hand. hUC-MSCs were transplanted into the trachea through the prepared endotracheal intubation at the glottis opening. Then the rats were allowed to recover from the anesthesia and return to their dam. Mortality induced by the iT administration procedure was not observed.

Protein content in BALF

BALF was centrifuged at 3000 rpm for 20 min at 4 °C, and the supernatant was collected into a new tube. The protein content of cell-free BALF was subsequently measured using the BCA Protein Quantitative Assay Kit (Beyotime Biotechnology, Shanghai, China) in accordance with the manufacturer's instructions and was used as an indication of endothelial and epithelial permeability.

Histological examination

The lung, heart, and kidney tissues were fixed in 4% PFA, washed with PBS, and then serially dehydrated in increasing concentrations of ethanol before being embedded in paraffin. Consecutive sections (5 µm thick) from each tissue block were subjected to hematoxylin and eosin (HE) staining, observed under a microscope (Olympus, BH2, Japan), and photographed (CellSens, Ver. 1.18, Japan). Alveolar structures in lung sections were quantified using the mean linear intercept (MLI) and septal count methods[17,39]. Briefly, the number of intercepts was counted in both the horizontal and vertical fields, and MLI was measured following the equation MLI = N × L/m, where m is the sum of all the intercepts, L is the length of the traverses, and N is the number of times the traverses were placed on the lung. To quantify the degree of PH-induced right ventricular hypertrophy (RVH), the thicknesses of the right ventricle (RV) free wall, left ventricle (LV) free wall, and interventricular septum (IVS) of excised hearts were measured, and RV/LV + IVS (Fulton's index) was calculated[12,21]. The kidney sections across the full coronal plane were stained for morphology assessment as previously described by Mohr *et al*[15]. Glomerular diameter and width of the nephrogenic zone were measured. Five measurements of each parameter in each of the three fields of view were recorded and then averaged. All the above parameters were measured using Image Pro Plus (V6.0, Media Cybernetics, United States).

Immunohistochemistry and immunofluorescence

Immunohistochemistry (IHC) and immunofluorescence (IF) were performed on 5 µm paraffin sections. After routine deparaffinization, heat-induced epitope retrieval was performed by immersing the slides in 0.01 M sodium citrate buffer (pH = 6.0). The sections were preincubated in 0.3% hydrogen peroxide for 10 min to remove the endogenous peroxidase activity and then in 0.1 M PBS containing 5% normal

goat serum for 1 h at room temperature to block the non-specific antibody binding. Subsequently, the samples were stained with rabbit anti-α smooth muscle actin (α-SMA) antibody (1:100; 19245S, CST, Danvers, MA, United States), or rabbit anti-von Willebrand factor (vWF) polyclonal antibody (1:50; 27186-1-AP, Proteintech, Rosemont, IL, United States) as primary antibodies overnight at 4 °C. For IHC, sections were then incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit/mouse IgG polymers (PK10006, Proteintech), developed in 3,3'-diaminobenzidine (Proteintech) and counterstained with hematoxylin following the manufacturer's recommendations. For IF, the samples were incubated with DyLight® 488 pre-adsorbed secondary antibodies (ab98498, Abcam, Cambridge, United Kingdom) for 1 h at 37 °C and cell nuclei were counterstained with DAPI for 15 min. The thickness of the microvessel muscle layers was measured using sections stained with anti-α-SMA and the medial thickness index (MTI) was calculated as follows: $(A_{ext} – A_{int})/A_{ext}$, where A_{ext} and A_{int} are the areas within the external and internal boundaries of the α-SMA layer, respectively[17]. Pulmonary microvessel density (MVD) was determined by counting the number of vWF-positive vessels (< 100 μm) *per* high-power field using Image J (V1.8, National Institutes of Health, United States)[16].

Malondialdehyde concentration in tissues
Frozen lung, heart, and kidney tissues were weighed, and 10% tissue homogenate was prepared at 4 °C using an electric tissue grinder (LC-TG-24, Lichen Keyi, Shanghai, China). Malondialdehyde (MDA) levels in the tissues were measured using colorimetric assay kits in accordance with the manufacturer's instructions (BC0025, Solarbio, Beijing, China). The absorbance of the organic layer was measured using spectrophotometry at 450, 532, and 600 nm (DNM-9602, Perlong, Beijing, China). MDA levels were expressed in mmol/g wet tissue and calculated as follows: $[12.9 \times (\Delta A532 - \Delta A600) - 2.58 \times \Delta A450] \times 50$.

Myeloperoxidase level in tissues
The tissues were homogenized to determine the expression of myeloperoxidase (MPO) in accordance with the manufacturer's instructions (A044-1-1, Jchio, Nanjing, China). Yellow compounds were produced on hydrogen supply from adjacent anisodamines, and the amount was measured at 460 nm to reflect the viability of MPO and the number of leukocytes. One unit of MPO activity was defined as the quantity of enzyme that degrades 1 μmol peroxide/min.

Quantitation of cytokine levels by enzyme-linked immunosorbent assay
The total protein content of lung tissue homogenate was measured and samples were analyzed using the rat interleukin (IL)-1β (KE20005, Proteintech), IL-10 (KE20003, Proteintech), tumor necrosis factor-alpha (TNF-α) (KE20001, Proteintech), and IL-6 (SEKR-0005, Solarbio, China) enzyme-linked immunosorbent assay (ELISA) kits to determine the level of inflammatory cytokines in accordance with the manufacturer's instructions. The absorbance was measured at 450 nm.

RNA extraction and RT-qPCR
Total RNA was extracted from the lung samples using TRIzol reagent (T9424-100 mL, Sigma, United States) according to the manufacturer's protocols. First-strand cDNA was synthesized from 1 μg of total RNA using the ReverTraAce RT-qPCR Master Mix kit (FSQ-201, TOYOBO, Osaka, Japan). RT-qPCR was performed using a Real-Time Thermocycler (Analytik Jena AG, qTOWER3G, Germany), and detection was performed using SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan) in a 20 μL reaction mixture to detect the mRNA levels of relative genes. All procedures were performed according to the manufacturer's protocol. The primer sequences for each gene analyzed using RT-qPCR are listed in Table 1. The cycling profile involved 40 cycles of the following: 95 °C for 5 s, 55 °C for 10 s, and 72 °C for 15 s. Data were analyzed using Sequence Detection Software 1.4 (Applied Biosystems, CA, United States). Relative fold changes were normalized to endogenous *β-actin* mRNA expression using the $2^{-\Delta\Delta Ct}$ method.

RNA-sequencing and analysis
The right lower lobe of the lung from the normoxia, hyperoxia, and hyperoxia + iT-MSC groups were collected for total RNA extraction, as mentioned above. Poly-A pull-down with Oligo(dT) was then used to enrich mRNAs from total RNA samples. Then, cDNA libraries were constructed *via* PCR amplification and sequenced using Illumina HiSeq X10 platform (Annoroad Genomics, Beijing, China). Adapter polluted reads and low-quality reads in the original sequence were filtered, and the obtained high-quality clean reads were mapped to the human reference genome (GRCh38) using hierarchical indexing for spliced alignment of transcripts 2 (HISAT2; version 2.2.1). Gene expression levels were measured according to the fragments *per* kilobase *per* million mapped fragments method. Differentially expressed genes (DEGs) were screened using DESeq2, with absolute log2 fold change ≥ 1 and adjusted *P* < 0.05 as criteria. Gene Ontology (GO) enrichment analyses were performed to find significantly enriched GO terms related to the DEGs.

Table 1 Primer sequences for quantitative reverse transcription-polymerase chain reaction

Genes		Sequence (5'– 3')	Length (bp)
MIP-1α	F	GCTTCTCCTATGGACGGCAA	20
	R	TGCCGGTTTCTCTTGGTCAG	20
MIP-1β	F	CTGCTTCTCTTACACCTCCCG	21
	R	AAAGGCTGCTGGTCTCATAGT	21
VEGF	F	GCGGATCAAACCTCACCAAAG	21
	R	TTGGTCTGCATTCACATCTGCT	22
PDGF	F	CTCTGCTGCTACCTGCGTCT	20
	R	TCGGGTCATGTTCAAGTCCA	20
β-actin	F	CTCTGTGTGGATTGGTGGCT	20
	R	CGCAGCTCAGTAACAGTCCG	20

MIP: Macrophage inflammatory protein; VEGF: Vascular endothelial-derived growth factor; PDGF: Platelet-derived growth factor; F: Forward primer; R: Reverse primer.

Western blot analysis

Lung tissue samples were lysed in radioimmunoprecipitation assay buffer (P0013B, Beyotime) containing a phosphatase inhibitor and protease inhibitor mixture. The samples were centrifuged at 12000 rpm for 20 min at 4 °C to remove the cellular debris. Protein concentrations were determined using a BCA protein assay kit (Beyotime). Equal quantities of proteins were separated *via* sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (ISEQ00010, Millipore, MA, United States). Membranes were blocked in 5% skimmed milk for 1 h at room temperature and then incubated with the following primary antibodies overnight at 4 °C: Rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:3000; 5174S, CST), rabbit anti-Janus kinase 2 (JAK2; 1:1000; 3230S, CST), rabbit anti-phospho-JAK2 (1:1000; 3776S, CST), rabbit anti-signal transducer and activator of transcription 3 (STAT3; 1:2000; 4904S, CST), rabbit anti-phospho-STAT3 (1:2000; 9145S, CST), and rabbit anti-HO-1 (HO-1; 1:1000, 43966S, CST). Membranes were washed and then incubated with HRP-conjugated goat anti-rabbit antibodies (1:5,000; SA00001-2, Proteintech) for 1 h at room temperature. The protein bands were visualized using the ECL chemiluminescence detection kit (Millipore) and analyzed with JP-K600plus (Jiapeng Technology Co., Shanghai, China). The band intensity was quantified using ImageJ software. The signal intensities of all target proteins were normalized to those of GAPDH. The experiments were performed in triplicate.

Statistical analysis

GraphPad Prism 8.0.1 (GraphPad Software, Inc., La Jolla, CA, United States) and SPSS Statistics 26.0 (SPSS, Inc., Chicago, IL, United States) were used for statistical analysis. The quantitative data were examined by normal distribution test and expressed as the mean ± standard error, followed by variance homogeneity test. One-way analysis of variance (ANOVA) was conducted to determine statistically significant differences among multiple homogeneous groups. For significant ANOVA results, Bonferroni's multiple comparison test was performed to analyze the differences between two groups. Results with P values less than 0.05 were considered significant.

RESULTS

Characterization of hUC-MSCs

Typical MSC-associated surface markers were identified by flow cytometry. The hUC-MSCs showed high expression of CD29, CD44, CD73, CD90, and CD105, and did not express endothelial cell marker (CD31), hematopoietic marker (CD45), and differentiated activated effector cell marker (CD271, Figure 1A). The cells were stained with Oil Red O (Figure 1B), Alizarin red (Figure 1C), and Alcian blue (Figure 1D). These results confirmed that the hUC-MSCs could differentiate into osteoblasts, adipocytes, and chondrocytes *in vitro*.

hUC-MSCs administration increases survival rate and improves lung development

Newborn mice were exposed to 80% O_2 or standard room air from P1 to P21. Treatment groups received a single dose of hUC-MSCs iT or iP on P7 (Figure 2A). The survival curve was recorded from the day of

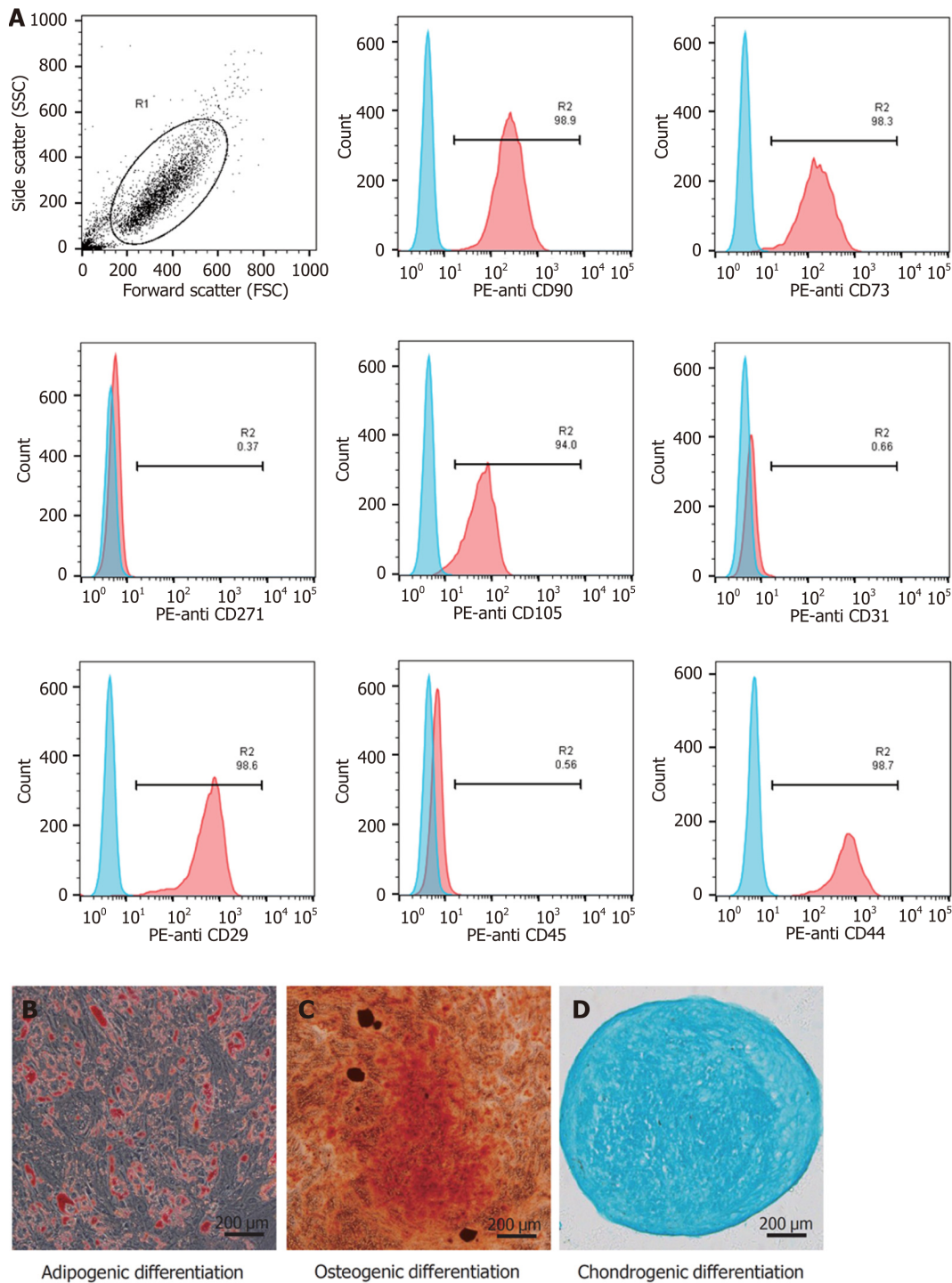**DOI**: 10.4252/wjsc.v14.i7.556 **Copyright** ©The Author(s) 2022.

Figure 1 Identification of human umbilical cord-derived mesenchymal stem cells. A: The human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) showed high expression of CD29, CD44, CD73, CD90, and CD105, and did not express CD31, CD45, and CD271; B–D: hUC-MSCs were positive for Oil Red O, Alizarin red, and Alcian blue staining under the corresponding induction conditions (scale bars = 200 μm).

injection (P7), which was decreased on hyperoxia. iT and iP administration of hUC-MSCs increased the survival of the rats exposed to O_2, although the difference was not statistically significant (Figure 2B). Remarkably slow growth and body weight gain were observed in rats exposed to hyperoxia from P7, and these were improved on hUC-MSCs administration on P14 and P21 (Figure 2C). There was no statistically significant difference between iT and iP administration.

The lung tissue sections stained with HE showed signs of impaired alveolar development in the animals exposed to hyperoxia, with marked interstitial thickening, fewer septations (hyperoxia *vs* normoxia, 19.55 ± 2.08 *vs* 32.44 ± 7.31, $P < 0.01$), and enlarged MLI (hyperoxia *vs* normoxia, 76.12 ± 7.7 μm *vs* 47.34 ± 10.48 μm, $P < 0.01$; Figure 2D). Treatment with hUC-MSCs significantly diminished the hyperoxia-induced increase in the MLI (60.16 ± 4.62 μm and 53.65 ± 6.85 μm for iT-MSC and iP-MSC,
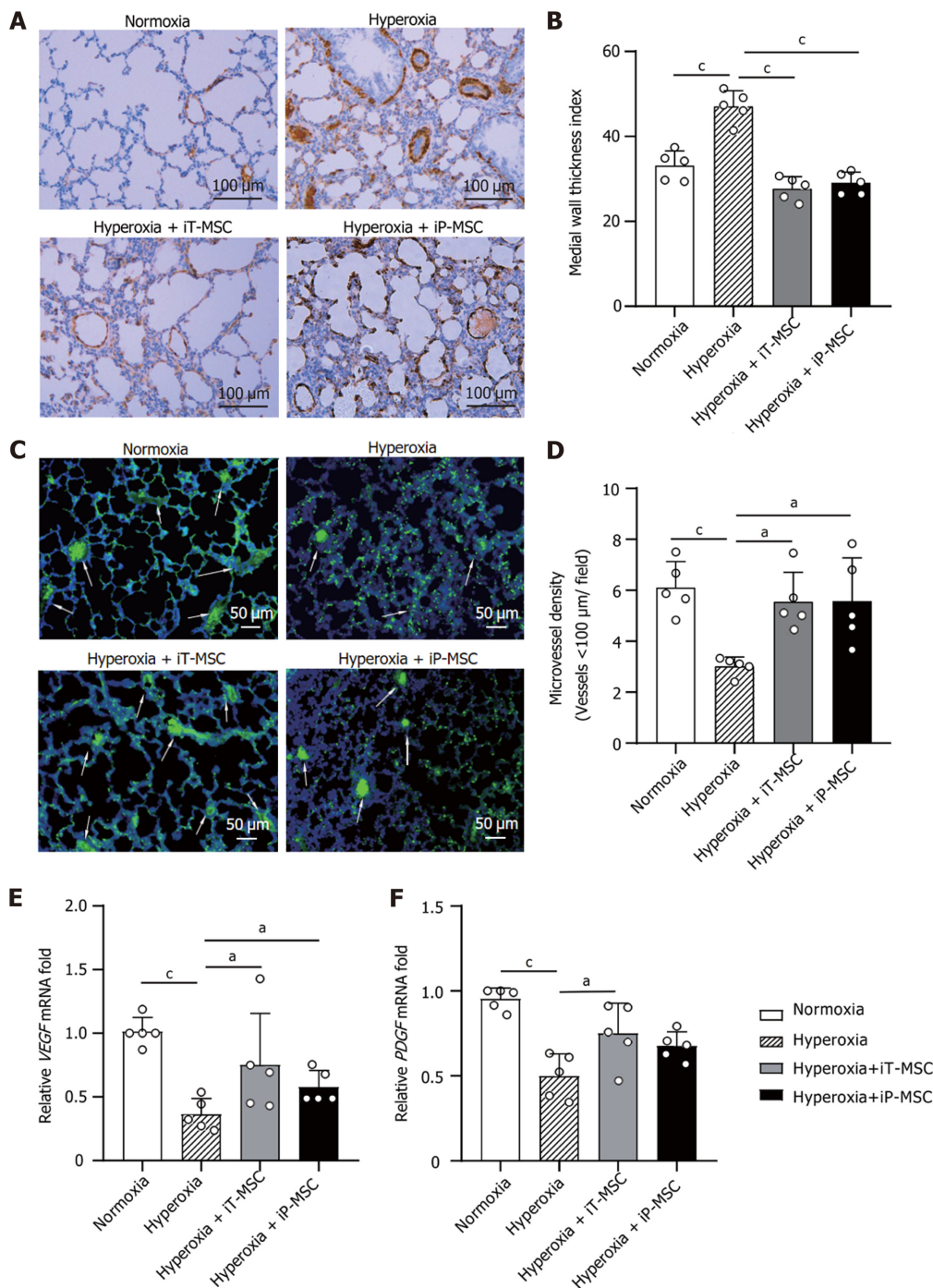
DOI: 10.4252/wjsc.v14.i7.556 **Copyright** ©The Author(s) 2022.
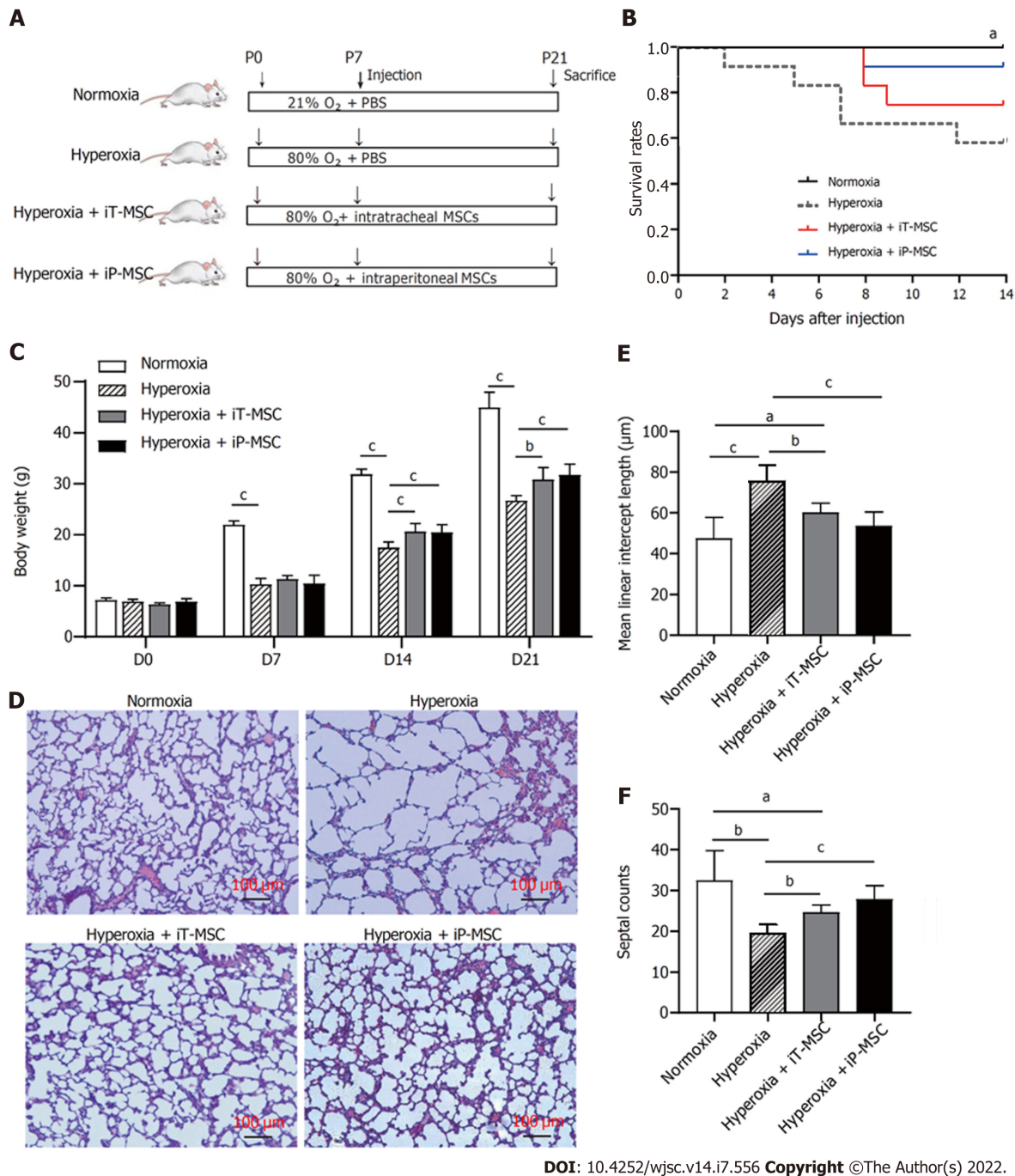
Figure 2 Human umbilical cord-derived mesenchymal stem cells administration increases survival rate and improves lung development.
A: Schematic diagram of the overall experimental design process and grouping; B: Kaplan-Meier survival curves of rat pups treated as indicated; C: Body weight alterations of rat pups treated as indicated; D: Representative images of harvested lung sections stained with hematoxylin and eosin for morphometric analyses (scale bars = 100 μm); E and F: Mean linear intercept and septal counts in lungs treated as indicated (*n* = 5 for each group, 10 fields/animal). [a]*P* < 0.05; [b]*P* < 0.01; [c]*P* < 0.001. iT: Intratracheal; iP: Intraperitoneal; P: Postnatal day; PBS: Phosphate-buffered saline; MSC: Mesenchymal stem cell.

respectively; Figure 2E) and decrease in septal counts (24.63 ± 1.85 and 27.84 ± 3.37 for iT-MSC and iP-MSC, respectively; Figure 2F).

hUC-MSCs treatment rescues hyperoxia-induced loss of peripheral pulmonary blood vessels and peripheral pulmonary arterial remodeling

To explore the effect of hUC-MSCs on hyperoxia-induced pulmonary vascular remodeling, lung sections were stained with an anti-α-SMA antibody (Figure 3A). The MTI for the peripheral pulmonary blood vessels was higher in hyperoxia-exposed animals than that in normoxia-exposed ones (47.18 ± 3.80 *vs* 33.12 ± 3.48, *P* < 0.001; Figure 3B). The hyperoxia + iT-MSC and hyperoxia + iP-MSC groups exhibited significantly induced vascular muscularization (27.65 ± 2.89 and 29.06 ± 2.52, respectively).

DOI: 10.4252/wjsc.v14.i7.556 **Copyright** ©The Author(s) 2022.

Figure 3 Human umbilical cord-derived mesenchymal stem cells treatment rescues hyperoxia-induced loss of peripheral pulmonary blood vessels and peripheral pulmonary arterial remodeling. A: Representative photomicrographs of lung sections of rats harvested at postnatal day 21 stained with α smooth muscle actin antibody in normal air or hyperoxia exposure groups, with or without human umbilical cord-derived mesenchymal stem cells administration (scale bars = 100 μm); B and D: Medial thickness index and microvessel density in lungs treated as indicated were calculated to assess hyperoxia-induced peripheral pulmonary vascular remodeling and loss of blood vessels in the peripheral microvasculature (*n* = 5 for each group, 5 fields/animal); C: Representative lung slides with von Willebrand factor- immunofluorescence staining obtained at 200 × magnification. White arrows highlight stained pulmonary vessels (scale bars = 50 μm); E and F: Vascular endothelial-derived growth factor and platelet-derived growth factor mRNA expression in the lung tissues in the indicated groups. [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$. iT: Intratracheal; iP: Intraperitoneal; VEGF: Vascular endothelial-derived growth factor; PDGF: Platelet-derived growth factor; MSC: Mesenchymal stem cell.

To determine the effect of hyperoxia exposure on peripheral pulmonary vessel number, we performed von Willebrand factor staining on lung sections (Figure 3C). The rats reared in hyperoxia yielded a significantly lower MVD than those reared in normoxia (3.01 ± 0.36 *vs* 6.11 ± 1.02 vessels/field, *P* < 0.001; Figure 3D). Treatment with hUC-MSCs significantly restored the hyperoxia-induced decrease in vascular density (5.54 ± 1.16 and 5.57 ± 1.70 for iT-MSC and iP-MSC, respectively). MTI and MVD of the lungs of the iT-MSC-treated animals did not differ significantly from those of the iP-MSC-treated

group.

Vascular endothelial-derived growth factor (VEGF) and platelet-derived growth factor (PDGF) expression were primarily detected in lung tissues using RT-qPCR. Decreased *VEGF* and *PDGF* mRNA expression was observed in the lungs of pups with hyperoxic lung injury, which was augmented upon hUC-MSC administration (Figure 3E and F).

hUC-MSCs treatment modulates hyperoxia-induced lung inflammation and oxidative stress

Total protein levels in BALF were measured as an indication of endothelial and epithelial permeability. Hyperoxia-exposed animals demonstrated elevated BALF protein concentrations compared to normoxia-exposed pups. hUC-MSCs administration (iT or iP) ameliorated the hyperoxia-induced high permeability of the lung epithelium (Figure 4A). The same trend was reflected in MPO expression, an indicator of the number of neutrophils in the lung (Figure 4B).

To further investigate the inflammatory response after hyperoxia exposure, we measured the expression of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 and the anti-inflammatory cytokine IL-10 in the lung tissues using ELISA. The increased TNF-α, IL-1β, and IL-6 expressions observed in the hyperoxia group were significantly attenuated in both the iT-MSC and iP-MSC groups (Figure 4C-E). Although there was no significant difference in IL-10 expression between the hyperoxic and normoxic groups, we observed an increase in IL-10 expression in rats that received hUC-MSCs iT compared with rats reared in hyperoxia (Figure 4F).

Furthermore, RT-qPCR results confirmed that hyperoxia exposure led to a significant increase in the mRNA expression of the pro-inflammatory mediators macrophage inflammatory protein *(MIP)-1α*, and *MIP-1β*. After infusion of hUC-MSCs, iT or iP, the expression of *MIP-1α* and *MIP-1β* also significantly decreased (Figure 4G and H). iP administration of hUC-MSCs was slightly more effective in reducing *MIP-1α* levels than iT administration.

MDA levels in tissues were measured to reflect the oxidative stress. Elevated MDA levels induced by hyperoxia exposure were significantly diminished upon hUC-MSC administration in hyperoxia-exposed rats (Figure 4I).

hUC-MSCs treatment modulates the lung transcriptome

We performed a comparative transcriptomic analysis of lung samples from the normoxia, hyperoxia, and hyperoxia + iT-MSC groups *via* RNA-sequencing (Xiuyue Biol, Shandong, China). Hyperoxia-exposed animals showed 1486 significantly enriched and 1720 suppressed mRNAs compared to their normoxic counterparts, while meeting the threshold of an absolute log2 fold change of ≥ 1 and an adjusted *P* < 0.05. Among the DEGs induced upon hyperoxia exposure, the expression of 880 suppressed genes was increased and that of 1314 upregulated genes was reduced upon hUC-MSCs administration, resulting in a shift in gene expression pattern toward normoxia. The top 100 DEGs are presented in a heat map (Figure 5A). GO analysis indicated that hUC-MSCs treatment blunted these hyperoxia-induced dysregulated genes involved in epithelial cell proliferation (*ENG, GPC3, ALDH1A2, SULF1*), regulation of vasculature development (*CYBB, PTPRC, CYR61, CDH5, ANXA3, NOTCH4*), positive regulation of cell adhesion (*ALOX15, CCDC80, CXCL12*), wound healing (*PLET1, POSTN, MACF1, DSP*), and leukocyte migration (*CCL6, ITGAL, CXCL3, LPL, CCL9, CHST4*), which are biological processes that likely contribute to BPD pathogenesis (Figure 5B). Additionally, five of the most significantly enriched cellular components were extracellular matrix, apical part of the cell, external side of the plasma membrane, receptor complex, and membrane microdomain (Figure 5C). The top five significantly enriched molecular functions were phospholipid binding, signaling receptor activator activity, cell adhesion molecule binding, actin binding, and sulfur compound binding (Figure 5D). Therefore, preliminary RNA-sequencing showed that hyperoxia exposure and hUC-MSCs transplantation caused significant changes in the gene expression, which might serve as novel targets for the treatment of hyperoxia-induced organ injury.

Beneficial effects of hUC-MSCs administration in heart and kidneys in hyperoxia exposed neonatal rats

To quantify the degree of PH-induced RVH, HE stained heart tissue sections were assessed (Figure 6). Exposure to hyperoxia increased the RV free wall thickness and IVS thickness (Figure 6A). The Fulton's index (RV/LV + IVS) showed an increase with hyperoxia exposure (Figure 6D). Both iT and iP administration of hUC-MSCs significantly ameliorated this increase.

Nephrogenesis was assessed on HE stained kidney sections. We observed that, compared to the normoxia group, hyperoxia exposure led to a significant reduction in nephrogenic zone width (237.9 ± 35.5 μm *vs* 145.3 ± 23.6 μm) and glomerular diameter (70.5 ± 5.33 μm *vs* 43.0 ± 8.5 μm). These morphological abnormalities in the heart and kidney were alleviated upon hUC-MSCs administration. In the hyperoxia + iT-MSC group, the averaged nephrogenic zone width and glomerular diameter were 196.7 ± 25.1 μm and 62.1 ± 5.2 μm, respectively. In hyperoxia-exposed rats receiving hUC-MSCs iP, the averaged nephrogenic zone width and glomerular diameter were 176.7 ± 27.8 μm and 61.9 ± 3.5 μm, respectively (Figure 6B, C, E, and F).
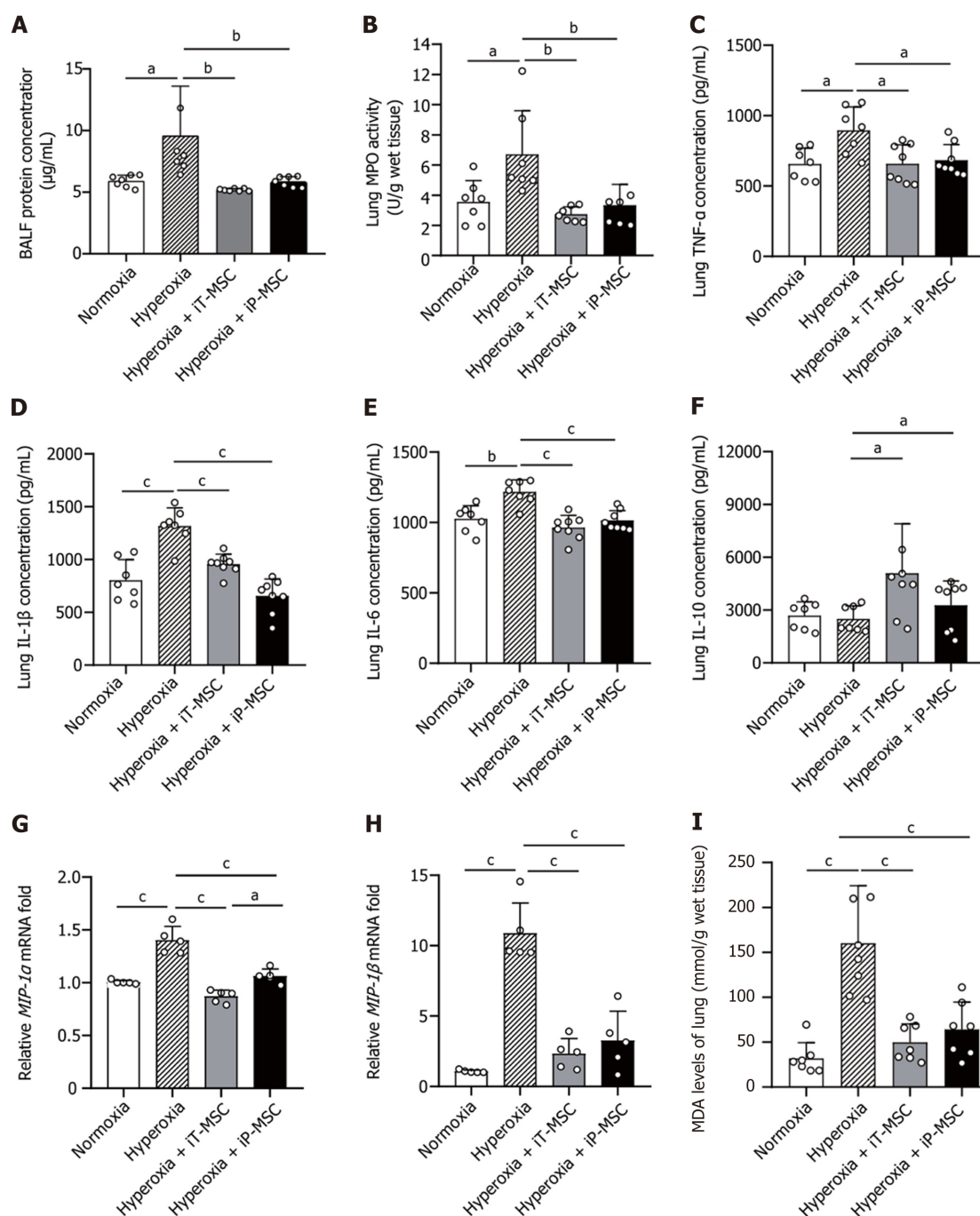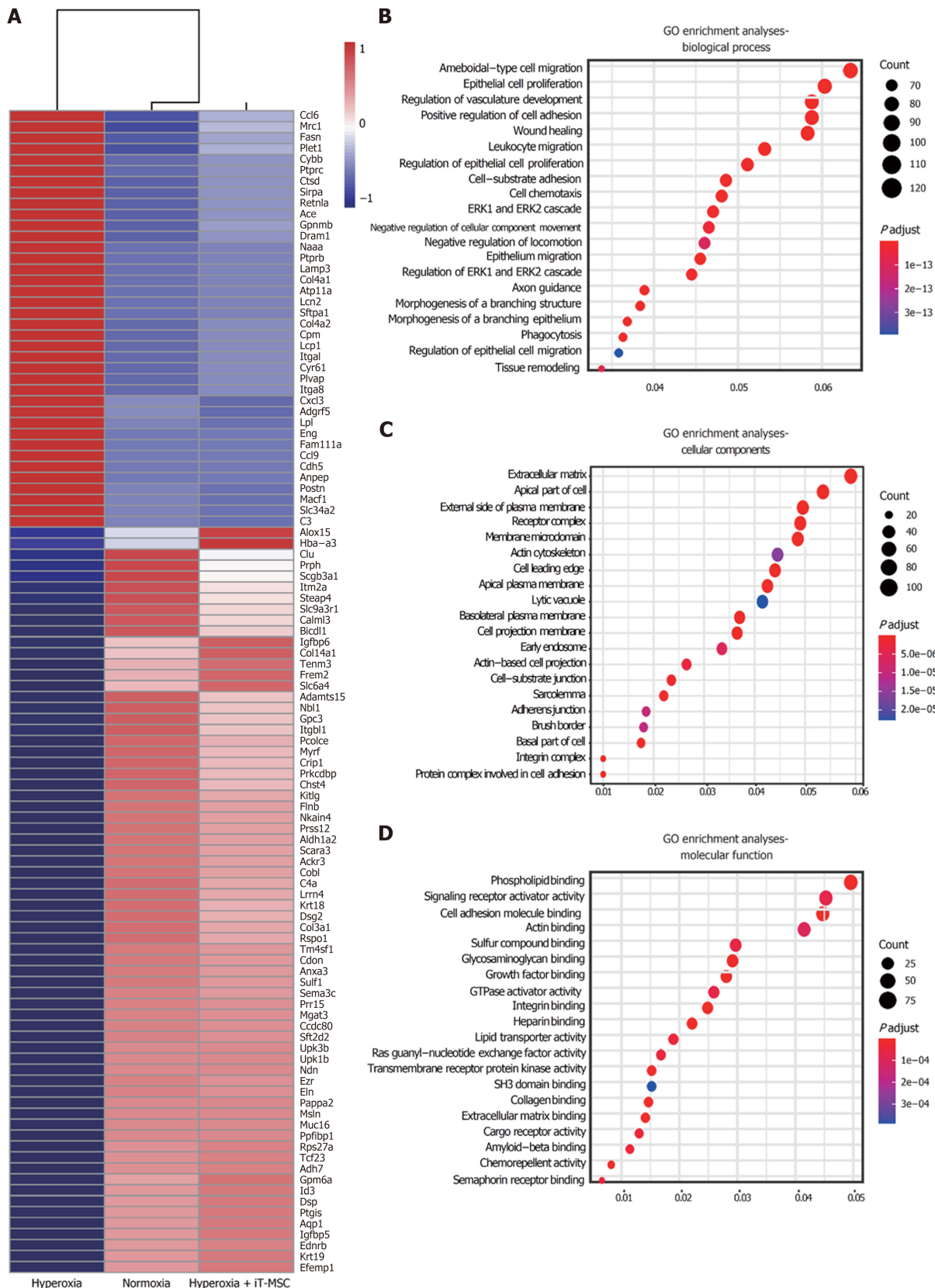
DOI: 10.4252/wjsc.v14.i7.556 **Copyright** ©The Author(s) 2022.

Figure 4 Human umbilical cord-derived mesenchymal stem cells treatment modulates the hyperoxia-induced lung inflammation and oxidative stress. A: Statistical analyses of overall protein concentration in bronchoalveolar lavage fluid in the four groups (*n* = 7); B-F: Statistical analyses of myeloperoxidase, tumor necrosis factor-alpha, interleukin (IL)-1β, IL-6, and IL-10 levels in the lung tissues in the indicated groups (*n* = 7); G and H: Macrophage inflammatory protein *(MIP)-1α* and *MIP-1β* mRNA expression in the indicated groups (*n* = 5); I: Malondialdehyde levels were measured to evaluate the degree of oxidative reaction in the lung tissues (*n* = 7). $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.001$. iT: Intratracheal; iP: Intraperitoneal; BALF: Bronchoalveolar lavage fluid; MPO: Myeloperoxidase; TNF-α: Tumor necrosis factor-alpha; IL: Interleukin; MIP: Macrophage inflammatory protein; MDA: Malondialdehyde; MSC: Mesenchymal stem cell.

Similarly, as *per* the degree of inflammatory and oxidative reactions, MPO expression and MDA levels in the hyperoxia group were elevated compared with those in normoxia in both heart and kidney tissues. Treatment with hUC-MSCs significantly diminished the hyperoxia-induced increase in MPO expression and MDA levels (Figure 6G-J). These parameters did not show a significant statistical difference between iT and iP hUC-MSC administration.

hUC-MSCs activate HO-1 expression and the JAK2/STAT3 signaling pathway to protect against hyperoxia-induced multiple organ injury

To further investigate the mechanism by which hUC-MSCs administration alleviated hyperoxia-induced

Figure 5 Human umbilical cord-derived mesenchymal stem cells treatment modulates the lung transcriptome. A: The heatmap shows the top 100 differentially expressed genes in lung tissues from the normoxia, hyperoxia, and hyperoxia + intratracheal-mesenchymal stem cell groups. The expression levels of up- and downregulated genes are shown in red and blue, respectively; B-D: Gene ontology analyses of the most significantly affected pathways related to biological process, cellular components, and molecular function. iT: Intratracheal; MSC: Mesenchymal stem cell; GO: Gene ontology.
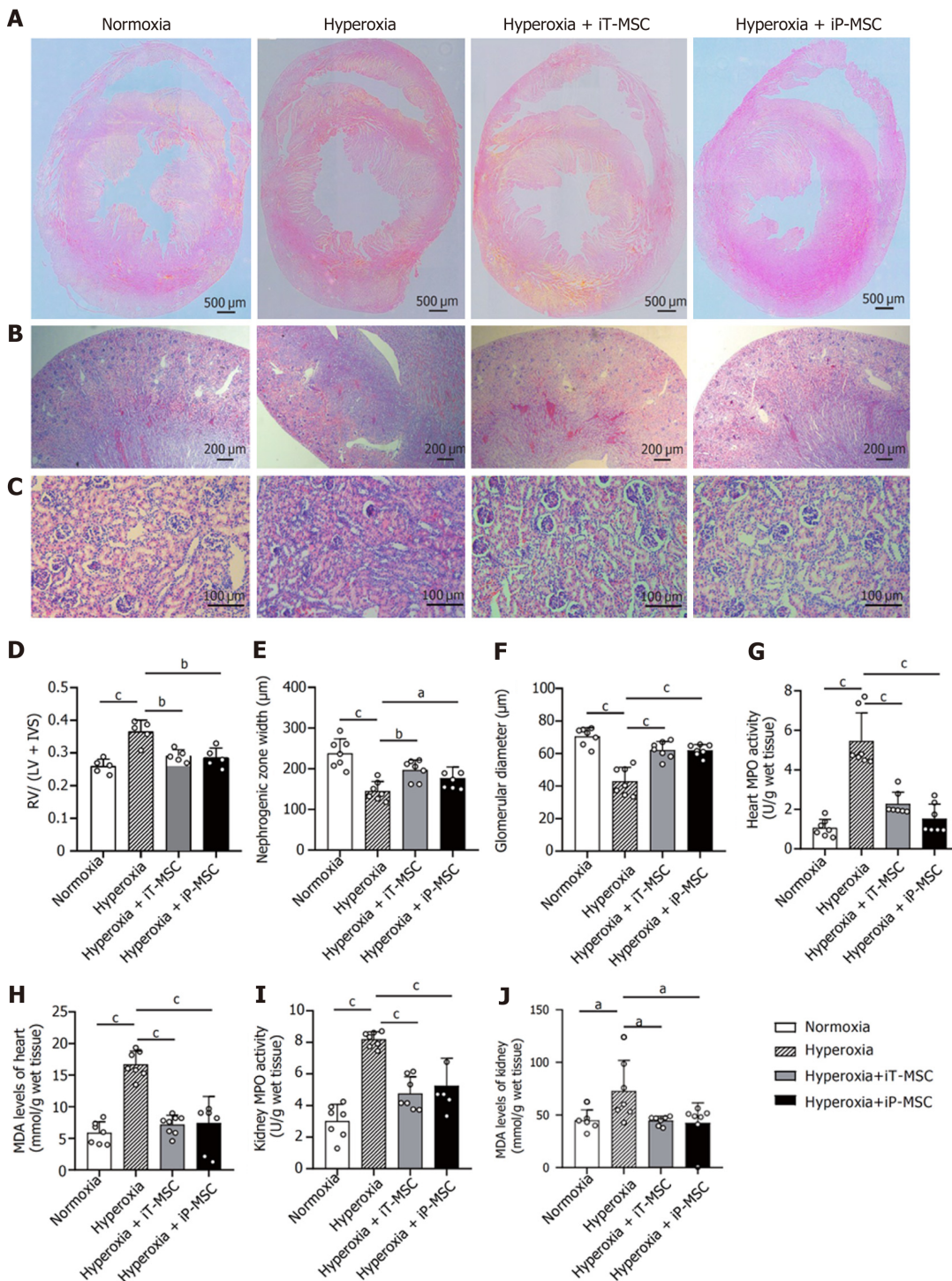
Figure 6 Beneficial effects of human umbilical cord-derived mesenchymal stem cells administration in the heart and kidneys of hyperoxic neonatal rats. A: Representative images of harvested heart sections stained with HE for morphometric analyses (scale bars = 500 μm); B and C: Representative photomicrographs of the hematoxylin and eosin stained sections of kidneys obtained at 40 × magnification (scale bars = 200 μm) and 200 × magnification (scale bars = 100 μm) respectively; D: Fulton's index (right ventricle/left ventricle + interventricular septum) was measured to quantify the degree right ventricular hypertrophy (*n* = 5); E and F: Nephrogenesis was assessed through measuring the width of the nephrogenic zone and the glomerular diameter (*n* = 7); G–J: Myeloperoxidase and malondialdehyde levels were measured to evaluate the degree of inflammatory and oxidative reaction in heart and kidney tissues respectively (*n* = 7). [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$. iT: Intratracheal; iP: Intraperitoneal; RV: Right ventricle; LV: Left ventricle; IVS: Interventricular septum; MPO: Myeloperoxidase; MDA: Malondialdehyde; MSC: Mesenchymal stem cell.

lung, heart, and kidney injury in BPD rats, we measured the expression of HO-1 and JAK2/STAT3 signaling pathways in the whole rat lungs, heart, and kidneys. Through gray value quantification, the results of western blotting showed that the expressions of phosphorylated JAK2 (pJAK2), JAK2, phosphorylated STAT3 (pSTAT3), and STAT3 in lungs, heart, and kidneys were significantly suppressed in the hyperoxia group. The expression of the above proteins increased significantly after

hUC-MSCs administration (Figure 7A–E). Hyperoxia exposure significantly increased the expression of HO-1, which was further enhanced in the hUC-MSCs treated groups (Figure 7A and F). These findings imply that the HO-1 and JAK2/STAT3 signaling pathways are associated with the benefits of hUC-MSC therapy in hyperoxic neonatal rats.

DISCUSSION

BPD is now recognized as a systemic disease associated with respiratory morbidity, cardiac dysfunction, and neural and renal development impairments[3,12,40,41]. Despite advances in the understanding of BPD pathogenesis, not all the mechanisms that lead to hyperoxia damage are completely understood. The use of allogeneic MSCs, derived from the umbilical cord[16,18], adipose tissue[42], or bone marrow [43], has been recognized as the most promising treatment for BPD. Many experiments and clinical trials have reported that MSC administration, iT[16,18], iP[44], intranasally[45], or intravenously[46], exerts beneficial outcomes with improved lung development, reduced inflammation, and decreased oxidative damage. Moreover, recent studies have demonstrated that MSC-derived exosomes (EXOs) or conditioned medium (CM) containing cytokines, growth factors, and nucleic acids play a critical role in mediating the therapeutic effects of MSCs[17,21]. Notably, we previously showed that iT drip of living human amnion-derived MSCs (hAD-MSCs) is more effective in treating hyperoxia-induced lung injury than hAD-MSC-EXOs or hAD-MSC-CM[42]. This phenomenon can be explained by the fact that living cells survive in the lungs for several days and secrete more exosomes.

Although the protective effects of MSCs or their exosomes on hyperoxia-induced lung injury have been explored by many researchers, the underlying mechanism has not been well studied, and few studies have focused on the therapeutic benefits of systemic multiple organ injury. Hence, based on the above findings, the protective effects of the local administration of hUC-MSCs on systemic multiple organ damage and its underlying mechanisms were investigated in the current study.

The most frequently used animal models of BPD are term-born mice or rats that are exposed to supplemental oxygen, to study the effects of hyperoxia exposure on preterm infants with respiratory distress[1,47]. In this study, we established an experimental newborn rat model *via* prolonged exposure to hyperoxia to induce lung, heart, and kidney injury. The present study showed that the administration of hUC-MSCs on P7 played an essential role in improving pulmonary alveolarization and modulating pulmonary angiogenesis in hyperoxia-exposed rats, although the survival rate did not significantly improve. Specifically, iT hUC-MSCs administration diminished the hyperoxia-induced increase in MLI and vascular smooth muscle thickness and augmented the hyperoxia-induced decrease in peripheral pulmonary vascular density in neonatal rats. We also determined the expression levels of VEGF and PDGF in rats. VEGF is a potent mitogen in endothelial cells that regulates angiogenesis and alveolar development[48]. PDGF is crucial for the alveolarization of normally developing lungs[49]. Our results suggested that treatment with hUC-MSCs augmented the hyperoxia-induced decrease in VEGF and PDGF expression in rats. Thus, we demonstrated that treatment with hUC-MSCs enhanced vascular and alveolar development in neonatal rats through the induction of growth factors.

Inflammation activated during oxidative stress is a common pathway leading to the BPD phenotype [1]. Increased pro-inflammatory cytokine levels found in tracheal aspirates and blood samples from premature infants correlate with an increased risk of BPD[50,51]. Recent studies have suggested that hyperoxia induces pro-inflammatory cytokines and cell infiltration in the alveolar spaces in neonatal Sprague Dawley rats[52]. MDA is the main product of lipid peroxidation and is recognized as a biological marker of oxygen stress injury[53,54]. In this study, rats reared in hyperoxia exhibited a significant increase in BALF protein content, as well as TNF-α, IL-1β, IL-6, MIP-1α, MIP-1β, MPO, and MDA levels, which decreased with hUC-MSC treatment. Additionally, we observed an increase in anti-inflammatory cytokine IL-10 expression in rats that received hUC-MSCs. These results support previous ones[16,55] and suggest that the therapeutic effects of surfactant and hUC-MSCs on hyperoxia-induced lung injury are mediated through the inhibition of pro-inflammatory cytokine production and oxidative stress reactions, as well as the induction of immunosuppressive soluble cytokines such as IL-10.

Subsequently, we performed a comparative transcriptomic analysis of differential gene expression in the lung tissues of rats in the normoxia, hyperoxia, and hyperoxia + iT-MSCs groups. It has been reported that hyperoxia dysregulates the expression of genes that modulate immune response[21,56], oxidative stress[57], cell migration, proliferation, and abnormal airway and pulmonary vascular contractility[58] in an experimental model of hyperoxia-induced BPD. Consistent with previous studies, our findings showed the DEGs in lung tissues induced upon hyperoxia exposure were enriched in pathways related to inflammatory responses, epithelial cell proliferation, and vasculature development. These dysregulated genes could contribute to the observed aberrant pulmonary alveolarization and angiogenesis, increased leukocyte infiltration, and enhanced pro-inflammatory cytokine production. Additionally, we found that hUC-MSCs infusion rescued the abnormally expressed genes, which possibly explains their therapeutic ability in hyperoxia-induced lung injury. Taken together, our findings may provide new evidence for the underlying mechanisms of BPD and MSCs treatment.

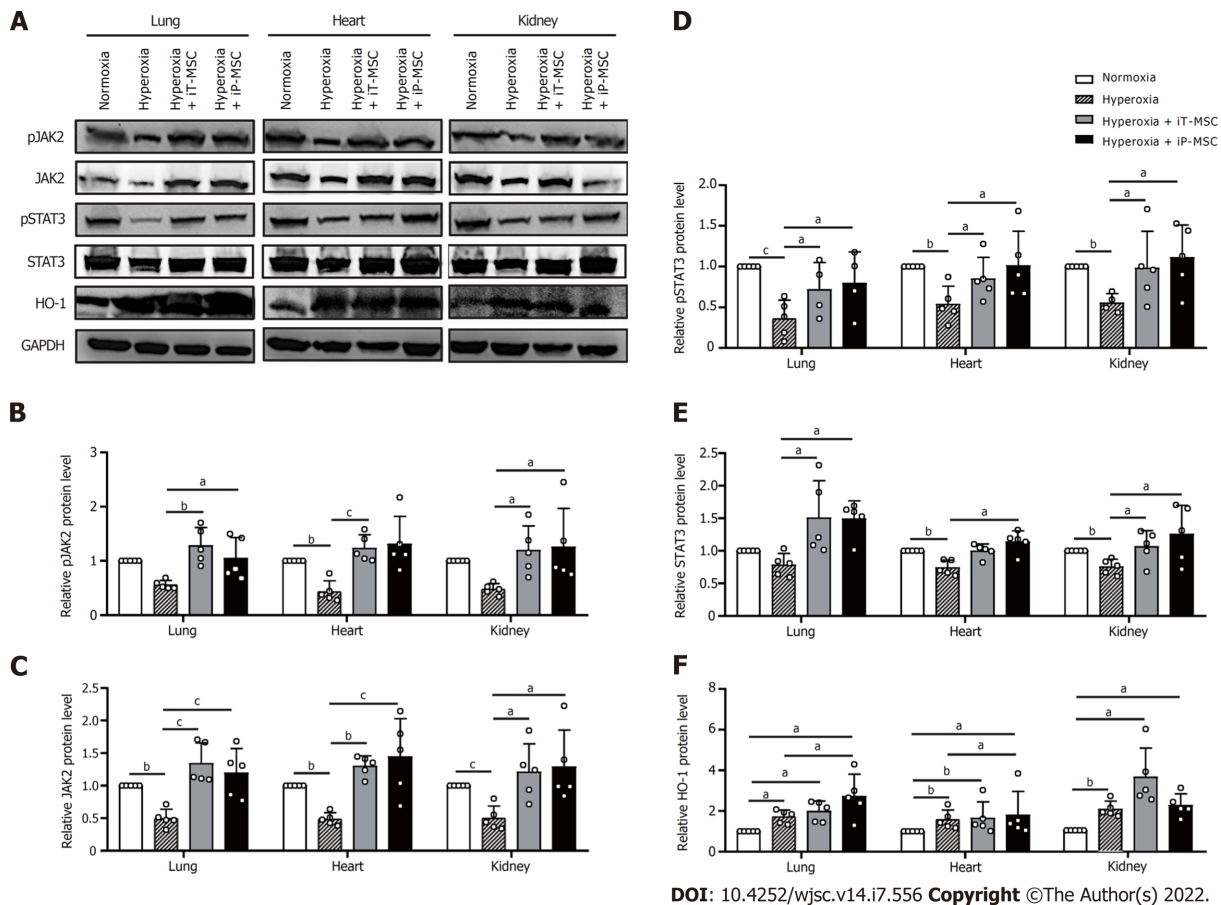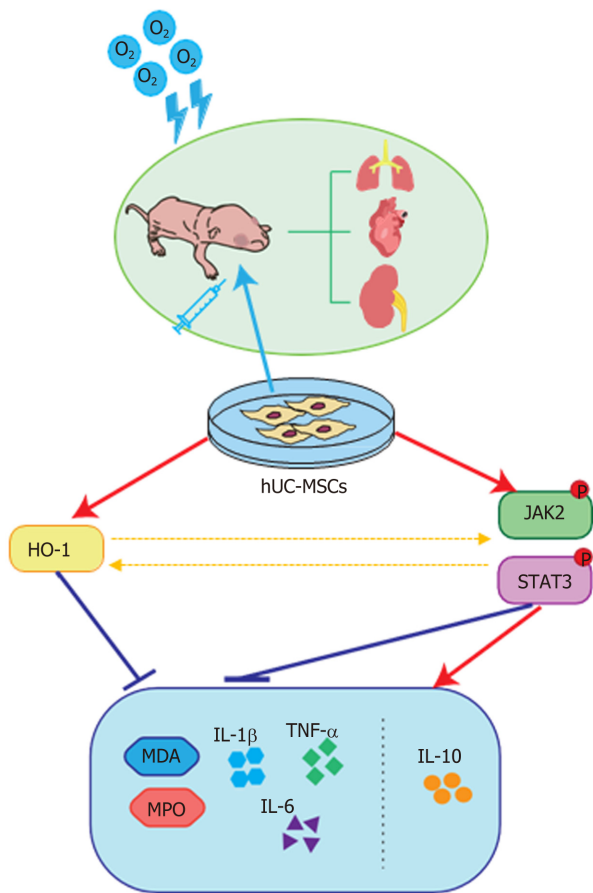**DOI**: 10.4252/wjsc.v14.i7.556 **Copyright** ©The Author(s) 2022.

Figure 7 Human umbilical cord-derived mesenchymal stem cells activated the heme oxygenase-1 and JAK2/STAT3 signaling pathway to protect against hyperoxia-induced multiple-organ injury. A: The protein extracted from tissue homogenates of the lungs, heart, and kidneys were collected for western blotting to detect heme oxygenase-1, JAK2, STAT3 protein expression levels, as well as their phosphorylation; B–F: Statistical analyses of protein expression levels of the above-mentioned genes. $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.001$. iT: Intratracheal; iP: Intraperitoneal; HO-1: Heme oxygenase-1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MSC: Mesenchymal stem cell.

As mentioned above, supraphysiological oxygen concentrations were found to lead to hyperoxia-related PH, ventricular hypertrophy, and renal development impairments, which have been recognized as strong contributors to poor outcomes in preterm infants with BPD[3,4,41]. Of concern, little is known about the treatment outcomes of MSCs in the heart and kidney injuries associated with hyperoxia. In our study, positive results with relieved RVH and nephrogenesis impairment, as well as reduced MPO and MDA levels in the heart and kidney tissues were obtained after iT or iP administration of hUC-MSCs in hyperoxic rat models. Consistent with previous reports, our results suggest additional therapeutic effects of hUC-MSCs administration on histological alterations and modulation of inflammatory cell infiltration and oxidative stress in extrapulmonary organs.

We conducted further experiments to investigate the mechanisms contributing to the therapeutic effects of hUC-MSCs in hyperoxia-exposed animals. The JAK/STAT3 pathway is one of the major pathways involved in many crucial biological processes and is closely associated with many immune and inflammatory diseases[31,59]. Recent studies have indicated that changes in the expression of JAK2 and STAT3 or their phosphorylated forms are related to IL-10 secretion[38,59,60]. Increased IL-10 secretion induced by MSCs in our study has been described previously. Increasing evidence suggests that the JAK2/STAT3 signaling pathway plays a key role in protection against pulmonary[32], cardiac [33,61], and renal injury[34]. The protective effects were inhibited by the selective inhibitors or JAK2/STAT3 siRNA. These studies provided powerful evidence indicating that the activated JAK2/STAT3 signaling pathway is involved in the protective phenotypic changes in injured organs. In line with previous studies, our results showed that JAK2 and STAT3 expression, as well as their phosphorylation, in the lungs, heart, and kidneys were suppressed to some extent in the hyperoxia group and significantly increased after hUC-MSCs treatment.

Among the special defense enzymes developed in mammalian cells to combat oxidative stress, HO-1, an essential enzyme in heme catabolism, has been postulated to play a key role in catalyzing the detoxification of oxidized proteins and modulating the cellular redox homeostasis[26]. In addition, increasing evidence suggests that the products released by the HO reaction possess anti-inflammatory, anti-apoptotic, and anti-proliferative properties[27,62,63]. In the present study, we observed increased HO-1

Figure 8 Proposed mechanism of therapeutic effects of human umbilical cord-derived mesenchymal stem cells in hyperoxia-induced multiple organ injury. The administration of human umbilical cord-derived mesenchymal stem cells at postnatal day ameliorates hyperoxia-induced lung, heart, and kidney development, and reduces inflammatory and oxidative responses by activating heme oxygenase-1 expression and the JAK/STAT3 pathway. O_2: Oxygen; hUC-MSCs: Human umbilical cord-derived mesenchymal stem cells; HO-1: Heme oxygenase-1; MPO: Myeloperoxidase; TNF-α: Tumor necrosis factor-alpha; IL: Interleukin; MDA: Malondialdehyde.

expression in the hyperoxia-exposed group, which is probably a spontaneous response induced by oxidative stress. Furthermore, our findings suggest that the infusion of hUC-MSCs into neonatal rats further enhanced HO-1 expression. No statistically significant difference in the expressions of these proteins was observed between iT and iP hUC-MSC administration. It has been reported that HO-1 contributes to attenuating hyperoxia-induced pulmonary inflammation, arterial remodeling, and RVH, which were significantly reversed in HO-1 knockout mice or upon tin protoporphyrin IX administration, a HO-1 inhibitor[29,30,64]. Detsika *et al*[65] establish HO-1 as a key regulator to attenuate glomerular injury by using HO-1 deficient rats or rats with HO-1 overexpression targeted to glomerular epithelial cells. Based on these studies, it is considered that elevated HO-1 expression also plays a crucial role in the protective effects of UC-MSCs in alleviating oxidative and inflammatory damage in the lung, heart, and kidney tissues. Moreover, recent studies have demonstrated that HO-1 expression is partly regulated by the JAK/STAT pathway[35]; in turn, the JAK/STAT pathway can be activated by enhanced HO-1 expression[36]. Taken together, we speculate that hUC-MSCs can sense inflammatory and oxidative stimulation and then trigger HO-1 expression, IL-10 secretion, and the JAK2/STAT3 signaling pathway, eventually reducing multiple abnormalities induced by hyperoxia (Figure 8).

Remarkably, the benefits of iT-hUC-MSC administration were equivalent to those of the iP-hUC-MSC administration group. Based on previous studies[66,67] and our investigation, we hypothesized that the beneficial effects of local iT administration of MSCs on hyperoxia-induced systemic multi-organ damage are ascribed to paracrine effects. Considering that the majority of low-birth-weight infants were endotracheal intubated at birth, our study supported that iT-MSC administration is a more convenient and effective administration method.

We acknowledge that there are several limitations to this study. Firstly, we only performed transcriptome sequencing of the whole-lung tissue, which consisted of several types of cells. Gene expression changes of sorted cell subtypes from multiple organs exposed to hyperoxia should be studied and validated in future research to assess the effect of MSCs on different target cell populations and address the underlying molecular mechanisms. Moreover, we observed that hUC-MSCs increased

HO-1 expression and activated the JAK2/STAT3 pathway in the lung, heart, and kidney tissues. However, we merely speculated that these changes in protein expression are involved in the therapeutic changes in the phenotype based on previous studies. Further in-depth mechanistic studies will be needed to validate and understand the molecular mechanisms modulated by hUC-MSCs.

CONCLUSION

In summary, the main findings of our study were that administration of hUC-MSCs significantly ameliorates not only lung injuries but also associated heart and kidney damage in hyperoxia-exposed infant rats. Remarkably, the therapeutic benefits of local iT instillation are equivalent to those of iP administration in hyperoxia-induced systemic multi-organ damage. Additionally, we also demonstrated that the underlying mechanism of the therapeutic effects is presumably the increase in HO-1 expression, IL-10 secretion, and the JAK2/STAT3 signaling pathway. Above all, it is desirable that the present study yield positive results to transform healthcare outcomes for a growing population of premature survivors.

ARTICLE HIGHLIGHTS

Research background
Increasing evidence has suggested that bronchopulmonary dysplasia (BPD) is not merely a lung disease, but a systemic condition with short-term and long-term multiple organ implications. Exposure to hyperoxia causes oxidative stress and contributes to the pathogenesis of these recognized implications, including respiratory morbidity, cardiac dysfunction, and impairments in renal development. Mesenchymal stem cells (MSCs) are multipotent stromal cells that have immunomodulatory, anti-inflammatory, and low immunogenicity properties and have shown great potential for the management of a range of different neonatal conditions, including BPD.

Research motivation
Although the protective effects of MSCs or their exosomes on hyperoxia-induced lung injury have been explored by many researchers, the underlying mechanism has not been addressed in detail, and few studies have focused on their therapeutic benefits on systemic multiple organ injury.

Research objectives
This study aimed to investigate whether intratracheal (iT) administration of human umbilical cord-derived MSCs (hUC-MSCs) could simultaneously attenuate hyperoxia-induced lung, heart, and kidney injuries in an experimental neonatal rat model, and elucidate the underlying regulatory mechanism.

Research methods
We established an experimental newborn rat model *via* prolonged exposure to hyperoxia to induce lung, heart, and kidney injury. Briefly, neonatal rats were exposed to hyperoxia (80% O_2), treated with hUC-MSCs iT or intraperitoneal (iP) on postnatal day 7 (P7), and harvested on postnatal day 21. The tissue sections of the lung, heart, and kidney were analyzed morphometrically. Protein contents of bronchoalveolar lavage fluid (BALF), pulmonary inflammatory cytokines, myeloperoxidase (MPO) expression, and malondialdehyde (MDA) levels were examined. Furthermore, RNA-sequencing, reverse transcription-quantitative polymerase chain reaction and western blot analysis were performed to explore underlying mechanisms.

Research results
The present study showed that the administration of hUC-MSCs on P7 played an essential role in improving pulmonary alveolarization and modulating pulmonary angiogenesis, as well as relieving right ventricular hypertrophy and nephrogenesis impairment in hyperoxia-exposed rats. Rats reared in hyperoxia exhibited a significant increase in BALF protein content, tumor necrosis factor-alpha, interleukin (IL)-1β, IL-6, and macrophage inflammatory protein (MIP)-1α, MIP-1β, MPO and MDA levels, which decreased with hUC-MSC treatment. Additionally, we observed an increase in anti-inflammatory cytokine IL-10 expression in rats that received hUC-MSCs. Transcriptomic analysis showed that hUC-MSCs administration blunted the hyperoxia-induced dysregulated genes, which were enriched in pathways related to inflammatory responses, epithelial cell proliferation, and vasculature development. Moreover, hUC-MSCs increased heme oxygenase (HO-1), JAK2, and STAT3 expression, and their phosphorylation in the lung, heart, and kidney. Remarkably, no significant difference was observed between iT and iP administration.

Research conclusions

Our results suggest additional therapeutic effects of hUC-MSCs administration on histological alterations, along with the modulation of inflammatory cell infiltration and oxidative stress in extrapulmonary organs. The therapeutic benefits of local iT instillation are equivalent to those of iP administration in systemic multi-organ damage. These therapeutic effects presumably result from an increase in HO-1 expression and the JAK2/STAT3 signaling pathway.

Research perspectives

In future studies, gene expression changes of sorted cell subtypes from multiple organs injured upon hyperoxia exposure will be studied. Further in-depth mechanistic studies are warranted to validate and understand the molecular mechanisms modulated by hUC-MSCs. Moreover, given the significance of changing the MSCs culture mode, the application of genetically modified and three-dimensionally cultured MSCs will be explored to improve therapeutic outcomes in future studies. These studies will lay the foundation for a more extensive and effective clinical application of MSCs in the treatment of BPD.

ORCID number: Na Dong 0000-0002-0129-5934; Pan-Pan Zhou 0000-0002-1019-6436; Dong Li 0000-0002-6496-4605; Hua-Su Zhu 0000-0001-6432-4555; Ling-Hong Liu 0000-0003-2637-0408; Hui-Xian Ma 0000-0002-8147-8166; Qing Shi 0000-0002-0921-3358; Xiu-Li Ju 0000-0001-5464-3106.

REFERENCES

1 **Morty RE**. Recent advances in the pathogenesis of BPD. *Semin Perinatol* 2018; **42**: 404-412 [PMID: 30384986 DOI: 10.1053/j.semperi.2018.09.001]

2 **Gilfillan M**, Bhandari A, Bhandari V. Diagnosis and management of bronchopulmonary dysplasia. *BMJ* 2021; **375**: n1974 [PMID: 34670756 DOI: 10.1136/bmj.n1974]

3 **Thébaud B**, Goss KN, Laughon M, Whitsett JA, Abman SH, Steinhorn RH, Aschner JL, Davis PG, McGrath-Morrow SA, Soll RF, Jobe AH. Bronchopulmonary dysplasia. *Nat Rev Dis Primers* 2019; **5**: 78 [PMID: 31727986 DOI: 10.1038/s41572-019-0127-7]

4 **Principi N**, Di Pietro GM, Esposito S. Bronchopulmonary dysplasia: clinical aspects and preventive and therapeutic strategies. *J Transl Med* 2018; **16**: 36 [PMID: 29463286 DOI: 10.1186/s12967-018-1417-7]

5 **Davidson LM**, Berkelhamer SK. Bronchopulmonary Dysplasia: Chronic Lung Disease of Infancy and Long-Term Pulmonary Outcomes. *J Clin Med* 2017; **6** [PMID: 28067830 DOI: 10.3390/jcm6010004]

6 **Katz TA**, Vliegenthart RJS, Aarnoudse-Moens CSH, Leemhuis AG, Beuger S, Blok GJ, van Brakel MJM, van den Heuvel MEN, van Kempen AAMW, Lutterman C, Rijpert M, Schiering IA, Ran NC, Visser F, Wilms J, van Kaam AH, Onland W. Severity of Bronchopulmonary Dysplasia and Neurodevelopmental Outcome at 2 and 5 Years Corrected Age. *J Pediatr* 2022; **243**: 40-46.e2 [PMID: 34929243 DOI: 10.1016/j.jpeds.2021.12.018]

7 **Hartnett ME**, Penn JS. Mechanisms and management of retinopathy of prematurity. *N Engl J Med* 2012; **367**: 2515-2526 [PMID: 23268666 DOI: 10.1056/NEJMra1208129]

8 **Madan A**, Penn JS. Animal models of oxygen-induced retinopathy. *Front Biosci* 2003; **8**: d1030-d1043 [PMID: 12700061 DOI: 10.2741/1056]

9 **White SL**, Perkovic V, Cass A, Chang CL, Poulter NR, Spector T, Haysom L, Craig JC, Salmi IA, Chadban SJ, Huxley RR. Is low birth weight an antecedent of CKD in later life? *Am J Kidney Dis* 2009; **54**: 248-261 [PMID: 19339091 DOI: 10.1053/j.ajkd.2008.12.042]

10 **Chow JC**, Chou HC, Hwang J, Chen CM. Anti-Tn Monoclonal Antibody Ameliorates Hyperoxia-Induced Kidney Injury by Suppressing Oxidative Stress and Inflammation in Neonatal Mice. *Mediators Inflamm* 2021; **2021**: 1180543 [PMID: 34720748 DOI: 10.1155/2021/1180543]

11 **Smit B**, Smulders YM, van der Wouden JC, Oudemans-van Straaten HM, Spoelstra-de Man AME. Hemodynamic effects of acute hyperoxia: systematic review and meta-analysis. *Crit Care* 2018; **22**: 45 [PMID: 29477145 DOI: 10.1186/s13054-018-1968-2]

12 **de Visser YP**, Walther FJ, Laghmani el H, Steendijk P, Middeldorp M, van der Laarse A, Wagenaar GT. Phosphodiesterase 4 inhibition attenuates persistent heart and lung injury by neonatal hyperoxia in rats. *Am J Physiol Lung Cell Mol Physiol* 2012; **302**: L56-L67 [PMID: 21949154 DOI: 10.1152/ajplung.00041.2011]

13 **Ramani M**, Bradley WE, Dell'Italia LJ, Ambalavanan N. Early exposure to hyperoxia or hypoxia adversely impacts cardiopulmonary development. *Am J Respir Cell Mol Biol* 2015; **52**: 594-602 [PMID: 25255042 DOI: 10.1165/rcmb.2013-0491OC]

14 **Menon RT**, Shrestha AK, Reynolds CL, Barrios R, Shivanna B. Long-term pulmonary and cardiovascular morbidities of neonatal hyperoxia exposure in mice. *Int J Biochem Cell Biol* 2018; **94**: 119-124 [PMID: 29223466 DOI: 10.1016/j.biocel.2017.12.001]

15 **Mohr J**, Voggel J, Vohlen C, Dinger K, Dafinger C, Fink G, Göbel H, Liebau MC, Dötsch J, Alejandre Alcazar MA. IL-6/Smad2 signaling mediates acute kidney injury and regeneration in a murine model of neonatal hyperoxia. *FASEB J* 2019; **33**: 5887-5902 [PMID: 30721632 DOI: 10.1096/fj.201801875RR]

16 **Chou HC**, Chang CH, Chen CH, Lin W, Chen CM. Consecutive daily administration of intratracheal surfactant and human umbilical cord-derived mesenchymal stem cells attenuates hyperoxia-induced lung injury in neonatal rats. *Stem Cell Res Ther* 2021; **12**: 258 [PMID: 33933128 DOI: 10.1186/s13287-021-02335-4]

17 **Porzionato A**, Zaramella P, Dedja A, Guidolin D, Van Wemmel K, Macchi V, Jurga M, Perilongo G, De Caro R, Baraldi E, Muraca M. Intratracheal administration of clinical-grade mesenchymal stem cell-derived extracellular vesicles reduces lung injury in a rat model of bronchopulmonary dysplasia. *Am J Physiol Lung Cell Mol Physiol* 2019; **316**: L6-L19 [PMID: 30284924 DOI: 10.1152/ajplung.00109.2018]

18 **Chang YS**, Choi SJ, Sung DK, Kim SY, Oh W, Yang YS, Park WS. Intratracheal transplantation of human umbilical cord blood-derived mesenchymal stem cells dose-dependently attenuates hyperoxia-induced lung injury in neonatal rats. *Cell Transplant* 2011; **20**: 1843-1854 [PMID: 23167961 DOI: 10.3727/096368911X565038]

19 **Pierro M**, Ionescu L, Montemurro T, Vadivel A, Weissmann G, Oudit G, Emery D, Bodiga S, Eaton F, Péault B, Mosca F, Lazzari L, Thébaud B. Short-term, long-term and paracrine effect of human umbilical cord-derived stem cells in lung injury prevention and repair in experimental bronchopulmonary dysplasia. *Thorax* 2013; **68**: 475-484 [PMID: 23212278 DOI: 10.1136/thoraxjnl-2012-202323]

20 **Pierro M**, Thébaud B, Soll R. Mesenchymal stem cells for the prevention and treatment of bronchopulmonary dysplasia in preterm infants. *Cochrane Database Syst Rev* 2017; **11**: CD011932 [PMID: 29125893 DOI: 10.1002/14651858.CD011932.pub2]

21 **Willis GR**, Fernandez-Gonzalez A, Anastas J, Vitali SH, Liu X, Ericsson M, Kwong A, Mitsialis SA, Kourembanas S. Mesenchymal Stromal Cell Exosomes Ameliorate Experimental Bronchopulmonary Dysplasia and Restore Lung Function through Macrophage Immunomodulation. *Am J Respir Crit Care Med* 2018; **197**: 104-116 [PMID: 28853608 DOI: 10.1164/rccm.201705-0925OC]

22   **Liang X**, Ding Y, Zhang Y, Tse HF, Lian Q. Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell Transplant* 2014; **23**: 1045-1059 [PMID: 23676629 DOI: 10.3727/096368913X667709]

23   **Kern S**, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 2006; **24**: 1294-1301 [PMID: 16410387 DOI: 10.1634/stemcells.2005-0342]

24   **Lin KC**, Yeh JN, Chen YL, Chiang JY, Sung PH, Lee FY, Guo J, Yip HK. Xenogeneic and Allogeneic Mesenchymal Stem Cells Effectively Protect the Lung Against Ischemia-reperfusion Injury Through Downregulating the Inflammatory, Oxidative Stress, and Autophagic Signaling Pathways in Rat. *Cell Transplant* 2020; **29**: 963689720954140 [PMID: 33050736 DOI: 10.1177/0963689720954140]

25   **Gutiérrez-Fernández M**, Rodríguez-Frutos B, Ramos-Cejudo J, Otero-Ortega L, Fuentes B, Vallejo-Cremades MT, Sanz-Cuesta BE, Díez-Tejedor E. Comparison between xenogeneic and allogeneic adipose mesenchymal stem cells in the treatment of acute cerebral infarct: proof of concept in rats. *J Transl Med* 2015; **13**: 46 [PMID: 25637958 DOI: 10.1186/s12967-015-0406-3]

26   **Gozzelino R**, Jeney V, Soares MP. Mechanisms of cell protection by heme oxygenase-1. *Annu Rev Pharmacol Toxicol* 2010; **50**: 323-354 [PMID: 20055707 DOI: 10.1146/annurev.pharmtox.010909.105600]

27   **Ryter SW**, Choi AM. Targeting heme oxygenase-1 and carbon monoxide for therapeutic modulation of inflammation. *Transl Res* 2016; **167**: 7-34 [PMID: 26166253 DOI: 10.1016/j.trsl.2015.06.011]

28   **Luo YY**, Wu SH, Lu HY, Li BJ, Li SJ, Sun ZY, Jin R, Chen XQ. Lipoxin A4 attenuates hyperoxiainduced lung epithelial cell injury *via* the upregulation of heme oxygenase1 and inhibition of proinflammatory cytokines. *Mol Med Rep* 2020; **21**: 429-437 [PMID: 31746387 DOI: 10.3892/mmr.2019.10821]

29   **Dunigan-Russell K**, Silverberg M, Lin VY, Li R, Wall SB, Li Q, Nicola T, Gotham J, Crowe DR, Vitiello PF, Agarwal A, Tipple TE. Club Cell Heme Oxygenase-1 Deletion: Effects in Hyperoxia-Exposed Adult Mice. *Oxid Med Cell Longev* 2020; **2020**: 2908271 [PMID: 32587658 DOI: 10.1155/2020/2908271]

30   **Fernandez-Gonzalez A**, Alex Mitsialis S, Liu X, Kourembanas S. Vasculoprotective effects of heme oxygenase-1 in a murine model of hyperoxia-induced bronchopulmonary dysplasia. *Am J Physiol Lung Cell Mol Physiol* 2012; **302**: L775-L784 [PMID: 22287607 DOI: 10.1152/ajplung.00196.2011]

31   **Owen KL**, Brockwell NK, Parker BS. JAK-STAT Signaling: A Double-Edged Sword of Immune Regulation and Cancer Progression. *Cancers (Basel)* 2019; **11** [PMID: 31842362 DOI: 10.3390/cancers11122002]

32   **Liu W**, Gao Y, Li H, Wang H, Ye M, Jiang G, Chen Y, Liu Y, Kong J, Liu W, Sun M, Hou M, Yu K. Intravenous transplantation of mesenchymal stromal cells has therapeutic effects in a sepsis mouse model through inhibition of septic natural killer cells. *Int J Biochem Cell Biol* 2016; **79**: 93-103 [PMID: 27521657 DOI: 10.1016/j.biocel.2016.08.013]

33   **Zhang L**, Wang X, Zhang H, Feng M, Ding J, Zhang B, Cheng Z, Qian L. Exercise-induced peptide EIP-22 protect myocardial from ischaemia/reperfusion injury *via* activating JAK2/STAT3 signalling pathway. *J Cell Mol Med* 2021; **25**: 3560-3572 [PMID: 33710777 DOI: 10.1111/jcmm.16441]

34   **Liu Y**, Wang L, Du Y, Chen Z, Guo J, Weng X, Wang X, Wang M, Chen D, Liu X. Effects of apigenin pretreatment against renal ischemia/reperfusion injury *via* activation of the JAK2/STAT3 pathway. *Biomed Pharmacother* 2017; **95**: 1799-1808 [PMID: 28962085 DOI: 10.1016/j.biopha.2017.09.091]

35   **Tron K**, Samoylenko A, Musikowski G, Kobe F, Immenschuh S, Schaper F, Ramadori G, Kietzmann T. Regulation of rat heme oxygenase-1 expression by interleukin-6 *via* the Jak/STAT pathway in hepatocytes. *J Hepatol* 2006; **45**: 72-80 [PMID: 16510205 DOI: 10.1016/j.jhep.2005.12.019]

36   **Yan X**, Cheng X, He X, Zheng W, Yuan X, Chen H. HO-1 Overexpressed Mesenchymal Stem Cells Ameliorate Sepsis-Associated Acute Kidney Injury by Activating JAK/stat3 Pathway. *Cell Mol Bioeng* 2018; **11**: 509-518 [PMID: 31719896 DOI: 10.1007/s12195-018-0540-0]

37   **Lee PJ**, Camhi SL, Chin BY, Alam J, Choi AM. AP-1 and STAT mediate hyperoxia-induced gene transcription of heme oxygenase-1. *Am J Physiol Lung Cell Mol Physiol* 2000; **279**: L175-L182 [PMID: 10893216 DOI: 10.1152/ajplung.2000.279.1.L175]

38   **Zhu HS**, Li D, Li C, Huang JX, Chen SS, Li LB, Shi Q, Ju XL. Prior transfusion of umbilical cord mesenchymal stem cells can effectively alleviate symptoms of motion sickness in mice through interleukin 10 secretion. *World J Stem Cells* 2021; **13**: 177-192 [PMID: 33708346 DOI: 10.4252/wjSC.v13.i2.177]

39   **Chen CM**, Chou HC, Lin W, Tseng C. Surfactant effects on the viability and function of human mesenchymal stem cells: *in vitro* and *in vivo* assessment. *Stem Cell Res Ther* 2017; **8**: 180 [PMID: 28774314 DOI: 10.1186/s13287-017-0634-y]

40   **Reich B**, Hoeber D, Bendix I, Felderhoff-Mueser U. Hyperoxia and the Immature Brain. *Dev Neurosci* 2016; **38**: 311-330 [PMID: 28152539 DOI: 10.1159/000454917]

41   **Xu X**, You K, Bu R. Proximal Tubular Development Is Impaired with Downregulation of MAPK/ERK Signaling, HIF-1α, and Catalase by Hyperoxia Exposure in Neonatal Rats. *Oxid Med Cell Longev* 2019; **2019**: 9219847 [PMID: 31558952 DOI: 10.1155/2019/9219847]

42   **Li Z**, Gong X, Li D, Yang X, Shi Q, Ju X. Intratracheal Transplantation of Amnion-Derived Mesenchymal Stem Cells Ameliorates Hyperoxia-Induced Neonatal Hyperoxic Lung Injury *via* Aminoacyl-Peptide Hydrolase. *Int J Stem Cells* 2020; **13**: 221-236 [PMID: 32323511 DOI: 10.15283/ijsc19110]

43   **Xie Y**, Chen F, Jia L, Chen R, Zhang VW, Zhong X, Wang D. Mesenchymal stem cells from different sources show distinct therapeutic effects in hyperoxia-induced bronchopulmonary dysplasia in rats. *J Cell Mol Med* 2021; **25**: 8558-8566 [PMID: 34322990 DOI: 10.1111/jcmm.16817]

44   **Chang YS**, Oh W, Choi SJ, Sung DK, Kim SY, Choi EY, Kang S, Jin HJ, Yang YS, Park WS. Human umbilical cord blood-derived mesenchymal stem cells attenuate hyperoxia-induced lung injury in neonatal rats. *Cell Transplant* 2009; **18**: 869-886 [PMID: 19500472 DOI: 10.3727/096368909X471189]

45   **Moreira A**, Winter C, Joy J, Winter L, Jones M, Noronha M, Porter M, Quim K, Corral A, Alayli Y, Seno T, Mustafa S, Hornsby P, Ahuja S. Intranasal delivery of human umbilical cord Wharton's jelly mesenchymal stromal cells restores lung alveolarization and vascularization in experimental bronchopulmonary dysplasia. *Stem Cells Transl Med* 2020; **9**: 221-234 [PMID: 31774626 DOI: 10.1002/sctm.18-0273]

46   **Álvarez-Fuente M**, Arruza L, Lopez-Ortego P, Moreno L, Ramírez-Orellana M, Labrandero C, González Á, Melen G, Cerro MJD. Off-label mesenchymal stromal cell treatment in two infants with severe bronchopulmonary dysplasia: clinical course and biomarkers profile. *Cytotherapy* 2018; **20**: 1337-1344 [PMID: 30327248 DOI: 10.1016/j.jcyt.2018.09.003]

47   **O'Reilly M**, Thébaud B. Animal models of bronchopulmonary dysplasia. The term rat models. *Am J Physiol Lung Cell Mol Physiol* 2014; **307**: L948-L958 [PMID: 25305248 DOI: 10.1152/ajplung.00160.2014]

48   **Yun EJ**, Lorizio W, Seedorf G, Abman SH, Vu TH. VEGF and endothelium-derived retinoic acid regulate lung vascular and alveolar development. *Am J Physiol Lung Cell Mol Physiol* 2016; **310**: L287-L298 [PMID: 26566904 DOI: 10.1152/ajplung.00229.2015]

49   **Lindahl P**, Boström H, Karlsson L, Hellström M, Kalén M, Betsholtz C. Role of platelet-derived growth factors in angiogenesis and alveogenesis. *Curr Top Pathol* 1999; **93**: 27-33 [PMID: 10339896 DOI: 10.1007/978-3-642-58456-5_4]

50   **Tullus K**, Noack GW, Burman LG, Nilsson R, Wretlind B, Brauner A. Elevated cytokine levels in tracheobronchial aspirate fluids from ventilator treated neonates with bronchopulmonary dysplasia. *Eur J Pediatr* 1996; **155**: 112-116 [PMID: 8775225 DOI: 10.1007/BF02075762]

51   **D'Angio CT**, Ambalavanan N, Carlo WA, McDonald SA, Skogstrand K, Hougaard DM, Shankaran S, Goldberg RN, Ehrenkranz RA, Tyson JE, Stoll BJ, Das A, Higgins RD; Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network. Blood Cytokine Profiles Associated with Distinct Patterns of Bronchopulmonary Dysplasia among Extremely Low Birth Weight Infants. *J Pediatr* 2016; **174**: 45-51.e5 [PMID: 27117196 DOI: 10.1016/j.jpeds.2016.03.058]

52   **Wang Y**, Yue S, Luo Z, Cao C, Yu X, Liao Z, Wang M. N-methyl-D-aspartate receptor activation mediates lung fibroblast proliferation and differentiation in hyperoxia-induced chronic lung disease in newborn rats. *Respir Res* 2016; **17**: 136 [PMID: 27769245 DOI: 10.1186/s12931-016-0453-1]

53   **Zhang J**, Yang Z, Zhang S, Xie Z, Han S, Wang L, Zhang B, Sun S. Investigation of endogenous malondialdehyde through fluorescent probe MDA-6 during oxidative stress. *Anal Chim Acta* 2020; **1116**: 9-15 [PMID: 32389192 DOI: 10.1016/j.aca.2020.04.030]

54   **Del Rio D**, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr Metab Cardiovasc Dis* 2005; **15**: 316-328 [PMID: 16054557 DOI: 10.1016/j.numecd.2005.05.003]

55   **Chen CM**, Chou HC. Human mesenchymal stem cells attenuate hyperoxia-induced lung injury through inhibition of the renin-angiotensin system in newborn rats. *Am J Transl Res* 2018; **10**: 2628-2635 [PMID: 30210699]

56   **Revhaug C**, Bik-Multanowski M, Zasada M, Rognlien AGW, Günther CC, Książek T, Madetko-Talowska A, Szewczyk K, Grabowska A, Kwinta P, Pietrzyk JJ, Baumbusch LO, Saugstad OD. Immune System Regulation Affected by a Murine Experimental Model of Bronchopulmonary Dysplasia: Genomic and Epigenetic Findings. *Neonatology* 2019; **116**: 269-277 [PMID: 31454811 DOI: 10.1159/000501461]

57   **Bhattacharya S**, Zhou Z, Yee M, Chu CY, Lopez AM, Lunger VA, Solleti SK, Resseguie E, Buczynski B, Mariani TJ, O'Reilly MA. The genome-wide transcriptional response to neonatal hyperoxia identifies Ahr as a key regulator. *Am J Physiol Lung Cell Mol Physiol* 2014; **307**: L516-L523 [PMID: 25150061 DOI: 10.1152/ajplung.00200.2014]

58   **Shrestha AK**, Gopal VYN, Menon RT, Hagan JL, Huang S, Shivanna B. Lung omics signatures in a bronchopulmonary dysplasia and pulmonary hypertension-like murine model. *Am J Physiol Lung Cell Mol Physiol* 2018; **315**: L734-L741 [PMID: 30047283 DOI: 10.1152/ajplung.00183.2018]

59   **Murray PJ**. The JAK-STAT signaling pathway: input and output integration. *J Immunol* 2007; **178**: 2623-2629 [PMID: 17312100 DOI: 10.4049/jimmunol.178.5.2623]

60   **Li L**, Zhang J, Chen J, Xu-Monette ZY, Miao Y, Xiao M, Young KH, Wang S, Medeiros LJ, Wang M, Ford RJ, Pham LV. B-cell receptor-mediated NFATc1 activation induces IL-10/STAT3/PD-L1 signaling in diffuse large B-cell lymphoma. *Blood* 2018; **132**: 1805-1817 [PMID: 30209121 DOI: 10.1182/blood-2018-03-841015]

61   **Qiao S**, Mao X, Wang Y, Lei S, Liu Y, Wang T, Wong GT, Cheung CW, Xia Z, Irwin MG. Remifentanil Preconditioning Reduces Postischemic Myocardial Infarction and Improves Left Ventricular Performance *via* Activation of the Janus Activated Kinase-2/Signal Transducers and Activators of Transcription-3 Signal Pathway and Subsequent Inhibition of Glycogen Synthase Kinase-3β in Rats. *Crit Care Med* 2016; **44**: e131-e145 [PMID: 26468894 DOI: 10.1097/CCM.0000000000001350]

62   **Otterbein LE**, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* 2003; **24**: 449-455 [PMID: 12909459 DOI: 10.1016/s1471-4906(03)00181-9]

63   **Brouard S**, Otterbein LE, Anrather J, Tobiasch E, Bach FH, Choi AM, Soares MP. Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. *J Exp Med* 2000; **192**: 1015-1026 [PMID: 11015442 DOI: 10.1084/jem.192.7.1015]

64   **Suliman HB**, Keenan JE, Piantadosi CA. Mitochondrial quality-control dysregulation in conditional HO-1$^{-/-}$ mice. *JCI Insight* 2017; **2**: e89676 [PMID: 28194437 DOI: 10.1172/jci.insight.89676]

65   **Detsika MG**, Duann P, Atsaves V, Papalois A, Lianos EA. Heme Oxygenase 1 Up-Regulates Glomerular Decay Accelerating Factor Expression and Minimizes Complement Deposition and Injury. *Am J Pathol* 2016; **186**: 2833-2845 [PMID: 27662796 DOI: 10.1016/j.ajpath.2016.07.009]

66   **Lee JW**, Fang X, Krasnodembskaya A, Howard JP, Matthay MA. Concise review: Mesenchymal stem cells for acute lung injury: role of paracrine soluble factors. *Stem Cells* 2011; **29**: 913-919 [PMID: 21506195 DOI: 10.1002/stem.643]

67   **Liang OD**, Mitsialis SA, Chang MS, Vergadi E, Lee C, Aslam M, Fernandez-Gonzalez A, Liu X, Baveja R, Kourembanas S. Mesenchymal stromal cells expressing heme oxygenase-1 reverse pulmonary hypertension. *Stem Cells* 2011; **29**: 99-107 [PMID: 20957739 DOI: 10.1002/stem.548]