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OPINION REVIEW

Banking of perinatal mesenchymal stem/stromal cells for stem cell-based personalized medicine over lifetime: Matters arising

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Abstract

Mesenchymal stromal/stem cells (MSCs) are currently applied in regenerative medicine and tissue engineering. Numerous clinical studies have indicated that MSCs from different tissue sources can provide therapeutic benefits for patients. MSCs derived from either human adult or perinatal tissues have their own unique advantages in their medical practices. Usually, clinical studies are conducted by using of cultured MSCs after thawing or short-term cryopreserved-then-thawed MSCs prior to administration for the treatment of a wide range of diseases and medical disorders. Currently, cryogenically banking perinatal MSCs for potential personalized medicine for later use in lifetime has raised growing interest in China as well as in many other countries. Meanwhile, this has led to questions regarding the availability, stability, consistency, multipotency, and therapeutic efficiency of the potential perinatal MSC-derived therapeutic products after long-term cryostorage. This opinion review does not minimize any therapeutic benefit of perinatal MSCs in many diseases after short-term cryopreservation. This article mainly describes what is known about banking perinatal MSCs in China and, importantly, it is to recognize the limitation and uncertainty of the perinatal MSCs stored in cryobanks for stem cell medical treatments in whole life. This article also provides several recommendations for banking of perinatal MSCs for potentially future personalized medicine, albeit it is impossible to anticipate whether the donor will benefit from banked MSCs during her/his lifetime.

Key Words: Mesenchymal stromal/stem cells; Adult mesenchymal stromal/stem cells; Perinatal mesenchymal stromal/stem cells; Perinatal tissue; Stem cell bank; Personalized medicine

Core Tip: This article reviews the current research related to the potential use of banked perinatal mesenchymal stromal/stem cells (MSCs) for future personalized medicine. This article also discusses the matters needing attention for cryopreservation of perinatal MSCs that are possible to be banked and stored over many years. Acknowledging the limitations and uncertainties of long-term cryopreservation of perinatal MSCs, several key recommendations need to be addressed in this article for future stem cell-based medical treatments.

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INTRODUCTION

Mesenchymal stromal/stem cells (MSCs) possess their unique properties that have attracted great attention in regenerative medicine and tissue engineering. Understanding of the biological properties of MSCs has been continued over a long period of time. Approximately 50 years ago, Fridenshteĭn *et al*[1] found a minor subpopulation of transplanted bone marrow (BM) cells cultured in the diffusion chambers that can act as osteogenic stem cells to show the differentiation of these cells towards osteogenesis. MSCs were initially identified in animal BM in a series of studies as fibroblast-like cells that were plastic-adherent and formed discrete fibroblast colonies[1-3]. Such fibroblast colony-forming cells were also shown to display high proliferative capacity and osteogenic potential[3]. Caplan first coined the name "mesenchymal stem cells" in 1991 on the basis of their *in vitro* capacity to give rise to bone, cartilage, *etc*.[4], but, to date, the stem cell properties of these cells remain controversial due to the multipotent uncertainty of MSCs *in vivo*. The essential features of MSC population have been identified as the minimal criteria by the International Society for Cell & Gene Therapy (ISCT) based on the specific phenotypic markers, adherence to plastic, and the potential of tri-lineage differentiation (adipogenesis, chondrogenesis, and osteogenesis)[5]. Given the unknown self-renewal and differentiation properties *in vivo*, later, Caplan[6] proposed to change the name of MSCs to more accurately reflect their main immunomodulatory and trophic potential not for multipotency of MSCs[7]. In 2019, to further consolidate and clarify the nomenclature of MSCs unless rigorous evidence for stemness exists, the ISCT MSC committee offers a position on the functional definition of mesenchymal stem *vs* stromal cells[8].

MSCs are well known to be isolated from various adult tissues including BM, adipose (AD) tissue, skeletal muscle, dental pulp, and blood[9,10], as well as vascularized tissues[11,12]. Given the immunomodulatory functions, paracrine capacity, and tropic aspects of MSCs, as well as the lacking of human leukocyte antigen (HLA)-DR, a major histocompatibility complex class II molecule, the potential therapeutic properties of MSCs in clinical trials are being explored for MSC-based regenerative medicine. Typically, MSCs are expanded *in vitro*, tested, cryopreserved, and banked for later use in preclinical and clinical studies[13-15]. Biobanking of MSCs from perinatal/neonatal tissues such as umbilical cord tissue, placental tissue, as well as placenta-associated amniotic fluid (AF) and amniotic membrane (AM) for potentially personalized medicine in the future has become more popular over the last few years in China. These newborn tissues would be routinely thrown away after birth. Importantly, these neonatal tissues are an abundant and easily available source of MSCs at birth. In this rapidly growing field, usually, stem cell banking companies involved in the stem cell industry in close collaboration with hospitals in China take over neonatal tissue collection, and preparation and cryobanking of the perinatal MSCs proposed for personal or family use for future stem cell therapies when a person develops a disease. Banking of perinatal MSCs has attracted renewed attention not only in China and probably in many other countries as well. However, serious concerns have been also raised pertaining to the maintenance of functionality and stability of stem cells along with therapeutic potential of MSCs at the time of release years. It is hard to predict the true likelihood of perinatal MSC transplantation later in whole lifetime and, noticeably, there is great uncertainty regarding whether or not these MSCs can be used to treat certain diseases after decades. As such, this opinion review article highlights several key observations in regard to the limitation of perinatal MSCs stored in stem cell cryobanks for later personal or family use in the future, which should be widely considered in the settings of cryostorage to minimize the possible side effects of these MSCs for future stem cell therapies.

Importantly, this review provides several practical recommendations for banking of perinatal MSCs to better serve patients who might be desperately in need for potential personalized medicine for future stem cell-based treatments.

PERINATAL MSCS *VS* ADULT MSCS IN THEIR BIOLOGICAL AND THERAPEUTIC PROPERTIES

MSCs from adult and perinatal sources exhibit differentiated biological and therapeutic properties. The preclinical and clinical data reported in the literature are varied to this end. With a focus on whether the perinatal MSCs are worth biobanking, the pros and cons of perinatal *vs* adult MSCs need to be addressed in this section.

Compared to adult MSCs, the newborn stem cells obtained from perinatal tissues rarely carry any infectious diseases and reduce risk of exposure to environmental toxins. Perinatal MSCs have their own advantages such as easy availability, lacking stem cell variability, and comparably low risk of donor environmental effects, as well as immune privileged property. The diversity of MSC differentiation potential is also observed among MSCs from different source tissues. For example, Hou *et al*[16] conducted an analysis of single-cell RNA-seq using MSCs derived from various tissues. By comparison, umbilical cord-derived MSCs (UC-MSCs), one of the most explored perinatal MSC types, and BM-derived MSCs (BM-MSCs) exhibited the highest osteogenic potential, while AD-derived MSCs (AD-MSCs) and BM-MSCs had the highest potential of adipogenesis and chondrogenesis, respectively[16]. Meanwhile, UC-MSCs showed the highest immunosuppression as well as the highest stemness among all MSC samples in this study by Hou *et al*[16]. Although human UC-MSCs and BM-MSCs exhibited similar immunosuppressive properties, the differences in immunomodulatory effects of UC-MSCs and BM-MSCs were also suggested in a previous study[17]. In contrast, BM-MSCs expressed more indolamine 2,3-dioxygenase (IDO) in *in vitro* inflammatory environment, while UC-MSCs expressed more prostaglandin E2, interleukine-6, and programmed death-ligands 1 and 2. In addition, there were more T helper 17 cells inhibited and more regulatory T cells induced by UC-MSCs compared with BM-MSCs in co-culture[17]. In an experimental model of sepsis, BM-MSCs but not UC-MSCs were proposed to improve survival rate in septic mice due to the enhanced immunoregulatory properties through a variety of mechanisms[18]. In an *in vitro* study, UC-MSCs showed a higher angiogenic capacity in comparison with BM-MSCs and AT-MSCs[19]. Park *et al*[20] conducted an *in vivo* study to test the angiogenic potential of perinatal chorion-derived MSCs (C-MSCs), which revealed that C-MSCs significantly increased the capillary formation in the C-MSCs injected myocardium compared to UC-MSCs and BM-MSCs. Transplantation of UC-MSCs and BM-MSCs also showed similar therapeutic effects for type I diabetes in a mouse model as well as in treated patients with type I diabetes, respectively[21]. Compared to the mice with BM-MSCs treatment, the UC-MSCs treated mice had seemingly higher β-cell mass post-transplantation, although no significant difference was observed between the two treatment groups[21]. There was no difference in the therapeutic efficacy of BM-MSCs and UC-MSCs at one-year follow-up, albeit both types of MSCs decreased the levels of haemoglobin A1c and fasting and postprandial C-peptide[21].

Overall, the relevant preclinical and clinical studies to date by the use of adult and perinatal MSCs have yielded comparable results, which may contribute to a fuller understanding of their therapeutic potential in laboratory and clinical settings. The biological and therapeutic properties of both perinatal and adult MSCs are summarized in Table 1.

PERINATAL MSC BANKING FOR PERSONALIZED MEDICINE OVER LIFETIME: CONCERNS AND UNCERTAINTIES

Over the past 20 years of MSC translational research, clinical experiences have shown that MSCs are seemingly unmet medical needs[22-24]. Translation of stem cell potential into medical practices still confronts many challenges. Clearly, these challenges include the long- and short-term therapeutic safety and efficacy of transplanted MSCs, the sufficient capability of homing and engraftment, the long-term cultivation associated alteration of MSC therapeutic properties, loss of stem cell potency with culture time, heterogeneous functions, and consistency and stability of MSCs or MSC-based therapeutic products. While clinical studies have shown the therapeutic benefits of using MSCs in various human diseases, including cardiomyopathy, autoimmune diseases, diabetes and diabetic complications, bone and cartilage repair, and fibrosis[9], rigorous clinical evidence for MSC therapeutics is still actually lacking. The positive, negative, or mixed clinical results have also been frequently observed in MSC clinical studies. Currently, MSCs based medicine still remains unproven and experimental. Therefore, it is possible to consider the limitation and uncertainty of perinatal MSC biobanking for personalized medicine in the context of the future therapeutic potential. Several important issues illustrated by the use of adult MSCs but not well discussed in perinatal MSCs are exemplified in this section to extend the

Table 1 Perinatal *vs* adult mesenchymal stem/stromal cells in their biological and therapeutic properties

	Perinatal mesenchymal stem/stromal cells	Adult mesenchymal stem/stromal cells
Availability	Readily available	Readily available
Ethical concerns	Minimal crucial ethical concerns and possible religious barriers	Minimal ethical concerns and impossible religious barriers
Immune privileged characteristics	Inactively controversial for immune privileged characteristics	Actively controversial for immune privileged characteristics
Differentiation	Multipotent differentiation	Multipotent differentiation
Variability	Low possibility of stem cell variability and multipotent plasticity	Possibility of stem cell variability and multipotent plasticity
Heterogeneity	Diverse heterogeneities/low homogeneity	Diverse heterogeneities/low homogeneity
Age-induced senescence	Unconcerned about age-induced senescence *in vitro*	Concerned about age-induced senescence *in vitro*
Environmental effects	Low risk of donor environmental effects (such as virus, bacterium, chemical toxins)	Risk of donor environmental affects (such as virus, bacterium, chemical toxins)
Therapeutic safety	Comparatively safe	Comparatively safe
Therapeutic efficacy	Relatively low therapeutic efficacy	Relatively low therapeutic efficacy
Therapeutic mechanisms	Lacking of precise therapeutic mechanisms	Lacking of precise therapeutic mechanisms
Cost for banking	Expensive for banking	Expensive for banking
Stem cell nomenclature	Controversial stem cell nomenclature	Controversial stem cell nomenclature

discussion regarding these relevant aspects of perinatal MSCs. The key issues have been identified to date, including the following.

Quality assurance before cryobanking for likely future personalized medicine

It is well known that functional properties and intrinsic multipotency of MSCs can be negatively affected by donor factors such as increased donor age, genetics, and health status. Banking of MSCs at their most potent state from perinatal tissues, the "best" cell source over one's lifetime, has been supposed for future use in need of regenerative therapies. Certainly, cryobanking of perinatal MSCs is a prerequisite in personalized medicine strategy; however, the great uncertainty remains concerning the final function of perinatal MSCs, the accessibility of MSCs (*e.g.*, a change in business circumstance), and therapeutic potential for the intractable diseases (*e.g.*, cancer) decades later. Due to no expiration date of the perinatal MSCs cryopreserved in biobanks, exploiting and developing new approaches to testing cellular variability and stability, functionality, and heterogeneity during the processing of preparation and banking of the end MSC products should be considered carefully for cell quality assessed over longer time periods. The quality assurance programs should be performed to ensure the quality of stem cell products during the whole banking process including the perinatal tissue collection, processing, testing, preparation, and storage, as well as additional analysis (Figure 1). It should be also considered that there should be minimal levels of differentiation of perinatal MSCs in the course of the overall culture period to preserve stem cells at their most multipotent state for future use. New quality control to meet the product standards is required to be developed during the process of banking of perinatal MSCs. Except for routine multiple experiments for cell quality assessment (*e.g.*, cell viability, proliferation, and differentiation potential), it is important to determine a panel of predicative *in vitro* tests for a system of quality assurance and these may be applied including but not limited to stem cell potency assay, spectral karyotyping, and genetic etiologies (Figure 1). Perinatal MSC potency assessment needs to be further validated for their therapeutic safety and effectiveness in the future. Specially, genetic etiologies associated with multi-factorial or monogenetic diseases may potentially influence stem cell safety. The wide analysis in identifying genetic/epigenetic etiologies is necessitated for therapeutic safety. For example, considering the importance of disease-associated individual single-nuleotide polymorphisms (SNPs), it is enabling to analyze and investigate whether stem cells carry disease-associated SNPs. Therefore, there is a critical need to further consider about whether it is worth biobanking of perinatal MSCs and whether it is to be stopped early if there is a family history of genetic conditions. Careful measuring and monitoring are extremely important before biobanking.

Reconsidering of pretreatment of perinatal MSCs before cryobanking

There are more and more *in vitro* and *in vivo* studies on pretreatment of MSCs, *e.g.*, pretreatment with cytokines or growth factors and hypoxia-priming, to improve their biological properties and therapeutic effectiveness. Acknowledging and understanding a beneficial role of pretreatment, the details given in this subsection describe the feature of pretreatment among MSCs from different source tissues including

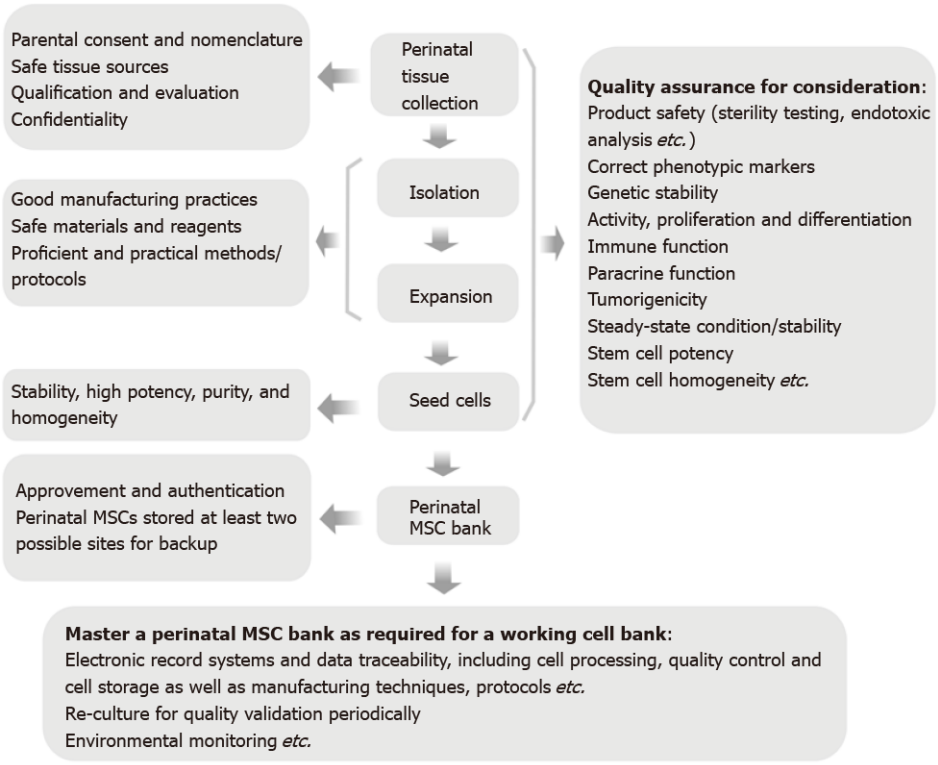

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Figure 1 Operating model of banking perinatal mesenchymal stromal/stem cells. Perinatal mesenchymal stromal/stem cells banking process includes the perinatal tissue collection, isolation, stem cell expansion, testing, preparation, and storage. The quality assurance programs are performed during the whole banking process. MSCs: Mesenchymal stromal/stem cells.

adult and perinatal sources, specifically, with the BM appearing the most common. This would be conducive to a better understanding of the stem cell therapeutic advantages and matters needing attention before banking of perinatal MSCs.

Biological properties could be altered by pre-conditioning of MSCs during *in vitro* expansion. Cell culture variables should be documented and, conventionally, growth medium containing fetal bovine serum and supplemented with basic fibroblast growth factor (bFGF) is considered to be the "gold standard" medium for primary human MSC expansion[25]. To remove animal components, serum-free medium throughout the process of cell preparation is being developed for clinical applications and MSC cryobanking. Usually, serum-free medium contains various cytokines/growth factors. Medium supplementation with bFGF is shown to increase the proliferation capacity of BM-MSCs over multiple passages[26]. Expansion is further improved in UC-MSCs by addition of bFGF in growth medium and the highest cell yield is detected in UC-MSCs among MSC lines pre-treated with bFGF including BM-MSCs and AD-MSCs during the whole culture period[27]. Additionally, despite the increased expression of HLA-DR induced by bFGF in AD-MSCs, the addition of bFGF in culture media seems not to affect the expression levels of HLA-DR in UC-MSCs and BM-MSCs in the course of passages[27]. Furthermore, the percentage of HLA-DR positive cells increases after inflammatory priming of all these three types of MSCs expanded in the bFGF-supplemented media[27]. As regards MSC differentiation, on the one hand, pretreatment with bFGF enhances BM-MSC chondrogenesis during chondrogenic pellet culture, resulting in upregulation of collagen type X and matrix metalloproteinase 13, the hypertrophic markers, in pellet cultures after 5 wk of endochondral ossification[26]. On the other hand, the supplementation with bFGF in medium has been also shown to have a negative effect on chondrogenesis of BM-MSCs[28,29] as well as their adipogenesis and osteogenesis[29,30]. Studies in laboratories demonstrate a time-dependent contribution of bFGF signaling to the reduced osteogenic/adipogenic differentiation of BM-MSCs throughout the culture[30,31]. For instance, the activity of alkaline phosphatase, a marker of osteogenesis, is significantly reduced during osteogenic differentiation in the case of addition of bFGF after passage 4 compared to the control at the same passage of MSCs and, similarly, adipogenesis potential is also significantly decreased by time during differentiation[30].

Pretreatment of MSCs has been observed to enhance the biological properties of the frozen and thawed MSCs. For example, one study reports that there are more apoptotic cells in the post-thawed cryopreserved BM-MSCs than the live BM-MSCs in active culture[32]. These frozen-thawed BM-MSCs are susceptible to complement-mediated lysis[33] and T-cell mediated apoptosis[34]. Interestingly, frozen-thawed MSCs primed by interferon-gamma (IFN-γ) in the culture medium for 48 h prior to

cryopreservation may partly avoid the lysis by activated T cells[34]. The mechanism of MSC-mediated immunosuppression has been previously proposed as IDO expression in BM-MSCs[35] as well as UC-MSCs[36] induced by IFN-γ. However, while pre-licensing MSCs with IFN-γ have been seen to enhance their IDO expression *in vitro*[37,38], the pre-licensed MSCs by IFN-γ, compared to the unstimulated MSCs, have let to the loss of their effectiveness in rescuing retinal ganglion cells in a retinal ischemia/reperfusion injury mouse model[37]. Together, the results of pretreatment with cytokines including but not limited to bFGF and IFN-γ indicate the different effects on the biological properties of MSCs depending on their existing microenvironment (*e.g.*, inflammation) or the specific pathological contexts.

Apart from pretreatment with cytokines, other approaches can also be proposed to boost the therapeutic potential of MSCs, for example, microenvironmental hypoxia-primed MSCs[39]. One previous study shows that, compared to standard 21% O_2 tension, 3% O_2 can increase clonogenic potential, *in vitro* migration, and stemness of MSCs from the Wharton's jelly (WJ) of human umbilical cord (WJ-MSCs)[40]. Another previous study indicates that umbilical cord blood (UCB)-derived MSCs (UCB-MSCs) primed with hypoxia and calcium ions exhibit improved self-renewal, migratory, anti-inflammatory and immunomodulatory capacities, resulting in further improving therapeutic potential for graft *vs* host disease in an animal model[41].

As mentioned above, in theory, pretreatment of MSCs from adult or perinatal sources with cytokines can improve their biological and functional properties. However, previous *in vitro* and *in vivo* studies could not prove the therapeutic efficacy of the freshly cultured or post-thawed MSCs with cytokine pre-treatment. Microenvironment conditions (*e.g.*, hypoxia) have become a promising strategy prior to cryopreservation, which is possible for the enhancement of MSC-based therapeutic potential. *In vitro* and *in vivo* studies indicate that pretreatment strategy may benefit patients in a disease specific context. However, without knowing the context of a specific pathology, it is not possible to assess and predict the function of perinatal MSCs pretreated with cytokines for stem cell medical treatments after many years. Therefore, whether pretreatment of perinatal MSCs should become the standardization for biobanking for their future applications as stem cell-based treatments remains to be further investigated.

Pre-selection for biobanking to decrease the heterogeneity of perinatal MSCs

As known, there are diverse heterogeneities of MSCs including inter-donor/individual, inter-tissue/source, inter-cellular, and inter-colony heterogeneities involved in the manufacturing and biobanking of MSCs. MSC heterogeneity reflects the diversity of MSC-associated environments or niches. The present paper extends the relevant discussion regarding the pre-selection required prior to banking with the aim to control product consistency for a stem cell bank for maximizing the "homogeneity" of perinatal MSCs. As well known, MSCs including adult and perinatal MSCs derived different tissues differ in their functional characteristics. Wegmeyer *et al*[42] conducted one previous study to evaluate the source- and donor-dependent differential stem cell properties and found UC-MSCs, AM-derived MSCs (AM-MSCs), and BM-MSCs exhibited comparable differences between each other. Interestingly, UC-MSCs and AM-MSCs exhibited different growth characteristics and morphologies as well as high inter-donor variability of AM-MSCs but not UC-MSCs. In contrast, another previous report indicated that, compared to UCB-MSCs, placental tissue MSCs, and WJ-MSCs, umbilical cord lining-derived MSCs showed the highest proliferation and migration rates and prolonged survival in immunodeficient mice [43]. Concerning immunosuppressive properties, in contrast to placenta MSCs, both UC- and BM-MSCs significantly reduced the proliferation of activated CD4+ and CD8+ T cells[44]. Additionally, the heterogeneous proangiogenic properties of perinatal and adult MSCs were observed in one previous study, which showed that both BM-MSCs and placental chorionic villi MSCs had significant proangiogenic activities on endothelial cells in matrigel *in vitro* compared to AD-MSCs and UC-MSCs[45]. Importantly, compared to dental pulp MSCs and AD-MSCs, WJ-MSCs showed the strongest therapeutic efficacy in reducing fasting glucose levels in type II diabetic mice in one previous *in vivo* study[46].

As mentioned above, due to inconsistency of MSCs, different perinatal MSCs populations may have heterogeneous functional properties, consequently leading to different therapeutic efficiency. Therefore, a pre-selection may be required before stem cell banking to identify relatively homogeneous perinatal MSCs for potential later use in life. As known, pretreatment with cytokines before banking may be a suitable approach to support therapeutic potential of perinatal MSCs in the future as exemplified by IFN-γ pre-licensing. Peltzer *et al*[47] conducted a previous study to create an *in vitro* "tolerogenic" niche priming mimicking placental environment, which showed that primed perinatal MSCs (UC-, UCB-, AM-, and chorionic-MSCs) with IFN-γ could enhance their immunomodulatory potential in a dose- and donor-dependant manner. This study by Peltzer *et al*[47] may suggest that an approach to screen the large number of perinatal MSCs with cytokine priming will be beneficial for the consistency of banking MSCs. Sorting of perinatal MSCs may be another effective approach for pre-selection, for example, pre-selection of MSC subpopulation using surface markers to increase the purity of the expanded MSCs. A series of markers need to be considered in sorting of perinatal MSCs, including cell surface markers related to MSC clonogenicity, potency, differentiation, and immunomodulatory properties[48]. Practically, a few conventional approaches to increasing the consistency of perinatal MSCs may be also considered, such as the use of the single colony of MSCs and specific biomaterials (see below), and the handing of MSCs by the standardized protocols for manufacturing consistency.

Biomaterial scaffold approach to maintain the maximal biological properties of perinatal MSCs

MSCs within the body have their own physical microenvironments or niches to maintain their unique biological properties. In order to mimic a specific stem cell niche, various biomaterials such as alginate, chitosan, collagen hyaluronic acid, silica gel, silk fibroin, poly(lactic acid), and graphene-base materials are being explored for use as scaffolds/surfaces supporting the growth and differentiation of MSCs *in vitro* and *in vivo*. By comparing the conventional techniques with monolayer culture of MSCs, biomaterial scaffold approach such as cell surface modification and encapsulation technology for tissue engineering exhibits improved MSC survival and proliferation, increased differentiation potential, and maintenance of stem cell stemness[49,50]. Further studies indicate that biomaterials can enhance the paracrine function of MSCs[51]. While technical challenges for development of biomaterials remain the degradability, viscoelasticity/elasticity, architecture property, and compatibility, the non-toxic 3D porous biomaterials are already widely used for delivery of encapsulated UC-MSCs or BM-MSCs in pre-clinical studies[52,53] as well as in clinical trials[54,55]. Interestingly, cryopreservation of microencapsulated stem cells in alginate hydrogel has been reported to suppress ice formation that contributes to the effect of cryoprotection[56]. Importantly, there are no significant differences in cell viability and multi-lineage differentiation potential between the MSCs post cryopreservation either encapsulated or without encapsulation[56]. Similar studies have been also conducted with MSCs cryopreserved in degradable hyaluronic-acid based hydrogel[57]. In addition, intravenous injection of freeze-thawed mouse MSC encapsulates with microgel into recipient mice shows similar levels of cell survival as fresh non-cryopreserved MSC encapsulates[58]. As mentioned above, previous and current studies are instructive to note that using special biomaterial scaffold may be considered as a suitable new strategy for banking of MSCs from different source tissues including but not limited to perinatal tissues.

Dimethyl sulfoxide and dimethyl sulfoxide-free cryoprotectants

Dimethyl sulfoxide (DMSO), as a key cryopreservation agent, is most often used in the cryostorage to protect cells from mechanical and osmotic stress due to the formation of ice crystals[59]. DMSO is also observed to have significant influence on the viability, phenotype, and proliferation of MSCs, as well as cellular epigenome, and to induce changes in cellular processes[60,61]. As such, low concentrations of DMSO and DMSO with combinations of non-cytotoxic biocompatible agents/substances are being explored for MSC culture and cryopreservation in preclinical and clinical studies. For example, one previous study demonstrated that human BM-MSCs modified to express tumor necrosis factor-related apoptosis inducing ligand were cryopreserved in a low concentration (5%) of DMSO (accepted as nontoxic concentrations of below 10%) with 95% human serum albumin without affecting their biological properties[62]. However, a question may be raised as to whether DMSO at a very low concentration may not be sufficient to prevent freezing damage to MSCs.

Currently, there are different types of effective and non-toxic cryoprotective agents/compounds used as suitable replacements for DMSO, such as glycerol, hydroxyethyl starch, trehalose, and dextran[59,61]. One study reports that DMSO-free cryopreservation solutions composed of sugars, sugar alcohols, and small-molecule additives have been showed to retain MSC post-thaw viability, cell surface markers, and proliferation and differentiation potential[60]. The osmolyte-based freeze solutions also exhibit a more normal alignment of the actin cytoskeleton of MSCs compared to DMSO frozen cells[60]. A non-toxic cryoprotective agent, a combination of trehalose and glycerol, has been tested in another study for cryopreservation of AD-derived stem cells, and the cells cryopreserved with this cryoprotective agent presented high cell viability and proliferation and migration capacity after thawing[63]. Overall, while various studies are being devoted to possible improvement of DMSO as a cryoprotectant, some alternatives to DMSO are being evaluated as cryopretectants for cryopreservation of MSCs from different tissue sources including perinatal tissues. DMSO together with alternatives to DMSO have not been tested enough to advocate their use for biobanking of MSCs stored over many years. Therefore, the use of DMSO alone as a cryopreservation agent may not insure the final functional properties of MSCs for stem cell-based treatments after many years and the new freezing solutions need to be intensively investigated for long-term therapeutic stem cell cryopreservation.

Ethical and regulatory concerns

Banking stem cells using neonatal birth-associated tissues or other related sources began with the establishment of banking cord blood for hematopoietic cell transplantation in the early 1990s[64]. Given that perinatal MSCs have the positive characteristics of both embryonic stem cells and adult stem cells, the ethical issue involvement may be related to the use of embryonic stem cells. In particular, there are further ethical and regulatory issues that will challenge banking of perinatal MSCs. In China, currently, there are so many exaggerate advertisements directly to consumers on banking of life-saving perinatal stem cells to insure infants or family members against serious illnesses in the future. Parents are encouraged to make decision for the preservation of their children' stem cells in a private stem cell bank for the future health of their children. Indeed, the current applications of MSCs as stem cell medical treatments are sometimes also exaggerated by the social media. Parents or requestors need to pay thousands of dollars for the commercial banks of perinatal MSCs as well as an annual fee for the maintenance of stem cells. Therefore, physicians and clinical investigators in hospitals who have

financial conflicts of interest associated with stem cell banking should disclose any financial relationship. Currently, there is a lack of clarity in country-level regulations in China for the management of stem cell banking and the guidance document for quality control on the banking of MSCs. Accordingly, technical and ethical guidelines are imperative for the appropriate governance and restriction of banking perinatal MSCs to insure standardization for MSCs with good quality for future stem cell therapies. Technical and ethical guidelines should also be in line with the international standardized regimens such as guidelines from the International Society for Stem Cell Research and the International Stem Cell Banking Initiative. Lastly, the most established private banks of perinatal MSCs in China, previously engaged in cord blood storage, are paid by the parents and these banks store the source of stem cells for future use by the donor and, probably, the donor's relatives.

Correspondingly, an additional challenge is whether the source of MSCs with potential applications in the future can be shared by the donor-unrelated recipients. In this regard, many public banks that store the perinatal MSCs derived from the donated newborn tissues should be established free charge of anything for banking. In accordance, these banks are open for research and they store stem cells for all recipients in future personalized medicine including the donors, the donor's families, and those unrelated with donors when they might be desperately in need due to life-threatening diseases. Due to no data available for the use of public banks of perinatal MSCs in China, clinical applications of cord blood stem cells from the banks are illustrated for the perspective in the future. One representative example is that the public cord blood banks have released at least 30 times for the usage rate of previously cryopreserved cord blood stem cells as compared to the private banks worldwide[65]. Almost over the past 30 years, cord blood banks have been successfully achieved for safe storage and rapid availability for cord blood stem cell-based treatments[66]. Another example is that, in a cord blood bank (MEDIPOST Co., Ltd., Seongnam-si, Gyeonggi-do, Korea), cell populations were evaluated and, among 557 UCB units, 128 units cryopreserved for more than 10 years were used for transplantation [67]. Therefore, the useful value of public or private stem cell banks to society or the donors is important for the stem cell medicine potential in the future.

Reculturing post-thawed cryopreserved perinatal MSCs from stem cell banks at the release time

Usually, the freshly cultured perinatal MSCs are seemingly to be more potent than freshly thawed cells in therapeutic properties of MSCs. Some studies suggest that perinatal MSCs may lose their functionality with cryopreservation, which could in turn affect the efficacy. Whether the characteristics of thawed perinatal MSCs *vs* cultured MSCs would be changed needs to be further discussed here. One previous study showed that freshly cultured UC-MSCs were superior to cryopreserved and thawed UC-MSCs in regard to cell viability[68]. Another previous study demonstrated that osteogenic and chondrogenic capacities were slightly reduced in cryopreserved human UC-MSCs for one year, compared to non-cryopreserved UC-MSCs from the same donor[69]. In contrast, one previous study showed that short-term cryopreservation and subsequent thawing of UC-MSCs could not alter the specific MSC surface markers and the proliferation capacity[70]. There was no obvious difference in fibroblast-like morphology and cellular viabilities between freshly frozen thawed and culture-rescued UC-MSCs[71,72]. Frozen thawed and culture-rescued UC-MSCs also displayed similar osteogenesis, chondrogenesis, and adipogenesis[71]. Similarly, one previous study by Narakornsak *et al*[73] to compare the post-thawed human AF MSCs (AF-MSCs) after one-month cryopreservation to the non-frozen AF-MSCs showed no statistical differences between these cells in MSC surface markers, cell proliferation, chondrogenic differentiation, and immune privileged properties. Still, one previous study to analyze immunomodulatory activity *in vitro* indicated no significant differences in the suppression of activated T cells in the freshly thawed UC-MSCs *vs* the freshly cultured UC-MSCs[70]. Comparatively, there were different observations reported by assessing the potential in immunosuppression between freshly thawed and cultured UC-MSCs[71], as showed the freshly culture-rescued UC-MSCs to be more potent in immunosuppression than the frozen-thawed UC-MSCs.

In animal experiments, immunomodulatory activity in an adjuvant-induced arthritis rat model and angiogenic potential using a mouse model for hindlimb ischemia were observed and *in vivo* studies showed no significant difference in immunomodulatory and angiogenic potential of freshly thawed and freshly cultured UC-MSCs[70]. Treatment with the freshly thawed UC-MSCs improved the regeneration of rotator cuff tendon in an animal model after injection at 2 and 4 wk, respectively, which was comparable to the therapeutic potential for tendon regeneration by the use of the culture-rescued UC-MSCs[72]. In a ventilator-induced lung injury animal model, the fresh UC-MSCs as well as the fresh BM-MSCs were found to enhance injury resolution and repair, while the cryopreserved UC-MSCs were comparably effective[74]. As mentioned above, these experimental and preclinical data were provided to support short-term cryopreserved perinatal MSCs (*e.g.*, UC-MSCs) for stem cell-based applications in translational medicine. However, long-term cryopreserved perinatal MSCs need to be further explored to achieve preclinical and clinical safety and efficiency after intervention. Accordingly, it is advisable to continuously culture post-thawed perinatal MSCs for at least an additional passage at the release time for restoration of stem cell biological properties and, meanwhile, it is also required to conduct extra assessment of safety and therapeutic effectiveness.

Short-term but not long-term cryopreservation of perinatal tissues for MSC preparation

Last but not least, a key issue has also been identified to date: Instead of banking perinatal MSCs, shot-term cryopreservation of perinatal tissues (*e.g.*, umbilical cord tissue) potentially for perinatal MSC storage for future stem cell-based treatments. Freezing of umbilical cords that yield post-thaw MSCs with high scalability is still controversial. One previous study reported that the frozen umbilical cord tissues for at least 2 wk were thawed and minced to generate a large number of MSCs by *in vitro* expansion[75]. UC-MSCs from cryopreserved and thawed umbilical cord tissues exhibited characteristics such as cellular phenotypes, immunosuppressive properties, and differentiation potentials similar to those of UC-MSCs from fresh tissues[75]. Another previous study reported that the fresh WJ cryopreserved for 30 d in liquid nitrogen can yield large numbers of post-thaw MSCs[76]. However, one previous study reported that although the cryopreserved umbilical cord tissue segments were thawed 5 years later to release MSCs after enzymatic digestion, those displayed poor recoveries and produced few viable UC-MSCs compared with fresh cord tissue samples[77]. Still, one previous study to assess the optimal isolation culture and cryopreservation methods to facilitate cord WJ-MSCs banking failed as no MSC cultures were obtained from the thawed cord WJ samples stored for 1 wk, 1 mo, or 6 mo in liquid nitrogen, respectively[78]. In addition, one study showed that cryopreservation of umbilical cord tissues for at least 1 mo in liquid nitrogen did not prevent the later collection of UC-MSCs by the explant method[79]. Currently, transplantation of UC-MSCs from fresh and short-term cryopreserved-then-thawed cord tissues is an issue of increasing interest. Similar to cord blood banks, perinatal umbilical cord tissue cryobanks are present in many other countries including United States, United Kingdom, Australia, and South Africa[80]. As a whole, given that cryopreservation cannot prevent the high cord quality, long-term restoration of perinatal umbilical cord tissue in banks may be unadvisable for potentially later use for post-thaw MSC preparation.

Collectively, when considering subsequent events, there are many limitations and uncertainties for stem cell medical treatments using perinatal MSCs banked over many years. Although most (if not all) aforementioned key issues, as summarized in Figure 2, are not specific to perinatal MSCs, such as cell quality insurance, heterogeneity, genetic etiologies, supplementation of scaffold, and use of cryoprotectants, these should be considered for the application of biobanking of perinatal MSCs. Research and development of different types of stem cell-based medication must be viewed with a forward-looking perspective. Importantly, when contemplating these issues including biological and technical concerns, this is a question as to whether any specific types of perinatal MSCs are worth biobanking. Without knowing the specific clinical applications, it is impossible to develop (*e.g.*, potency assay), and without a potency assay, it is not possible to assess the quality of perinatal MSCs stored by cryopreservation over many years. The question will always be raised as to what functions perinatal MSCs banked over many years can still actually play for the possible applications in their clinical practices. Realizing that short-term banking and restoration of perinatal MSCs is being routinely applied particularly in regenerative medicine and tissue engineering, long-term banking of perinatal MSCs as well as perinatal tissues for stem cell medical treatments within the lifetime should not be encouraged.

DISCUSSION

This opinion review focuses on several important points of view that are currently attracting people's attention. The review article does not diminish the clinical translational perspective of MSCs from either adult or neonatal tissues. Given the current situation of banking perinatal MSCs, the aim of this article is to contribute a balanced, comprehensive, and critical view in the settings of cryobanking of perinatal MSCs to probably maximize their potentially therapeutic activity in the future. As described above, there is no expiration date of cryostorage of the perinatal MSCs and it is not known about future timeline of stored perinatal MSCs for potential therapeutic applications. Therefore, the key issues have been identified in this article and, on the one hand, it is possible that the stored perinatal MSCs would be used for potential personalized medicine for a child when he/she develops a disease later on. On the other hand, it is too remote to assess the therapeutic benefit in the next decades. For example, by the time when a baby reaches the age of 50, no one knows whether these cells could be accessible and useful for personalized medicine at that time because there is a potential change for therapeutic benefit of perinatal MSCs. Although the short-term cryopreserved-then-thawed perinatal MSCs or the recultured MSCs are being widely used in pre-clinical and clinical studies, there are no data provided to support applications of long-term low-temperature storage of perinatal MSCs in banks, *e.g.*, cryopreservation of MSCs over a period of more than 10 years. Furthermore, it is not clear about how much the advanced techniques in science would be developed, for example, genome editing and precision medicine technologies to provide the best tools, probably, as opposed to MSC-based therapies looking forward 50 years. Science still drives the development of the advanced techniques and, eventually, the emerging advanced technologies are likely to influence the direction of therapeutic strategies in MSC-based translational medicine.

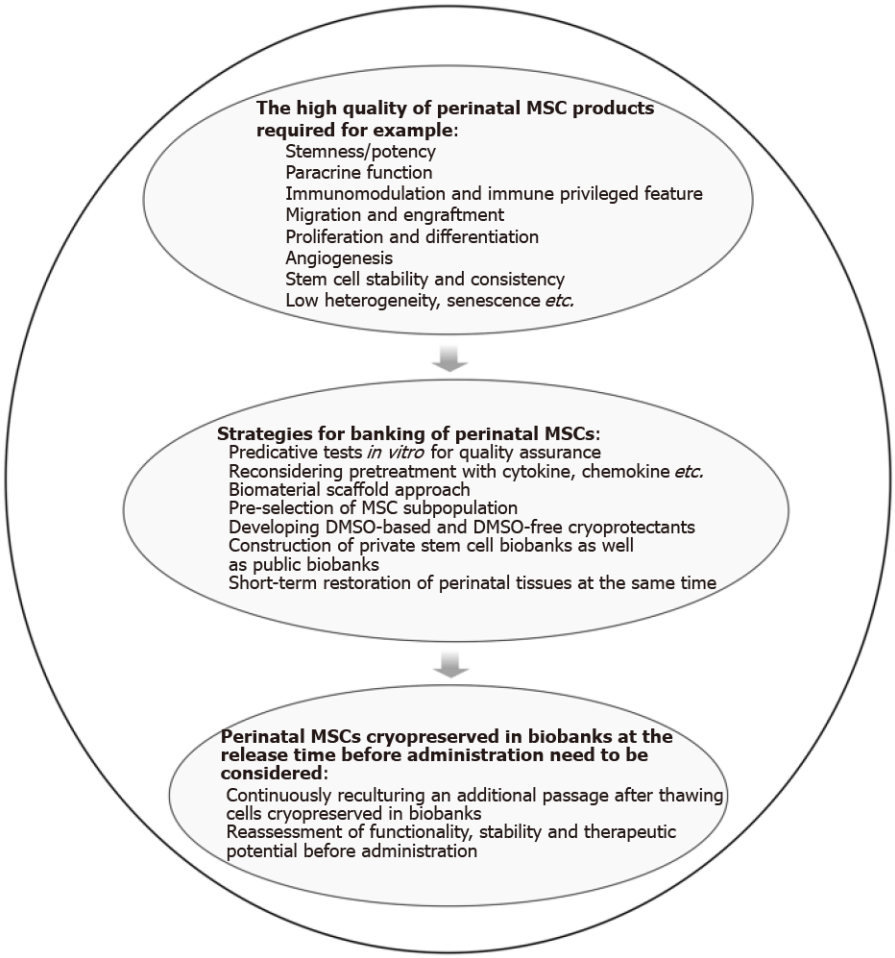

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Figure 2 Strategies and administrative requirements for high-quality stem cell banks. MSCs: Mesenchymal stromal/stem cells; DMSO: Dimethyl sulfoxide.

To date, banking perinatal stem cells and stem cell-derived newborn tissues have raised growing interest potentially for future stem cell applications. Due to less information available in scientifically understanding stem cell biology *in vivo* from the transplanted patients, such as stem cell proliferation, differentiation, immunomodulation, homing, and fate of MSCs, the true function and the precise mechanisms of the therapeutic benefit remain largely unclear. More importantly, given the unknown context of a specific pathology in the future, it should be noted as to whether perinatal MSCs are worth biobanking for a long period of time to achieve clinical efficiency. People believe that banking of perinatal MSCs may serve patients well one day in the foreseeable future, ideally for several years, when they may really need for the personalized medicine. But instead, the great uncertainties remain for potential use of the cryopreserved perinatal MSCs for stem cell-based treatments in the unforeseeable future. Acknowledging the limitations and uncertainties of banking perinatal MSCs for future potential personalized medicine, the following key recommendations should be addressed and the most of the recommendations proposed do not apply to the perinatal MSCs only.

For the sake of therapeutic safety and effectiveness in the future, a series of predicative tests *in vitro* for quality assurance as well as the additional analysis of genetic/epigenetic etiologies should be considered before cryobanking of perinatal MSCs. The novel strategic approaches, for example, biomaterial scaffold techniques, should be encouraged to use for maintaining the maximal biological and functional properties of perinatal MSCs in the routine banking practices.

Emerging practicable technologies would also be applied to yield a number of the desired stem cell types, *i.e.*, homogeneous and consistent perinatal MSCs, and, consequently, it is essential to develop novel biological technologies with a high yield of stem cells for cryobanking. The appropriate governance is required and banking of the perinatal MSC-based therapeutic products should comply with accreditation standards and the international standardized guidelines.

The public banks are to be built as a priority to better serve all recipients who might be desperately in need in the future and, therefore, the roles played by the public banks should not be underestimated. Lastly, instead of banking perinatal MSCs, short-term but not for long-term restoration of perinatal tissues, for instance umbilical cord tissue, may be suggestive of a possible approach of cell preparation

for stem cell-based medicine.

CONCLUSION

MSCs can provide therapeutic benefit given their unique biological characteristics, but, to date, there is still much to learn about stem cell science and medicine. MSCs derived from diverse tissues have their different functional properties and perinatal MSCs have their own advantages. Therapeutic properties of perinatal MSCs have been shown by multiple preclinical and clinical studies; however, banking of perinatal MSCs for personalized medicine in whole life remains to be unforeseeable. Therefore, it is extremely important for parents, physicians, and clinical investigators to be aware that there are limitations and uncertainties of banking perinatal MSCs for the future personalized medicine. Based on the above consideration, this opinion review article is conducted to address the concerns raised and provides several practical recommendations for banking perinatal MSCs for future potential personalized medicine. Accordingly, different strategic approaches can be employed in this rapidly growing field to improve this process, making perinatal MSCs available for future stem cell based therapies when needed to avoid banking poor-quality MSCs discarded as novel medical waste products in the future.

FOOTNOTES

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REVIEW

Obesity and cancer stem cells: Roles in cancer initiation, progression and therapy resistance

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Abstract

Obesity, the global pandemic since industrialization, is the number one lifestyle-related risk factor for premature death, which increases the incidence and mortality of various diseases and conditions, including cancer. In recent years, the theory of cancer stem cells (CSCs), which have the capacity for self-renewal, metastasis and treatment resistance, has been bolstered by increasing evidence. However, research on how obesity affects CSCs to facilitate cancer initiation, progression and therapy resistance is still in its infancy, although evidence has already begun to accumulate. Regarding the ever-increasing burden of obesity and obesity-related cancer, it is pertinent to summarize evidence about the effects of obesity on CSCs, as elucidating these effects will contribute to the improvement in the management of obesity-related cancers. In this review, we discuss the association between obesity and CSCs, with a particular focus on how obesity promotes cancer initiation, progression and therapy resistance through CSCs and the mechanisms underlying these effects. In addition, the prospect of preventing cancer and targeting the mechanisms linking obesity and CSCs to reduce cancer risk or to improve the survival of patients with cancer is considered.

Key Words: Obesity; High-fat diet; Cancer stem cells; Carcinogenesis; Metastasis

Core Tip: Obesity increases the incidence and mortality of various cancers; however, research on how obesity affects cancer stem cells (CSCs) is still in its infancy. In this review, we discuss the association between obesity and CSCs, with a particular focus on how obesity promotes cancer initiation, progression and therapy resistance through CSCs and the mechanisms underlying these effects. In addition, the prospect of preventing cancer and targeting the mechanisms linking obesity and CSCs to reduce cancer risk or to improve the survival of patients with cancer is considered.

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INTRODUCTION

For millions of years, humans and their predecessors have evolved under the pressure of undernutrition, which selects a genotype that enables overeating, low energy expenditure, a high degree of calorie absorption and efficient energy storage in adipose tissue[1]. Therefore, with the development of the social economy in the past few decades, overnutrition and an increasingly sedentary lifestyle tip the balance from a few calories consumed but more expended to more calories consumed but little expended, leading to the pandemic of excess body weight, which is mainly measured by body mass index (BMI). Over the past four decades, the prevalence of overweight and obesity has nearly tripled globally. Between 1975 and 2016, the worldwide prevalence of obesity increased from less than 1% to 6%-8% among children, from 3% to more than 11% among men and from 6% to 15% among women[2]. Based on data from the Global Burden of Disease (GBD) 2015, overweight or obesity affects over 2.1 billion people, or nearly 30% of the global population[3]. Obesity was estimated to increase the economic burden by approximately 2 trillion United States dollars, or 2.8% of the global gross domestic product, and to lead to the loss of an estimated 5-20 years of life expectancy, representing one of the most serious unmet public health challenges of the 21st century[4-6].

Malignancy, a set of diseases caused by the interplay between genetic and environmental or behavioral factors, ranks as the third leading cause of premature death and disability attributable to excess body weight worldwide following cardiovascular disease and type 2 diabetes mellitus[7]. Recent studies have demonstrated that excess body weight is associated with higher risks of several types of cancer, including esophageal adenocarcinoma, multiple myeloma, and cancers of the gastric cardia, colon, rectum, biliary tract system, pancreas, breast, endometrium, ovary, and kidney[8]. In 2019, the estimated number of high BMI-related cancer cases accounted for 4.59% and 4.45% of all cancer-cause deaths and disability-adjusted life years, respectively[9]. Obesity can not only increase the risk of tumorigenesis but also promote the progression and metastasis of developed cancer and can affect the therapeutic efficacy and survival of patients with cancer[10].

Regarding the altered biological processes that occur in the context of obesity that contribute to cancer, the majority of studies have focused on common themes, including inflammation, hypoxia, angiogenesis and altered energy metabolism, which influence the proliferation and survival of cancer cells[10]. However, in recent years, emerging challenges in cancer management have promoted the proposal of many theories to explain the initiation and progression of cancer; one of them is the hypothesis of cancer stem cells (CSCs), which has been bolstered by an accumulating body of evidence [11]. CSCs, also referred to as treatment-refractory, tumor-initiating cells, constitute a small subpopulation of cancer cells within tumors capable of self-renewal, which can divide and differentiate into various tumor cell types (intratumoral heterogeneity). They can secrete antiapoptotic factors, undergo epithelial-to-mesenchymal transition (EMT), and display a higher performance of drug efflux pumps. Therefore, CSCs are preferentially aggressive and pose a high risk of therapy resistance and disease relapse[11]. With the rapidly increasing incidence of cancer attributable to obesity, a better understanding of the roles of obesity in CSC biology is of paramount significance. However, research in this area is in its infancy. In this review, we discuss the association between obesity and CSCs, with a particular focus on how obesity promotes cancer initiation, progression and therapy resistance through CSCs and the mechanisms underlying these effects. In addition, the prospect of prevention and targeting mechanisms linking obesity and CSCs to reduce cancer risk or to improve the survival of patients with cancer is considered.

OBESITY AND CSCS IN CANCER INITIATION

In the context of an increase in the global prevalence of obesity, large-scale epidemiological studies have demonstrated a compelling increased risk of tumorigenesis in individuals with obesity, and several landmark studies have summarized this evidence. To evaluate the strength and validity of the evidence for the association between adiposity and the risk of developing or dying from cancer, an umbrella review of the literature comprising 204 meta-analyses of large studies with limited heterogeneity or evidence of bias was published in 2017, which concluded that the associations for 11 cancers were supported by strong evidence, while others could be genuine, but substantial uncertainty remains[8]. In 2016, data from a meta-analysis reported by the International Agency for Research on Cancer supported relative risks of 1.5 to 1.8 in obesity for these tumor sites[12]. In GBD 2019, 13 cancer types were also found to be affected by a high BMI[9]. Although some inconsistencies in cancer types contributing to obesity were reported across these studies, a consistent and compelling association has been demonstrated in many cancer types, including esophageal adenocarcinoma, multiple myeloma, and cancers of the gastric cardia, colon, rectum, biliary tract system, pancreas, breast, endometrium, ovary, and kidney (Figure 1). In the majority of these cancers, the CSC theory has been established in tumorigenesis. For example, in the intestine, inactivation of the adenomatous polyposis coli (APC) gene can lead to the rapid and lethal generation of adenomas in intestinal stem cells (ISCs) but not in non-stem cells[13]. Breast cancer was found to originate from a rare population of mammary gland progenitor cells, the depletion of which significantly impaired tumor growth[14]. Therefore, to increase the risk of these cancers, obesity may disturb the normal biology of stem/progenitor cells residing in these tissues, which is conducive to their transformation.

Except for compelling epidemiological evidence, no attempts have been made to investigate the biology of cancer-related adult stem cells in populations with obesity. Multiple animal models have been developed to recapitulate the effects of obesity or a pro-obesity diet on the initiation of cancer and have suggested that a high-fat diet (HFD) can promote tumorigenesis in the colorectum, prostate and liver[15-17]. However, the cellular origin of cancer was not defined in these studies. In recent years, research revealing the links between obesity or HFD and adult stem cells has increased (Table 1)[18-28]. Although discrepancies exist, the majority of these studies reported one or more of the following findings: Obesity or a HFD increases the depth or number of crypts in the intestine; non-stem cell progenitors in a HFD setting acquire stem cell attributes; the number and the capacity to form organoids of stem cells or progenitor cells are increased by a HFD; stem cells undergo autonomous changes in response to a HFD that poise them for niche-independent growth. Although studies exploring the initiation of carcinogenesis from these stem cells are very limited, the alterations in stem cells reported in these studies can predispose them to transformation. First, obesity or a HFD expands the pool of cells - both stem cells and progenitor cells - that can serve as the cellular origin of nascent cancers. Second, stem cells from mice on a HFD become functionally uncoupled from their niche in the organoid assay and *in vivo*, consistent with the hallmarks of cancer cells. Third, several studies have shown the possible links between these perturbations and tumorigenesis. For instance, when injected with azoxymethane, aberrant crypt foci (ACF), an early-appearing lesion of colon carcinogenesis, were increased in male mice fed a HFD[21]. In another study, more spontaneous intestinal low-grade adenomas and carcinomas were observed in HFD-fed mice than in standard diet-fed mice[19].

At present, elucidating how obesity and a pro-obesity diet contribute to the cellular origin of cancer in the intestine is the central focus of research, and data on stem cells in other tissues are very limited. Several reasons can explain such a tissue preference for studying the impact of obesity and HFD physiology on the initiation of cancer. First, robust epidemiological evidence has been accumulated for the increased risk of colon cancer in obese populations, and a better understanding of the altered biology of ISCs that occurs in the context of obesity will provide immeasurable public health benefits [10]. Second, the stem cell theory advanced most rapidly in ISCs, from which the histological architecture of the intestine has been well established[29]. Third, ISCs reside in the base of the intestinal crypt and directly interact with luminal nutrients, bacteria, and other intraepithelial and subepithelial cells, making the intestine an ideal system for studying the pathophysiological changes on a HFD[29]. Finally, the natural orifice of the intestine makes in situ manipulations for tumor induction or diagnostic tests easier than other *in vivo* cancer models[30]. Despite all of this, as consistent and compelling associations have been demonstrated between obesity and more than 10 cancer types, elucidating how stem cells in tissues other than the intestine are perturbed by obesity or HFD holds the same importance. Currently, the majority of obesity models are induced by HFD; however, other dietary patterns, such as a high-sugar diet and Western-styled diet, have also been shown to be obesogenic, and the effects of these dietary patterns on the initiation of cancer warrant further studies. Furthermore, the alterations in stem cell biology in tissues with increased cancer incidence warrant further investigation in obese human beings, not just in animals.

Table 1 Murine models of high-fat diet-induced obesity and cancer stem cells

Tissue	Findings	Ref.
Intestine	Increased crypt depth and villus height; increased number of Olmf4-positive ISCs; increased size of the enterospheres that developed from ISCs	[18]
Intestine	Increase in crypt depth; non-stem progenitor intestinal cells gain more stemness features and self-renewal; 50% increase in the number of Olfm4+ ISCs; 23% decrease in the number of Paneth cells; more likely to initiate mini-intestines; organoids had higher frequencies of Lgr5+ ISCs; ISCs by themselves had an increased capacity to initiate organoids	[19]
Intestine	Combined with Pten inactivation, obesity is insufficient to drive Lgr5+ ISC-derived tumorigenesis	[20]
Intestine	Increased aberrant crypt and crypt foci; increased proliferation of colonocytes per mouse	[21]
Intestine	Higher number of Lgr5+ stem cells per crypt	[22]
Intestine	Increased number of ISCs and progenitor cells; crypts are further likely to form mini-intestine organoids in a 3D culture	[23]
Intestine	Increased intestinal epithelial cell proliferation	[24]
Intestine	Reprograms Bmi1+ cells to function and persist as stem-like cells in mucosal homeostasis and tumor development	[25]
Intestine	Increased number of crypts; increased total numbers of ISCs and percentage of ISCs in S-phase; reduced numbers of Paneth and goblet cells	[26]
Lung	Increased number of AT2 cells; higher stem cell colony forming efficiency	[27]
Esophagus	Increased numbers of epithelial progenitors in Barrett's esophagus	[28]

ISC: Intestinal stem cell; Lgr5: Leucine-rich repeat-containing G protein-coupled receptor 5; AT2: Alveolar type-2.

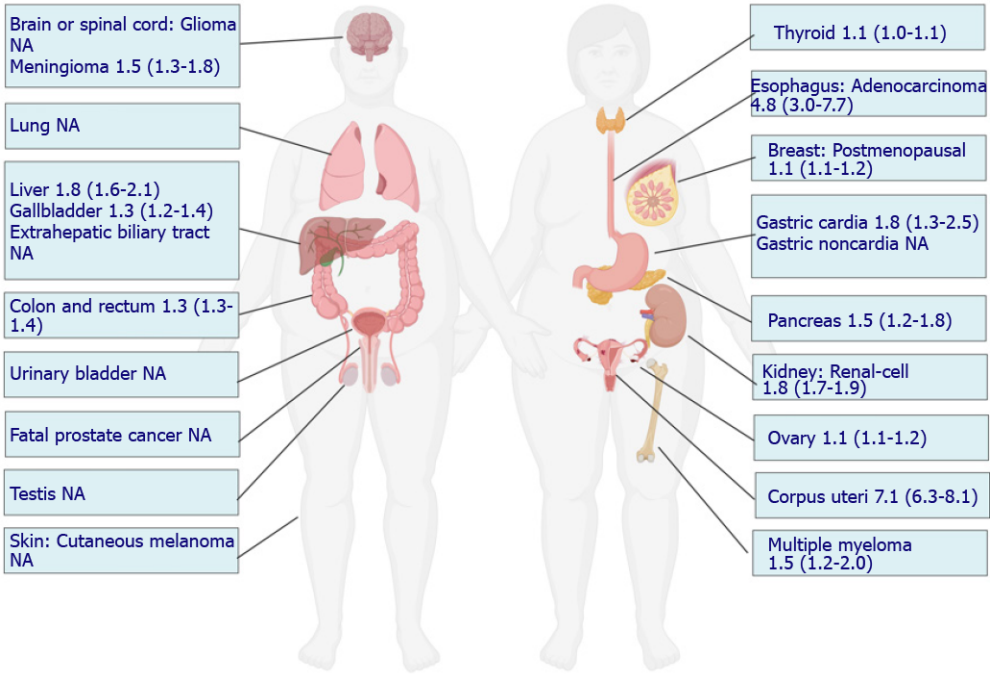

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Figure 1 Relative risk of individual cancers at high body mass index. Data were obtained from a meta-analysis reported by the International Agency for Research on Cancer working group. The number represents the relative risk and its 95% confidence interval of the highest body mass index category *vs* normal body mass index. NA: Not available.

OBESITY AND CSCS IN CANCER PROGRESSION AND THERAPY RESISTANCE

In addition to promoting tumorigenesis, obesity might also promote the progression of established cancers, affect the efficacy of present forefront antitumor therapies and shorten the survival of patients with cancer. For instance, a meta-analysis including 86490 patients treated for clinically localized prostate cancer showed a moderate and consistent relationship between obesity and biochemical recurrence, and there was a 10% increase in biochemical recurrence per 5 kg/m^2 increase in BMI[31]. In the Carolina Breast Cancer Study phase 3, a high waist-to-hip ratio was found to be associated with a

high risk of metastasis[32]. Poor survival was also reported in overweight or obese patients with colorectal, endometrial and breast cancer[33-35]. In addition to the increased likelihood of recurrence, the poor prognosis of obese patients with cancer also results from the reduction in the efficacy of antitumor therapies[36]. Mechanistically, the link between obesity and increased recurrence, therapy resistance and poor survival is likely multifactorial, with some differences related to more advanced stages being attributed to reduced participation in routine screening or the systemic effects of obesity on drug pharmacokinetics and metabolism[37,38]. In addition to these explanations, emerging evidence has shown that the activation of stem cell programs in cancers can lead to progression, metastatic growth and therapy resistance[11].

The key roles of obesity in the activation of stem cell programs have attracted much attention in recent years; however, as in studies on the effects of obesity on cancer-initiating cells, the promotion effects of obesity on cancer through CSCs are also mainly limited to animal models, which are utilized to investigate how specific obesity-related factors induce the stemness of cancer cells. Knowledge about CSCs in obese patients with cancer is still not clear. For example, obesity increases inflammation in the tumor microenvironment (TME) through local and systemic adipokines, proinflammatory cytokines or hormones, which modulate the stemness of cancer cells[39]. In a mouse model of hepatocellular carcinoma, diet-induced obesity increased inflammatory signaling *via* STAT3, and this finding was associated with larger tumors with a cancer-stem-cell-like phenotype[40]. Prolonged culture of breast cancer cells, which developed from a fat-rich environment, with adipocytes increased the proportion of cells expressing stem-like markers *in vitro* and the abundance of cancer cells with metastatic potential *in vivo*[41]. Regarding the involvement of CSCs in therapy resistance, leptin was found to interfere with the efficacy of 5-fluorouracil (5-FU) in colon tumor stem cells by increasing cell viability and reducing 5-FU-induced DNA damage[42].

EMT is a reversible cellular process during which epithelial cells transiently acquire mesenchymal phenotypes, such as an elongated, fibroblast-like morphology as well as an increased capacity for migration and invasion[43]. It is now widely accepted that EMT has well-established roles in cancer metastasis[43]. In the majority of carcinomas, only CSCs exhibit aspects of EMT-program activation[44]. Various extracellular stimuli, including obesity-related factors, have been implicated in the induction of EMT programs. For instance, esophageal cancer cells cocultured with visceral adipose tissue taken from obese patients resulted in the induced expression of genes involved in EMT, which was also noted in tumor biopsies from obese patients[45]. Cytokines and growth factors released by adipose stem cells (ASCs) can induce EMT-like changes in various cancer cells[10]. The adipokine leptin has also been found to activate EMT programs to enhance the proliferation and metastasis of breast cancer cells[46]. Therefore, obesity can propel primary tumor cells toward EMT events, leading to malignant progression.

THE LINKS BETWEEN OBESITY AND CSCS

As discussed above, CSCs participate in every step of tumorigenesis promoted by obesity. Understanding the key links between obesity and CSCs, therefore, offers important potential to decrease the incidence and improve the outcomes of obese patients with cancer. Several main factors are considered to connect obesity and cancer: Components of pro-obesity diets, metabolic and hormonal alterations associated with obesity, dysfunctional adipose tissue in the TME, low-grade obesity-related inflammation, self-renewal and stemness pathways, and microbiome dysbiosis. Each of these factors is intimately linked to and cross-talks with each other. For example, fatty acids in pro-obesity diets accumulate in adipocytes, leading to expansion and dysfunction of adipose tissue, which is intimately linked to endocrine and paracrine dysregulation, such as increased circulating insulin, insulin-like growth factor-1 (IGF-1) and leptin. All of these alterations activate and maintain a prolonged low-grade inflammatory state, predisposing individuals with obesity to an increased cancer risk and poor outcomes[47-49]. Although these factors were mainly investigated in nonspecific conditions, their involvement in CSC biology is also beginning to accumulate evidence (Figure 2).

Components of pro-obesity diets

Although the mechanistic links are not completely understood, nutrient-sensing signaling activated by components of a pro-obesity diet has been shown to influence stem cell behavior and tumorigenesis. This was also indirectly suggested in a leptin-receptor-deficient (*db/db*) mouse model, which becomes obese on control diets and does not rely on a HFD. In *db/db* mice, the number of ISCs was reduced, while ISC function was not affected, highlighting that components of a pro-obesity diet can regulate stem cells independently of obesity[19]. As early as 1989, Blakeborough *et al*[50] reported that diets high in fat support a *Bacteroides*-dominated colonic microflora and increase the excretion of secondary bile acids to augment free radical production, which may overcome the antioxidant defense mechanisms of stem cells, causing DNA damage, tumorigenesis and proliferation of transformed stem cells. Since then, numerous studies have suggested for decades that a pro-obesity diet engages many diverse pathways in stem cells in various tissues that collectively contribute to tumorigenesis. For instance, a HFD increases

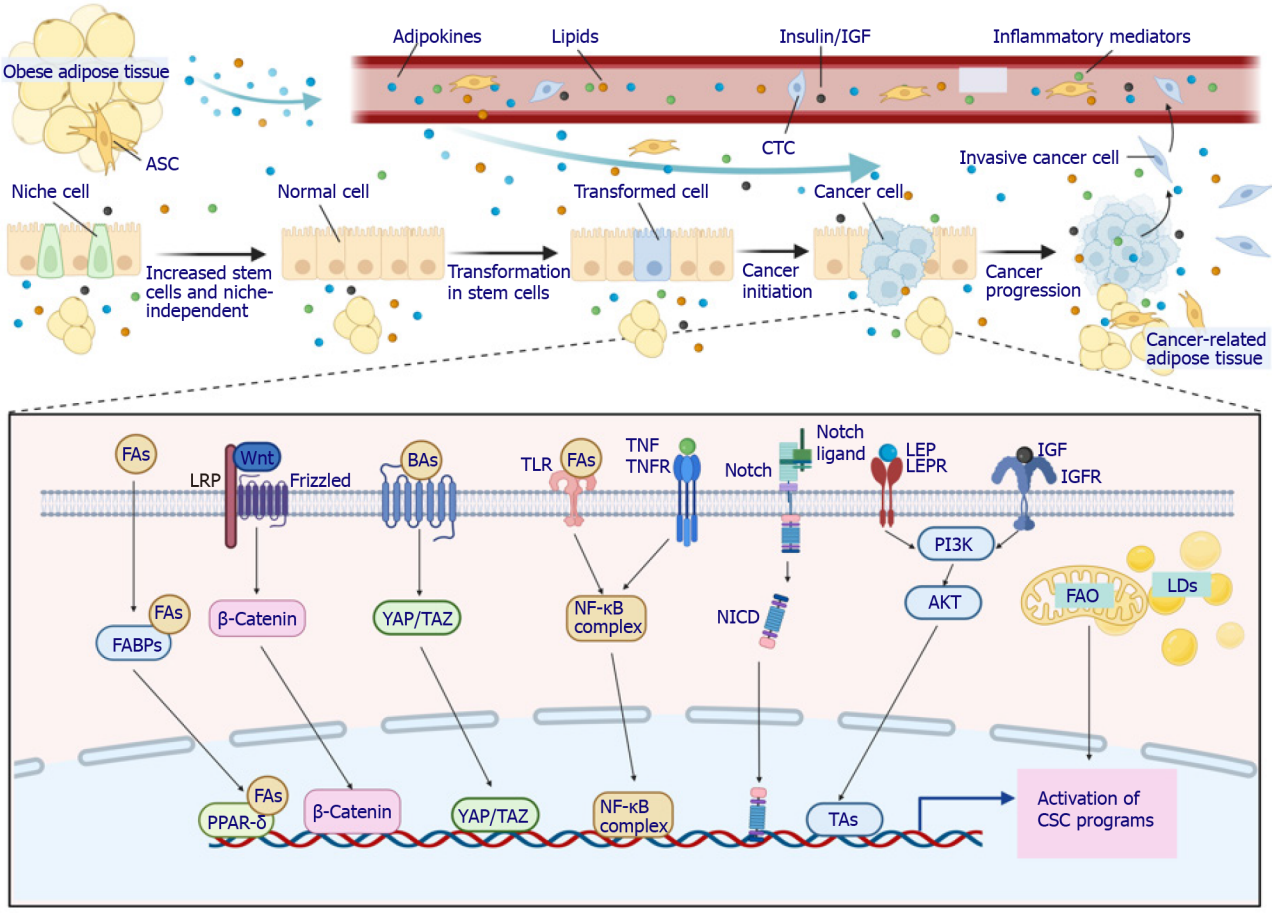

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Figure 2 Mechanisms linking obesity and cancer stem cells. In the obese microenvironment, the stem cell pool is increased and loses niche dependence, predisposing these stem cells to transformation. Several main factors are considered to connect obesity and cancer stem cells (CSCs): Components of pro-obesity diets, metabolic and hormonal alterations associated with obesity, dysfunctional adipose tissue in the tumor microenvironment, low-grade obesity-related inflammation, self-renewal and stemness pathways, and microbiome dysbiosis. These factors are intimately linked to and cross-talk with each other, synergistically leading to the activation of CSC programs through various signaling pathways. ASC: Adipose stem cell; BAs: Bile acids; CSC: Cancer stem cell; CTC: Circulating tumor cell; FAs: Fatty acids; FABPs: Fatty acid binding proteins; FAO: Fatty acid oxidation; IGF: Insulin-like factor; IGFR: Insulin-like receptor; LDs: Lipid droplets; LEP: Leptin; LEPR: Leptin receptor; LRP: Low-density lipoprotein receptor-related protein; NCID: Notch intracellular cytoplasmic domain; PI3K: Phosphatidylinositol 3-kinase; PPAR-δ: Peroxisome proliferator-activated receptor δ; TAZ: Transcriptional coactivator with PDZ-binding motif; TFs: Transcription factors; TLR: Toll-like receptor; TNF: Tumor necrosis factor; TNFR: Tumor necrosis factor receptor; YAP: Yes-associated protein; NF-κB: Nuclear factor-kappaB.

leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) expression and promotes tumor growth in a xenograft model independent of obesity. Mechanistically, dietary fats stimulate vitamin A-bound serum retinol-binding protein 4 and retinoic acid 6, which are implicated in colon stem cell self-renewal, to activate the JAK-STAT3 pathway and boost Lgr5 expression and the tumorigenicity of ISCs [51].

The representative components of a pro-obesity diet, that is, fatty acids such as palmitic acid or oleic acid, were found to enhance the number and self-renewal potential of ISCs and to permit organoid body formation without supporting signaling from their niche cells in a well-designed study[19]. The molecular mechanism by which fatty acids deregulate ISCs to promote tumorigenesis was also delineated in this study. It was proposed that fatty acids can be transported to the nucleus by fatty acid-binding proteins or can be produced directly by lipid metabolism in the nucleus, where they increase the number and self-renewal of ISCs *via* peroxisome proliferator-activated receptor δ (PPAR-δ), a nuclear receptor that senses fatty acid derivatives, the synthetic activation of which mimics both the *in vivo* and *in vitro* impact of a HFD and fatty acid treatment[19]. In contrast, loss of PPAR-δ completely abrogated the effects of fatty acids on Lgr5+ ISC function with respect to organoid-initiating capacity [19]. The downstream signaling mediating the effects of PPAR-δ activation was attributed to WNT/β-catenin, as demonstrated by increased β-catenin staining and upregulation of its target genes (Jag1, Jag 2 and Bmp4) in ISCs and progenitors from HFD- and PPAR-δ agonist-treated mice[19]. Free fatty acids produced by obese fat lipolysis also serve as ligands for Toll-like receptor 4 (TLR4) on cancer cells to activate nuclear factor-kappaB (NF-κB), leading to an increase in CSCs[52]. Another critical element in a pro-obesity diet that is significantly elevated in some obese individuals, cholesterol, was also demonstrated to affect stem cell function, thus promoting tumorigenesis. In *Drosophila*, dietary

cholesterol modulated the differentiation of ISCs by stabilizing the Delta ligand and Notch extracellular domain and altering their trafficking in endosomal vesicles, while a low-sterol diet slowed the proliferation of enteroendocrine tumors initiated by Notch pathway disruption[53]. In a rodent animal model, evidence also showed that dietary cholesterol acts as a mitogen for ISCs, while disruption of cholesterol homeostasis dramatically enhances tumor formation in APC_{min} mice[54].

Although bile acids are not elements in a pro-obesity diet, their excretion is essential for the digestion and absorption of dietary fat and is increased when a HFD is consumed. Bile acids are endogenous agonists of the G protein-coupled bile acid receptor, the activation of which augments Yes-associated protein 1 (YAP1) signaling, leading to increased stem cell number and proliferation and enhanced organoid-forming capability of ISCs[55]. In addition, bile acids, such as tauro-β-muricholic acid and deoxycholic acid, can antagonize intestinal farnesoid X receptor (FXR), a master regulator of bile acid homeostasis. Antagonizing FXR in the intestinal lumen enhances the proliferation and DNA damage of stem cells, initiating the transformation of ISCs to a malignant phenotype and promoting adenoma-to-adenocarcinoma progression[56]. Conversely, selective activation of intestinal FXR by its agonist can restrict abnormal ISC growth and skew differentiation toward goblet cells, thus curtailing HFD-induced intestinal cancer progression[56].

Metabolic alterations

Aerobic glycolysis has long been viewed as the main metabolic characteristic of cancer cells. However, in recent years, CSCs have been found to be intimately dependent on lipid metabolism to maintain their self-renewal capability. Therefore, in addition to the abovementioned studies that investigated the regulation of CSC function by lipids as elements of a pro-obesity diet, numerous studies have explored the effects of lipids on CSCs at the cellular metabolism level. Metabolism of fatty acids and cholesterol, including *de novo* biosynthesis, storage and fatty acid oxidation (FAO), supports the stemness, proliferation and chemotherapy resistance of CSCs[57]. Metabolic analysis demonstrated that lipid synthesis, including *de novo* lipid biosynthesis, lipid desaturation, and cholesterol synthesis, displays high activity in CSCs, indicating that lipid synthesis plays critical roles in stemness maintenance[58]. Human breast cancer-derived data suggest that FAO promotes cancer cell stemness and chemoresistance. Blocking FAO resensitizes them to chemotherapy and inhibits CSCs in mouse breast tumors *in vivo*[59]. Furthermore, cytarabine-resistant acute myeloid leukemia cells, which are enriched in leukemic stem cells, exhibited increased FAO[60]. FAO is also responsible for the stemness and chemotherapy resistance in gastric cancer induced by mesenchymal stem cells (MSCs)[61]. To meet the critical functions of lipids in CSCs, lipid droplets, organelles that store neutral lipids, accumulate and are more abundant in CSCs in numerous types of cancer[57]. Although evidence connecting lipid metabolism and CSCs is increasingly accumulating, whether obesity can augment the lipid metabolic alteration in CSCs is not clear because studies on the metabolic adaptations of CSCs in obese environments are limited. However, the incidence of hyperlipidemia is higher in obese populations, and in obese individuals, CSCs more readily reside in a fat-rich TME, which may provide more lipids to CSCs. Therefore, theoretically, lipid metabolic alterations in obesity support the stemness of cancer cells, although further studies are warranted to validate such effects.

Another common metabolic alteration of obese patients is insulin resistance, leading to increased levels of circulating insulin and IGF-1, which contribute to the increased risk and mortality of several cancers in obese individuals. Mice with diet-induced obesity exhibited increased concentrations of plasma glucose, insulin, and IGF-1, which were significantly correlated with increased proliferation and self-renewal of ISCs, as well as decreased Paneth cell numbers[26]. In addition, insulin significantly increased the capacity of organoid formation *in vitro*[26]. Reports have suggested that the PI3K/AKT pathway is the major contributor to the abnormal renewal of ISCs endowed by insulin/IGF-1[62]. Therefore, insulin/IGF-1 signaling was suggested to mediate the effects of obesity on the function of stem cells, which is conducive to their transformation. Even insulin/IGF-1 levels in newborns are associated with the risk of future breast cancer, possibly resulting from an increased total number of stem cells[63]. Parallel to their function in normal stem cells, evidence suggests the roles of insulin/IGF-1 in cancer progenitor/stem cells from solid and hematopoietic malignancies. Insulin/IGF-1 and their receptors are overexpressed or overactivated in human thyroid, hepatic and breast CSCs and participate in the self-renewal, EMT and chemoresistance of cancer cells[64]. These emerging discoveries will undoubtedly promote renewed efforts aimed at targeting the insulin/IGF-1 system that contribute to CSC biology.

Hormonal alterations

Adipose tissue has long been viewed as an energy reservoir; however, this perspective has changed in recent years, as numerous bioactive adipokines, including more than 50 different metabolic and hormonal factors, cytokines and chemokines, were reported to be released by adipose tissue[65]. Two of the major adipose tissue-derived hormones are leptin and adiponectin, which have opposite effects. In contrast to lean adipose tissue, which mainly secretes the antimitogenic adipokine adiponectin in obesity, increased preadipocytes yield high levels of leptin, which has proangiogenic and promitogenic effects[66,67].

Leptin acts as a growth factor for many tissues, such as the mammary gland, lung, liver, and colonic epithelium[68]. The links between leptin and CSCs have been comprehensively studied in breast cancer [69]. In a diet-induced obese mouse model, mammary epithelial polarity was disrupted, which can contribute to overactivation of the PI3K/AKT pathway downstream of the paracrine effect of leptin expressed by neighboring adipocytes. Leptin expands the pool of stem/progenitor cells in the breast epithelium and causes mitotic spindle misalignment, which is an early step in tumor initiation[70]. The leptin receptor was found to be expressed on breast CSCs, and in orthotopically transplanted breast cancer, leptin can promote CSC enrichment[71,72]. Inactivation of the leptin receptor attenuated the expression of CSC transcription factors and reduced the self-renewal of cancer cells in tumor sphere assays[71]. Leptin-mediated cancer initiation, progression and therapy resistance through CSCs in other cancers have also been extensively investigated[69]. For instance, leptin was found to initiate the early transformation of colon cancer. ACF multiplicity, as early-appearing lesions of tumorigenesis, was increased by a HFD in *ob/ob* mice or in a genetic mouse (*db/db*) model with leptin receptor deletion[73, 74]. However, in *db/db* mice fed a control diet, the function of ISCs and the activity of PPAR-δ and Wnt/β-catenin signaling were not changed, indicating that obesity and elements in a pro-obesity diet may cause different alterations in the function of stem cells[19]. In addition, no leptin receptor was found on colonic stem cells, and leptin did not increase the pool of Lgr5+ stem cells, suggesting that leptin may be dispensable in the early stages of colon carcinogenesis[22]. Collectively, these findings indicate that although the crucial role of leptin in CSCs may be affected by the cellular origin of cancer, the potential of leptin pathways in cancer initiation and progression will lead to future areas of therapeutic management.

Although data are scarce, other adipokines with altered secretion in obese adipose tissue were also shown to affect the function of CSCs. For example, DeClercq *et al*[22] specifically investigated the effect of a HFD on colonic stem cell maintenance during cancer initiation and found that the number of stem cells and their proliferation capacity were significantly increased, while the incidence of apoptosis was decreased. The authors proposed that these effects are the result of decreased adiponectin signaling based on the findings that the reduction in stem cell number and increase in apoptosis were diminished in organoid cultures from obese mice treated with an adiponectin receptor agonist[22]. In addition, following a decrease in adiponectin signaling, obesity can increase tumorigenesis in the intestine[22]. Resistin, another adipokine, was highly associated with the transcription of genes related to CSCs in low malignant breast cancer cells and noncarcinogenic breast epithelial cells[75]. These adipokines with different effects on CSCs and their therapeutic translational potential need further research.

Dysfunctional adipose tissue in the TME

Despite the systemic effects of adipose tissue on CSCs through the secretion of circulating metabolic and hormonal factors, adipose tissue also constitutes an important part of the microenvironment of several cancers, and its dysfunction resulting from obesity is considered a critical determinant of cancer progression[76]. For example, cancer-associated adipose tissue obtained from obese patients with breast cancer was found to increase inflammatory breast cancer aggressiveness *via* the regulation of CSC markers[77]. Coculture of breast cancer cells with human-derived adipocytes increased the abundance of mammosphere-forming cells and stem-like cancer cells *in vitro* and increased tumor-initiating cells and metastasis in mouse models[41]. Mechanistic investigations demonstrated that immature adipocyte contact activates Src, thus promoting embryonic stem cell transcription factor upregulation, including Sox2, c-Myc, and Nanog, to mediate CSC expansion[41]. Moreover, Sox2-dependent induction of miR-302b further stimulated c-Myc and Sox2 expression and potentiated cytokine-induced CSC-like properties[41]. However, adipose tissue from different anatomical sites may have different effects on CSCs. For example, serial transplantation of pluripotent stem cells cultured in conditioned medium of breast cancer cells into mammary fat pads evoked the same features of breast cancer, while this result was perturbed following subcutaneous transplantation, indicating that mammary adipose tissue can synergize with secretory factors produced by cancer cells to transform normal cells into CSCs, while subcutaneous adipose tissue cannot[78]. Such performance differences may be caused by the various metabolic characteristics of adipose tissue from different anatomical sites determined by sex steroid hormones[79].

Among various adipose tissue cell types, ASCs are key players in adipose tissue. Under obese conditions, the biology of ASCs is dramatically altered, and ASCs can be recruited to sites of inflammation, including tumors[80]. ASCs are able to produce a large variety of circulating growth factors, cytokines and adipokines, which play important roles in CSC function. In addition to their systemic effects, ASCs represent an important cellular component in the TME. Therefore, in a breast cancer patient-derived xenograft model, cancers grown in the presence of ASCs had increased numbers of CD44+CD24− CSCs in the peripheral blood and had a higher tendency to form metastases[81]. This effect may be mediated by leptin, as the stable knockdown of leptin in obese ASCs led to a significant reduction in circulating CSCs[81]. In addition, ASCs reshape the TME and support the generation of CSCs, which are associated with radioresistance and chemotherapy resistance[82].

Low-grade obesity-related inflammation

In recent decades, the contribution of inflammation to cancer initiation, progression and therapy resistance has regained enormous interest, and the association between inflammation and CSCs has also been explored extensively[83]. Obesity has long been considered a facilitator of mild, chronic, systemic inflammation. Along with the expansion of adipose tissue in obesity, hypoxia causes adipocyte stress and malfunction, recruiting different types of immune cells[84,85]. Both adipocytes and immune cells release numerous adipokines, cytokines, chemokines and hormones, which perpetuate the inflammatory state[84,85]. Therefore, it is reasonable to speculate that obesity can affect CSCs through low-grade inflammation. Although chronic inflammation is not induced consistently in obese mouse models, which may be related to the differences in feeding patterns and other combined interventions, current evidence indicates that inflammation might have a role in alterations in stem cells leading to tumorigenesis. Inflammatory mediators were found to be increased in the intestinal mucosa in mice with obesity or those fed a HFD, resulting from cytokine release by myofibroblasts and immune cells[86]. Local inflammation has been demonstrated to expand colon cell progenitors or stem cells and to induce their proliferation in the intestine. For example, HFD-induced obesity elevated both the colonic proliferative zone and stem cell zone in a pig model, and the proliferative zone was associated with an increase in the innate inflammatory markers TLR4, NF-κB, IL-6, and lipocalin-2[87]. In addition, activation of NF-κB, the central pathway downstream of the majority of proinflammatory cytokines, following local inflammation enhanced the reprogramming of non-stem enterocytes to acquire stem-cell-like properties, which expanded the pool of stem cells and generated tumor-initiating cells[88]. HFD-induced obesity promoted the phosphorylation of GSK3β and then increased the nuclear translocation of β-catenin, thereby activating the expression of WNT signaling target genes[89]. These effects were diminished by the deletion of tumor necrosis factor-alpha (TNF-α), indicating the role of inflammation induced by TNF-α in colon tumorigenesis associated with obesity[89]. Another inflammatory mediator, prostaglandin E2, was also found to be elevated by HFD in the circulation or in local tissues, leading to an increased number and division rate of stem cells[22].

Other proinflammatory mediators have also been demonstrated to facilitate CSC expansion. For example, IL-6 can induce malignant features in human ductal breast carcinoma stem/progenitor cells [90]. IL-8 treatment leads to breast cancer cells partially acquiring some stem-like cell attributes, thereby increasing their aggressiveness[91]. Chemokine (C-C motif) ligand 2, derived from cancer-associated fibroblasts, stimulates the stem cell-specific, sphere-forming phenotype in breast cancer cells and CSC self-renewal[92]. Although these studies were not carried out under obese conditions, these proinflammatory mediators are consistently elevated in obese individuals and are upregulated upon contact with cancer cells. The enrichment of CSCs may be partially attributed to these cytokines, as a few studies indeed found that proinflammatory cytokines, including IL-6, IL-8 and monocyte chemoattractant protein 1, are overexpressed in cancer-associated adipose tissue from obese patients and induce the stemness of cancer cells, while such effects were not found in nonobese patients[77]. Considering the importance of localized and systemic inflammation in the induction and maintenance of stemness in cancer cells and the definite association between inflammation and obesity, elucidating how these inflammatory pathways increase the risk of cancer incidence, progression and therapy resistance *via* CSCs holds great promise to decrease the burden of cancer in obesity.

Self-renewal and stemness pathways

Stem cells are proposed to reside in a distinctive microenvironment, that is, the stem cell niche, which induces and maintains the self-renewal and differentiation of stem cells. In the TME, niche signals also play critical roles in cancer cells acquiring more stemness. Although the pathways responsible for establishing a CSC phenotype are diverse and differ among cancer entities, developmental signaling pathways, including the Notch, WNT, Hedgehog and Hippo pathways, are commonly altered in CSCs and interact with each other and with other common oncogenic signaling pathways and have key regulatory functions that support the maintenance and survival of these cells, making them prime targets for anti-CSC therapy[82]. Obesity, HFD and abnormal adipocytes may engage in these self-renewal and stemness pathways directly or indirectly through increased local and systemic levels of many cytokines and adipokines. For example, in ISCs and progenitors from mice fed a HFD, the expression of Jag1 and Jag2, which are ligands for the Notch pathway and are normally expressed by neighboring niche cells, was increased by the activation of WNT/β-catenin, indicating that a HFD drives ISCs to niche independence[19]. Within the ISC niche, MSCs were expanded and secreted predominant levels of Wnt2b in the colon of HFD-fed mice, which promoted the growth of tumorigenic properties and accelerated the expression of CSC-related markers in colon organoids[93]. CSCs isolated from obese mice also exhibited enhanced Notch2 expression[94]. However, such direct evidence supporting the association between obesity and alterations in stemness pathways is scarce, and more studies are needed to test this model. Nonetheless, some emerging data demonstrate alterations in these stemness pathways in obesity-induced cancers. For instance, HFD consumption could upregulate the expression of β-catenin proteins in a mouse xenograft tumor model[95]. Notch signaling activity was increased in breast cancer cells following coculture with obesity-altered ASCs[96]. In addition, YAP, the major player in the Hippo pathway, dictates mitochondrial redox homeostasis to facilitate obesity-associated breast

cancer progression[97]. Although the contributions of CSCs were not examined in these studies, regarding the definitive effects of these signaling pathways on CSCs, it is reasonable to speculate that the upregulated activity of these pathways in obesity may promote the progression of cancer through CSCs and that targeting these pathways may be more promising in obesity-induced cancers.

Microbiome dysbiosis

The epithelial barrier surfaces of our body host a diverse microbial community, or microbiota, that is composed of a variety of microorganisms, such as bacteria, fungi, and viruses[98]. Substantial studies have reported that obesity or HFD markedly affects the composition of the commercial microbiota, especially in the intestine[48]. Obesity-induced perturbation of the gut microbiota has been demonstrated to influence stem cell phenotypes. For example, structural changes in the microbiota were associated with HFD-induced myeloid progenitor skewing of the differentiation capacity of hematopoietic stem cells[99]. The intestinal tract bacteria *Lactobacillus* induces the release of adiponectin by niche cells through NF-κB activation[100]. In pigs with HFD-induced obesity, the elevation of the proliferative zone and stem cell zone was associated with increased abundance of the gut bacterial phyla *Proteobacteria* and *Firmicutes*[87]. In addition to these initial data suggesting the association between obesity-related microbiome dysbiosis and the possible development of CSCs, evidence linking the microbiome in obesity to CSC function is lacking. Whether obesity-induced alterations in the composition of the host microbiota affect initiation, progression and therapy resistance and the underlying mechanisms need more investigation with the hope of providing more strategies for cancer prevention and treatment.

CLINICAL SIGNIFICANCE

If the above-discussed links between obesity and CSCs are founded on convincing evidence, an obvious question is whether targeting both can prevent or improve the outcomes of cancer. At present, targeting both obesity and CSCs has great challenges; however, progress is gradually being made. For example, bariatric surgery has been popularized globally, leading to more weight loss and longer maintenance than diet and lifestyle changes[101]. Intriguingly, in these patients with obesity who received bariatric surgery, a decrease in overall cancer diagnoses was observed[102,103]. However, surgical intervention does not guarantee the recovery of obese patients to a normal state and is typically a harmful method. Theoretically, obesity prevention represents the most promising and scientific solution, which requires joint efforts and cooperation from throughout the whole world[104]. However, under obesity pandemic conditions, exploring strategies to lower the incidence of obesity-related cancer represents the primary goal. Unfortunately, no experience has been gained. As low-grade inflammation plays a central role in linking obesity and cancer, anti-inflammatory therapy may be a promising direction, which has been validated in the prevention of colorectal cancer[105]. For obese patients with established malignancies, there is an urgent need to improve therapeutic efficacy and long-term survival. As mentioned above, various mechanisms have been proposed to link obesity and CSCs; thus, whether blocking these connections can prevent or delay the initiation and progression of cancer needs further study. Encouragingly, such strategies have been explored extensively, and some of them have already advanced into clinical use. For example, the clinical development of therapeutics targeting CSC-associated developmental signaling pathways has resulted in improved patient outcomes[82]. Some inflammatory factor-targeting therapies also show promising results in improving outcomes in patients with cancer[106]. Lifestyle interventions, such as reduced dietary intake and increased physical activity, were demonstrated to cause weight loss, leading to altered expression of inflammatory factors and maintenance of stem cell homeostasis[107,108]. Therefore, what awaits us next is to validate their efficacy in obese people with cancer.

CONCLUSION

As stated above, notwithstanding the clear and compelling link between obesity and CSCs, as well as an understanding of the mechanisms connecting them, this research area is still in its infancy. Most scientific research exploring the association of CSCs and obesity originates from mouse models or was inferred indirectly from the conclusions of different research fields. Therefore, there are still many major questions waiting for answers. For example, how can the profound differences in cancer incidence across different anatomical sites influenced by obesity be explained? Do the differences in the microenvironment across adult stem cell niches contribute to these variations? Regarding alterations in stem cell biology, how much overlap is there among animal models and obese patients? What role does the microbiome play in mediating the induction, maintenance and therapy response of CSCs? Does obesity differentially regulate normal and malignant stem cells, and how does it do so? How does obesity influence the crosstalk between CSCs and immunoediting? To what extent are stem cells conditioned in

obesity reversed when obesity is improved? Does obesity enhance the establishment of premetastatic niches? Can strategies aimed at targeting the mechanisms linking obesity and CSCs prevent the initiation and delay the progression of cancer?

Despite a wealth of unknowns, it is clear that obesity increases the stem cell pool and induces biological modulation in these cells, which predisposes these stem cells to transformation. Regarding the increased prevalence of obesity and its convincing association with cancer, programs are urgently needed to decrease the incidence of obesity. At this time, primary obesity prevention through public health policies, including dietary and lifestyle changes, represents a compelling approach toward a reduction in the incidence of obesity and its associated cancer. Identifying obese patients with increased cancer risk and developing appropriate management of obesity or applying cancer prevention methods such as anti-inflammatory agents in these populations represents another compelling approach toward a reduction in the burden of cancer in obesity. Several mechanisms have been proposed to explain the association between obesity and CSCs, and large amounts of agents targeting these pathways have been developed. Testing them in patients with obesity and comparing their efficacy with that in nonobese individuals are important components of future translational research and clinical trials.

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FOOTNOTES

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REVIEW

Clinical application prospects and transformation value of dental follicle stem cells in oral and neurological diseases

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Abstract

Since dental pulp stem cells (DPSCs) were first reported, six types of dental SCs (DSCs) have been isolated and identified. DSCs originating from the craniofacial neural crest exhibit dental-like tissue differentiation potential and neuro-ectodermal features. As a member of DSCs, dental follicle SCs (DFSCs) are the only cell type obtained at the early developing stage of the tooth prior to eruption. Dental follicle tissue has the distinct advantage of large tissue volume compared with other dental tissues, which is a prerequisite for obtaining a sufficient number of cells to meet the needs of clinical applications. Furthermore, DFSCs exhibit a significantly higher cell proliferation rate, higher colony-formation capacity, and more primitive and better anti-inflammatory effects than other DSCs. In this respect, DFSCs have the potential to be of great clinical significance and translational value in oral and neurological diseases, with natural advantages based on their origin. Lastly, cryopreservation preserves the biological properties of DFSCs and enables them to be used as off-shelf products for clinical applications. This review summarizes and comments on the properties, application potential, and clinical transformation value of DFSCs, thereby inspiring novel perspectives in the future treatment of oral and neurological diseases.

Key Words: Dental follicle stem cells; Oral disease; Neurological disease; Tissue engineering; Regeneration; Immunoregulation

Core Tip: This review is intended to summarize and comment on the properties, application potentials, and clinical transformation value of dental follicle stem cells (DFSCs). Stem cells derived from dental SCs (DSCs) originating from the craniofacial neural crest exhibit dental-like tissue differentiation potentials and neuro-ectodermal features, making them a promising alternative for the treatment of oral and neurological diseases. Moreover, in contrast to other DSCs, DFSCs from the early-developing tissues exhibit a number of superior properties, including larger tissue volume, higher cell proliferation rate, more similar biological profiles to progenitor cells of origin, and better anti-inflammatory effects, *etc.* These advantages are part of the critical mechanism by which DFSCs exert therapeutic effects and are relevant for large scale scaling and industrial generation for clinical applications. Moreover, cryopreservation preserves the biological properties of DFSCs and enables them to be used as off-shelf products for clinical applications. Therefore, DFSCs could have great clinical prospects and translational value in oral and neurological diseases with natural advantages.

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INTRODUCTION

Stem cells are undifferentiated cells characterized by the ability of self-renewal, clonality, and differentiation into various types of cells[1]. With the development of cell biology and modern medicine, the application of stem cells has brought a new approach for restoring tissue defects and treatment of some refractory diseases[2,3]. To date, regenerative medicine has become an important branch of modern medical science and has played an increasingly important role in clinical treatment. Stem cells are a vital element of regenerative medicine, and different stem cell types have their own advantages and drawbacks[4]. With stem cell research deepening, it is crucial to explore the appropriate stem cells to solve clinical problems and obtain better clinical outcomes.

Stem cells for application in regenerative medicine are divided into the following two categories: Pluripotent and multipotent. Pluripotent stem cells include natural embryonic stem cells (ESCs) originating from the inner cell mass of the blastocyte and artificially induced pluripotent SCs (iPSCs)[5]. Multipotent stem cells refer to adult stem cells that exist in different tissues of the body, and their main function is general homeostasis and repair of injured tissues by differentiation[6]. Adult stem cells are also known as postnatal stem cells and mainly originate from either epithelial cells or mesenchymal cells [4].

Mesenchymal SCs (MSCs) are typical adult stem cells derived from mesenchymal tissues. In addition to the ability of self-renewal, high proliferation, and multidirectional differentiation, MSCs also present high immunomodulation and antiapoptotic capacity, to achieve the purpose of promoting tissue regeneration and disease treatment. Compared with ESCs, MSCs are multipotent but limited in terms of differentiation ability. However, the acquisition and clinical application of ESCs are also dramatically restricted to ethical, legal, safety, and source constraints[7]. Moreover, since iPSCs were first generated in 2006 by Takahashi *et al*[8] with four factors, a growing number of researchers have focused on the clinical application prospects and transformation value of iPSCs and their specialized differentiation cells. Nevertheless, iPSCs also present some worrying aspects as follows: (1) iPSCs have pluripotency, similar to ESCs, as well as the ability to cause possible teratomas while the specialized differentiated final product contains undifferentiated cells[9]; (2) there is possibility of tumor formation by integrated oncogenes, insertional mutagenesis, and disrupting tumor suppressor genes[9]; (3) epigenetic memories and genomic aberrations have been detected in reprogrammed cells[10]; and (4) human skin fibroblast-derived iPSCs have a 72% ultraviolet light-related damage, and human blood-derived iPSCs have a high prevalence of acquired BCL6 corepressor mutations (26.9% of lines)[11].

The first human trial using retinal pigment epithelium derived from iPSCs for the treatment of age-related macular degeneration was started in Japan in 2014 but was later suspended[12]. Recently, another study has reported a distinctive case of immature teratoma after the patient underwent autologous iPSC-derived cell therapy for diabetes. Two months after the cells had been injected into the deltoid muscle, a teratoma formed in the injection area[13]. Safety remains the most important criterion of a cellular product for clinical applications[9]; thus, more safety-related quality detections with iPSC-derived cell therapy should be performed. In contrast to ESCs, iPSCs, and iPSCs specialized differentiation cells, several studies have demonstrated that MSCs exhibit good safety profiles, making MSCs

the most widely used cell type for clinical applications at present[14-16]. To date, various MSCs have been discovered in different tissues, including bone marrow, umbilical cord, umbilical cord blood, placenta, amniotic fluid, hair follicle, adipose tissue, and dental tissues[17-26].

Since dental pulp stem cells (DPSCs) were first reported by Gronthos *et al*[27] in 2000, a growing interest has been observed toward the potential of dental stem cells (DSCs) for the treatment of oral and neurological diseases[28,29]. Six types of human DSCs have been isolated and identified at different stages of tooth development[30]. For instance, DPSCs, stem cells from apical papilla (SCAPs)[31], stem cells from human exfoliated deciduous teeth (SHEDs)[32], periodontal ligament stem cells (PDLSCs)[33], and gingival mesenchymal stem cells (GMSCs)[34] can be gained after tooth eruption, while dental follicle stem cells (DFSCs) are a special kind of DSCs, which can be obtained at the early developing stage of the tooth prior to eruption[35] (Figure 1).

The dental follicle is an ectomesenchyme-derived and loose connective tissue originating from the cranial neural crest. During the bud stage of tooth development, the dental follicle is formed and surrounds the dental papilla and enamel organ, which plays a critical role in tooth eruption *via* regulating bone resorption and formation[36]. In the late bell stage, the dental follicle gives rise to the supporting tissues of the tooth-periodontium, including cementum, alveolar bone, and periodontal ligament[37]. Compared with other dental tissues, dental follicle tissue has the obvious advantage of large tissue volume, which is the premise of obtaining a sufficient number of cells to meet the needs of clinical application. Meanwhile, the number of DFSCs obtained in the same passage is far greater than that of DPSCs, accordingly being more suitable for large-scale expansion and industrial generation[38]. Due to the origin of dental follicle tissue in the early stage of tooth development, it has been demonstrated that DFSCs have the following advantages over DPSCs. First, DFSCs exhibit a significantly higher cell proliferation rate and colony-formation capacity than DPSCs[38], which further suggests that DFSCs may be better able to meet the needs of clinical transformation in terms of quantity and quality. Second, DSCs originate from neural crest cells. DFSCs have more similar protein profiles with cranial neural crest cells (CNCCs) than DPSCs, and possess high potency in odontogenic differentiation *in vitro*[39], which demonstrates that DFSCs may have better transformation advantages for the treatment of neurological and oral diseases. Third, DFSCs have better inhibitory effects on the proliferation of proinflammatory lymphocytes and better promote the proliferation of anti-inflammatory Treg cells than SHEDs and DPSCs[40], which indicates that DFSCs have better immunoregulation capacity. Of note, the advantages in terms of quantity, quality, differentiation, and immunoregulatory properties, as mentioned above, are part of the critical mechanism by which MSCs exert therapeutic effects. In this regard, DFSCs appear to be the candidate cells with natural advantages for regenerative medicine compared with stem cells from other dental tissues. Indeed, as a kind of dental tissue-derived stem cells originating from the neural crest, DFSCs may be more advantageous in promoting oral tissue regeneration, including periodontium, dental pulp, and tooth regeneration, as well as in treating neurological injury and neurodegenerative diseases, such as spinal cord and brain injury, as well as Parkinson's disease (PD) and Alzheimer's disease.

ORAL DISEASES AND FUNCTIONAL UNIT REGENERATION *VIA* DENTAL FOLLICLE STEM CELLS

Periodontitis and periodontal regeneration

The periodontium is a complex functional unit that plays a critical role in the oral cavity[41]. Periodontitis is a common and chronic inflammatory disease caused by plaque in the teeth[42]. As the disease progresses, gingival recession, loss of soft tissue attachment, and even intrabony defects can occur, ultimately resulting in premature tooth loss[43]. To date, while numerous conventional clinical treatments for periodontitis have been shown to control inflammation and aggressive progression, these strategies have not been able to achieve periodontal regeneration. Previous preclinical studies have demonstrated that the transplantation of stem cells presented the potential and provided new hope for periodontal regeneration[44,45].

The combination of DFSCs and hydroxyapatite scaffold forms a cementum-like matrix *in vivo* after transplantation into mice[35]. Scaffold plays a critical role in tissue engineering, providing support for transplanted stem cells and enhancing the therapeutic effects of tissue regeneration. The cell sheet technique prevents extracellular matrix degradation and provides a novel scaffold-free cell delivery strategy[46]. The extracellular matrix contains numerous growth factors and provides support to the cells without the need for additional scaffolds[47]. In addition, cell viability and function can be restored without the digestion operation step. A complex of dental follicle cell sheets forms periodontal tissue-like structures, including cementum-like structures and periodontal ligament with abundant blood vessels after transplantation into the subcutaneous areas of nude mice[48], demonstrating that DFSC sheets have the potential to achieve the goal of periodontal regeneration.

The tooth root development requires stimulation from Hertwig's epithelial root sheath (HERS), and DFSCs differentiate into cementoblasts during the epithelial-mesenchymal interaction process[49]. Accordingly, it has been demonstrated that the formation of cementum and periodontal ligament-like

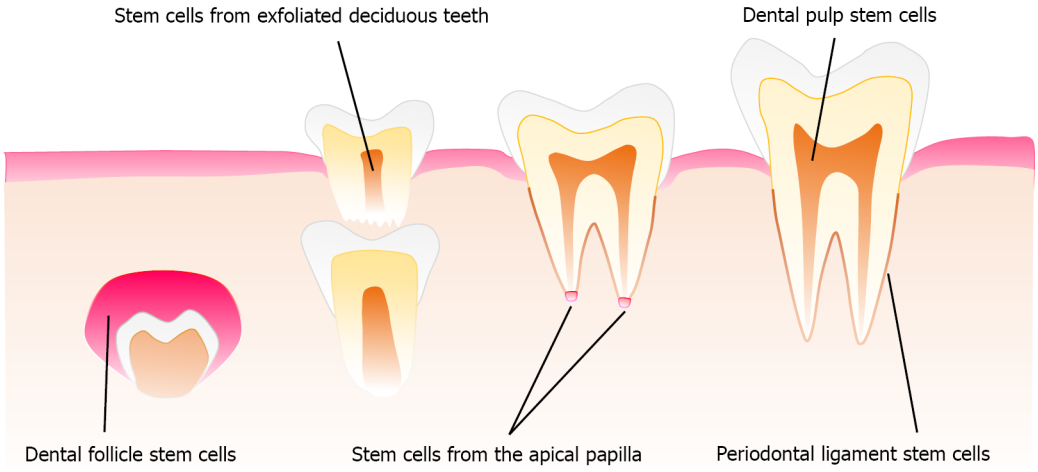

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Figure 1 Main dental stem cells derived from different dental tissues. This image shows the six types of human dental stem cells isolated and identified at different stages of tooth development. Dental follicle stem cells are obtained at the early developing stage of the tooth prior to eruption.

tissue was enhanced at 5 wk after implantation into rat submentum when DFSCs had been pre-exposed to HERSCs[50], suggesting that establishing a microenvironment similar to tooth root development is important for periodontal regeneration. Moreover, appropriate microinflammation preconditioning is important for improving the regeneration and immunoregulation capacity of DFSCs and their secreted exosomes. Lipopolysaccharides (LPS) upregulated the expression of osteogenic and adhesion-related proteins in DFSC sheets, which showed good performance in canine periodontal regeneration[51]. Furthermore, LPS enhanced the paracrine activity and immunomodulatory effect of DFSCs, and LPS-preconditioned DFSC-derived small extracellular vesicles (sEV) were beneficial for repairing lost alveolar bone in rats[52]. A later study further clarified that LPS-preconditioned DFC-sEV inhibited intracellular reactive oxygen species (ROS) as an antioxidant; it reduced the NF-κB receptor activator ligand/osteoprotegerin ratio of PDLSCs by inhibiting ROS/Jun amino-terminal kinase (JNK) signaling under inflammatory conditions and promoted macrophages to polarize toward the M2 phenotype *via* ROS/extracellular signal-regulated kinase (ERK) signaling[53].

For clinical applications, autologous PDLSCs have been used in clinical studies (ClinicalTrials.gov Identifier: NCT01357785; www.isrctn.com Identifier: ISRCTN13093912), and the results showed no significant differences in the effect on intrabony lesions between PDLSCs groups and scaffold-only groups[54,55]. Although some previous studies have demonstrated that PDLSCs might be the first choice for periodontal regeneration[28,43], DFSCs can give rise to periodontal supporting tissues, including cementum, alveolar bone, and periodontal ligaments. Moreover, previous comparative studies have revealed that DFSCs exhibit a stronger capacity for the regeneration of cementum and periodontal attachment than PDLSCs[48,56]. Thus, DFSCs can be considered a better candidate cell source for periodontal regeneration.

Dental pulp necrosis and pulp regeneration

Dental pulp necrosis is an irreversible inflammatory dental disease that causes destruction and loss of the pulp tissue, resulting in the loss of teeth and even abscesses of the jaw[57]. Bacterial infections play a key role in the development of dental pulp necrosis. Bacterial invasion and colonization were observed in the pulp necrotic areas with caries exposure and symptomatic irreversible pulpitis. Additionally, bacterial penetration of blood vessels occurred, which may spread bacterial infections[58]. Before it leads to more serious consequences, endodontic treatment must be performed to remove the damaged pulp[59,60]. However, the tooth becomes more fragile and susceptible to caries, periapical infection, and fracture after endodontic treatment because of the loss of blood and nutrition supply[59].

Stem cell-based dental pulp regeneration has the objective of developing new methods to replace the conventional treatment of dental pulp necrosis. Scaffolds, stem cells, and growth factors have been used for dental pulp regeneration. Findings from animal studies have shown that DPSCs seeded in a collagen scaffold with dentin matrix protein 1 were able to induce the formation of dental pulp-like tissues in immunodeficient mice[61], and DPSCs pellets stimulated by bone morphogenetic protein 2 promoted the dentin formation onto the amputated pulp of dog teeth[62]. Later, autologous pulp stem cells and granulocyte colony-stimulating factor with a clinical-grade atelocollagen scaffold were transplanted into the dog pulpectomized teeth, promoting pulp/dentin regeneration[63]. A pilot clinical study using a similar strategy has also observed complete pulp regeneration in humans[64]. Moreover, a scaffold-free translation strategy has also been used for pulp regeneration. DPSC aggregates derived from the autologous canine tooth pulp induced the regeneration of three-dimensional pulp tissue equipped with

blood vessels and sensory nerves 12 mo after treatment[65]. Consequently, DPSCs are derived from the pulp tissue and it appears that DPSCs have the potential to promote dentin-pulp regeneration, while DFSCs have a similar capacity.

Previous studies have demonstrated that providing an inductive microenvironment with a suitable scaffold could achieve dentin and even dental pulp regeneration. Treated dentin matrix (TDM) is derived from animal or human dentin matrix treated with ethylenediamine tetraacetic acid, containing abundant collagen, noncollagenous proteins, and growth factors. Both rat and human TDM induce and support complete dentin regeneration, in addition to inducing transplanted DFSCs to differentiate into odontoblasts and express dentin sialoprotein and matrix protein 1[66,67]. The cell sheet technique promotes dental pulp regeneration. DFSC sheets were substituted for DFSCs and, in combination with TDM, were implanted subcutaneously into the dorsum of mice. New dentin pulp-like tissues were observed after eight weeks post-transplantation[68]. Moreover, native dental pulp extracellular matrix (NDPE) can be used to obtain prefabricated-shaped dental pulp. Dentin-pulp complex-like tissues and columnar odontoblasts-like layers arranged along the interface between newly formed predentin matrix could be found after DFSCs-NDPE-TDM transplantation to the jaw of miniature swine for 12 wk[69]. Therefore, DFSCs could also exhibit the capacity to regenerate the dentin-pulp tissues with suitable inductive scaffolds.

Tooth root and whole-tooth regeneration

The regeneration of whole teeth is a major objective and promise of oral regenerative medicine. To date, two main strategies have been used to achieve this goal. The first strategy is a combination of mesenchymal and epithelial cells to construct a bioengineered tooth germ, which is then transplanted into the alveolar socket. Parts of animals showed whole-tooth eruption around 3.5 mo after tooth-germ implantation[70]. However, several barriers should be addressed in future studies of bioengineered teeth, such as indiscriminate shape and smaller size than natural teeth[4,70-73]. The second strategy includes the direct reconstruction of the functional units, such as bio-tooth root with periodontal tissue-like structures and dentin-pulp-like tissues.

As with the combination of implants and crowns to replace missing teeth, researchers have explored implant-like scaffolds to reconstruct the root. As mentioned above, TDM has the potential to be a suitable scaffold for root reconstruction. Yang *et al*[68] used calcified human dentin to model an alveolar microenvironment and used DFSC sheets-TDM-DFSC sheets to reconstruct the pulp-root-periodontium structure. TDM could induce and support DFSC sheets to develop new dentin-pulp and cementum-periodontium-like tissues after subcutaneous transplantation into nude mice for 8 wk[68]. Moreover, with seed DFSCs, the complex of aligned PLGA/Gelatin electrospun sheet/TDM/NDPE generated tooth root-like tissues after 12 wk of transplantation in porcine jaws[69]. Shape-optimized TDM scaffolds with DFSCs were transplanted into the alveolar bone of swine, and ceramic crowns were installed. These bio-tooth roots not only regenerated histologically but also allowed masticatory functions and remained stable for 3 mo[74]. In nonhuman primates, a novel functional biological root complex was constructed based on DFSC sheets and *in vitro* three dimensional (3D) suspension culture. This complex was then transplanted into rhesus monkeys and gradually restored occlusal function and long-term masticatory function for 2 years during the evaluation period[75]. In this regard, DFSCs with suitable scaffolds have the potential to be suitable stem cells for whole-tooth reconstruction based on bio-booth root regeneration.

THERAPEUTIC POTENTIALS OF DENTAL FOLLICLE STEM CELLS IN NEUROLOGICAL DISEASES

Neural differentiation of dental follicle stem cells

As mentioned above, dental follicle is an ectomesenchyme tissue that originates from the cranial neural crest. DFSCs not only express the markers typical of MSCs [*e.g.*, cluster of differentiation (CD) 44, CD90, CD105] but also express neural cell markers (*e.g.*, Nestin, β-III tubulin, and CNpase)[76-78] and even embryonic stem cell markers (octamer-binding transcription factor 4 and sex-determining region Y-box 2)[79], indicating that DFSCs may retain some of the neural and embryonic features and can differentiate into neural-like cells. DFSCs display neural-like cell morphology with small neurite-like cell extrusions with a neuronal differentiation strategy[76]. Dental pulp comprises blood vessels, neural fibers, and connective tissue, and DPSCs exhibit extraordinary capacity to differentiate into neural-like cells and represent a potential source for neuronal regeneration therapies[80]. However, a comparative study has demonstrated that DFSCs possess more similar protein profiles to CNCCs than DPSCs[39]. Moreover, compared with DPSCs, the expression of CNpase, neurofilament protein, Nestin, and β-III tubulin of DFSCs was upregulated significantly after treatment in the same neural-induction condition [78]. From this perspective, DFSCs may be a better candidate cell type for neural differentiation and even for the treatment of neurological diseases based on pre-differentiation.

Therapeutic potentials of dental follicle stem cells in neurotrauma and neurodegenerative disorders

Spinal cord injury (SCI) is a severe neurological trauma that causes the impairment of sensory and motor functions[81]. The acute stage of injury is directly caused by trauma, including compression, contusion, and shear injury forces. Proinflammatory cells are then activated, releasing abundant inflammatory cytokines, which induce a cascade of secondary injury[82]. Many neurons, astrocytes, and other neural cells die in the injured area due to necrosis or apoptosis during the secondary injury[83]. Until now, current clinical strategies have not achieved satisfactory outcomes due to irreversible damage to neural cells. Stem cell-based therapies hold the promise of developing new approaches for the treatment of SCI. Several types of stem cells have been used for transplantation, such as neural SCs (NSCs), ES/iPS-derived NSCs, and MSCs[84]. However, the sources of NSCs are limited and also face ethical issues. ES/iPS-derived cells exhibit some of the aforementioned worrying aspects. Thus, MSCs may now be a better candidate cell type for SCI. Researchers have compared the therapeutic effects of MSCs from bone marrow and dental pulp. Transplantation of DPSCs promoted marked recovery of locomotor function in the hind limbs, while transplantation of bone marrow SCs (BMSCs) resulted in substantially less recovery of locomotor function in rats with complete SCI. The main mechanisms include inhibition of apoptosis in neurons, astrocytes, and oligodendrocytes; promotion of regeneration of disjunct axons; and differentiation into mature oligodendrocytes to replace lost cells[85]. Later, another comparative study explored the differences among DFSCs, SCAPs, and DPSCs for the treatment of SCI. Findings from an animal study demonstrated that all three types of DSCs, especially DFSCs, have the potential to promote functional recovery after SCI by reducing the inflammatory response, promoting neurite regeneration, reducing progressive hemorrhagic necrosis, and differentiation into mature neurons and oligodendrocytes but not astrocytes[77]. Moreover, scaffolds with DFSCs were also used to repair the spinal cord defect; for example, aligned poly-ε-caprolactone/poly-lactide-co-glycolic acid electrospun material allowed nerve fibers to pass through, and induced DFSCs to differentiate *in vivo*[86].

PD is a common and progressive neurodegenerative disorder characterized by tremors, rigidity, and bradykinesia[87,88]. The aggregates of ubiquitin and α-synuclein-positive protein, Lewy bodies, and the loss of dopaminergic neurons in the substantia nigra pars compacta are the main characteristics that define PD[89]. The incidence of the disease rises steeply with age, affecting approximately 1% of the population between the ages of 70 years and 79 years[90]. Although pharmacological approaches (such as amantadine and levodopa) and nonpharmacologic strategies (deep brain stimulation, exercise, and physical therapy) have been used in PD treatment[91,92], these therapeutic strategies only delay the progression of the disease and relieve the symptoms but do not achieve regeneration of dopaminergic neurons. There has been considerable excitement about the use of MSCs to treat neurodegenerative diseases *via* secretion of anti-inflammatory factors [*e.g.*, indoleamine (2,3)-dioxygenase (IDO), prostaglandin E2 (PGE2)], growth factors [*e.g.*, vascular endothelial growth factor (VEGF), glial cell line-derived neurotrophic factor (GDNF)], and exosomes to achieve neuroinflammation attenuation and neural regeneration[93,94]. Various cell types, including BMSCs, adipose derived stem cells, umbilical cord mesenchymal stem cells (UCMSCs), and DSCs, and their neural-primed cells or accessory products (such as exosomes) have been used to treat PD[95-99]. The locomotive defect was recovered in PD rats after neural-primed SHEDs were transplanted and differentiated into neurons and dopaminergic neurons *in vivo*. The transplanted cells resided in the brains of rats and formed functional connections [98]. DFSCs have also been transplanted for the treatment of PD and survived in the transplanted regions of PD mice for more than 6 wk after surgery. DFSCs not only increased the number of dopaminergic neurons around them but also differentiated into tyrosine hydroxylase-positive cells, indicating that DFSCs may be a potential source in the exploration of possible therapeutic roles in PD [99]. In addition, other DSCs have been used to treat stroke, Alzheimer's disease, and a variety of peripheral nervous system diseases[100,101]. Based on the biological properties of DFSCs, they may also present promising therapeutic effects in the treatment of the aforementioned neurological diseases.

IMMUNOREGULATION OF DENTAL FOLLICLE STEM CELLS

Both in oral and neurological diseases, the immunomodulatory capacity is one of the most crucial functions of MSCs to facilitate the repair or regeneration of damaged tissues[51,77]. MSCs can regulate the proliferation, activation, maturation, and function of innate and adaptive immune cells *via* cell-to-cell direct contact, soluble cytokines, and exosomes[102]. As a kind of MSCs, DFSCs also present immunomodulatory characteristics. In acute lung injury models, DFSCs could suppress the production of proinflammatory cytokines, such as monocyte chemoattractant protein (MCP)-1, interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α; decrease the proportion of proinflammatory macrophage M1 phenotype; increase the level of anti-inflammatory cytokine IL-10 and the proportion of anti-inflammatory M2 phenotype both in bronchoalveolar lavage fluid *in vivo* and *in vitro* experiments[103]. Furthermore, DFSCs also exhibit great therapeutic potential in autoimmune diseases and chronic inflammatory disorders. DFSCs suppress the proliferation of T lymphocytes and lymphocyte apoptosis but increase the number of Tregs. DFSCs also reduce the secretion level of TNF-α but upregulate the level of IL-10 in peripheral blood mononuclear cells (PBMCs) of patients with rheumatoid arthritis[104].

Moreover, in inflamed mononuclear cell samples of patients with Crohn's disease, DFSCs also downregulate lymphocyte proliferation, CD4 + IL22BP T cell ratio, and the secretion of TNF-α and IL-6, but increase the frequency of Tregs and the level of IL-10[105]. From the case studies mentioned above, we can briefly summarize that DFSCs exert the common immune modulatory capacity *via* suppressing the proinflammatory immune cells [*e.g.*, T-helper (Th) 1, Th17, macrophage M1] and cytokines (*e.g.*, TNF-α, IL-1, IL-6) and increasing the number of anti-inflammatory immune cells (*e.g.*, Tregs, macrophage M2) and cytokines (*e.g.*, IL-4 and IL-10). Therefore, it is necessary to design experiments to examine the ratio or level changes in inflammatory cells and cytokines after co-culture of DFSCs with activated PBMCs, which can help us to screen the cells with superior immune properties and obtain better clinical outcomes.

CRYOPRESERVATION OF DENTAL FOLLICLE STEM CELLS

Stem cell-based therapies have been investigated for tissue engineering and treatment of many diseases for several decades, and cryopreservation can be used to effectively preserve stem cells. Cryopreservation is the process of gradually cooling cells or tissues to sub-zero temperatures, and finally preserving them in the gas phase liquid nitrogen (-150°C to -196°C) for an extended period. In this state, the biological activity of the cells is stopped, and their viability can be restored by careful thawing when needed[106]. Human DFSCs isolated from fresh and cryopreserved dental follicles show similar biological characteristics, such as proliferation ability, surface markers, and tri-linage differentiation capacities[107]. Moreover, the two types of DFSCs possess the same osteogenic differentiation potential and immunomodulatory properties for bone tissue engineering, resulting in the inhibition of adaptive immune response, which demonstrates that the stemness and immunomodulatory capacity of long-term-preserved dental follicle tissues can be restored[108]. Another study compared the biological characteristics between cryopreserved DFSCs and the cells from cryopreserved dental follicles. After 3 mo of cryopreservation, the cells from the cryopreserved dental follicles showed similar levels of stemness and apoptosis-related genes and exhibited similar osteogenic and adipogenic differentiation capabilities to cryopreserved DFSCs[109]. In this regard, both cryopreservation of DFSCs from fresh dental follicle tissues and direct cryopreservation of dental follicle tissues can preserve the biological properties of the cells. As a consequence, cryopreservation technology enables DFSCs to the off-the-shelf products for clinical applications.

Cryopreservation addresses the preservation of DFSCs. Another problem is that it is questionable whether allogeneic stem cells exhibit the same therapeutic effects as autologous stem cells. A previous study compared the therapeutic effects of allogeneic and autologous PDLSCs on periodontal tissue regeneration in a miniature pig model of periodontitis. Significant periodontal tissue regeneration was achieved in both transplanted groups without significant difference due to low immunogenicity and marked immunosuppression of T-cell antigen *via* PGE2[110]. Furthermore, allogeneic DSCs likely did not affect the therapeutic effects because of their inherent characteristics. However, more experiments should be performed to compare the outcomes of allogeneic and autologous DFSCs in different diseases.

In addition to cell cryopreservation, some biological materials can be cryopreserved. For instance, after being cryopreserved in liquid nitrogen with cryoprotectant for several months, the cryopreserved TDM exhibited superior mechanical properties, more dentin-related proteins, and a larger pore diameter than the fresh TDM. The cryopreserved TDM was also able to induce dental follicle cells to regenerate new dentin-pulp-like tissues[111], suggesting that the cryopreservation techniques address the preservation of biological materials and also enable them to be used as off-the-shelf scaffold for tissue engineering.

CONCLUSION

In this review, it was found that among MSCs, stem cells derived from dental tissue originating from the craniofacial neural crest exhibit dental-like tissue differentiation potential and neuro-ectodermal features, which makes them a promising alternative for the treatment of oral and neurological diseases (Figure 2). Moreover, in contrast to other DSCs, those from the early-developing tissues exhibit several superior properties, including larger tissue volume, higher cell proliferation rate and colony-formation capacity, more similar biological profiles to progenitor cells of origin, and better anti-inflammatory effects. These advantages are part of the critical mechanism by which MSCs exert therapeutic effects and are relevant for large-scale scaling and industrial generation for clinical applications. Cryopreservation preserves the biological properties of DFSCs and enables them to be used as off-shelf products for clinical applications. Therefore, DFSCs could have great clinical prospects and translational value in oral and neurological diseases with natural advantages.

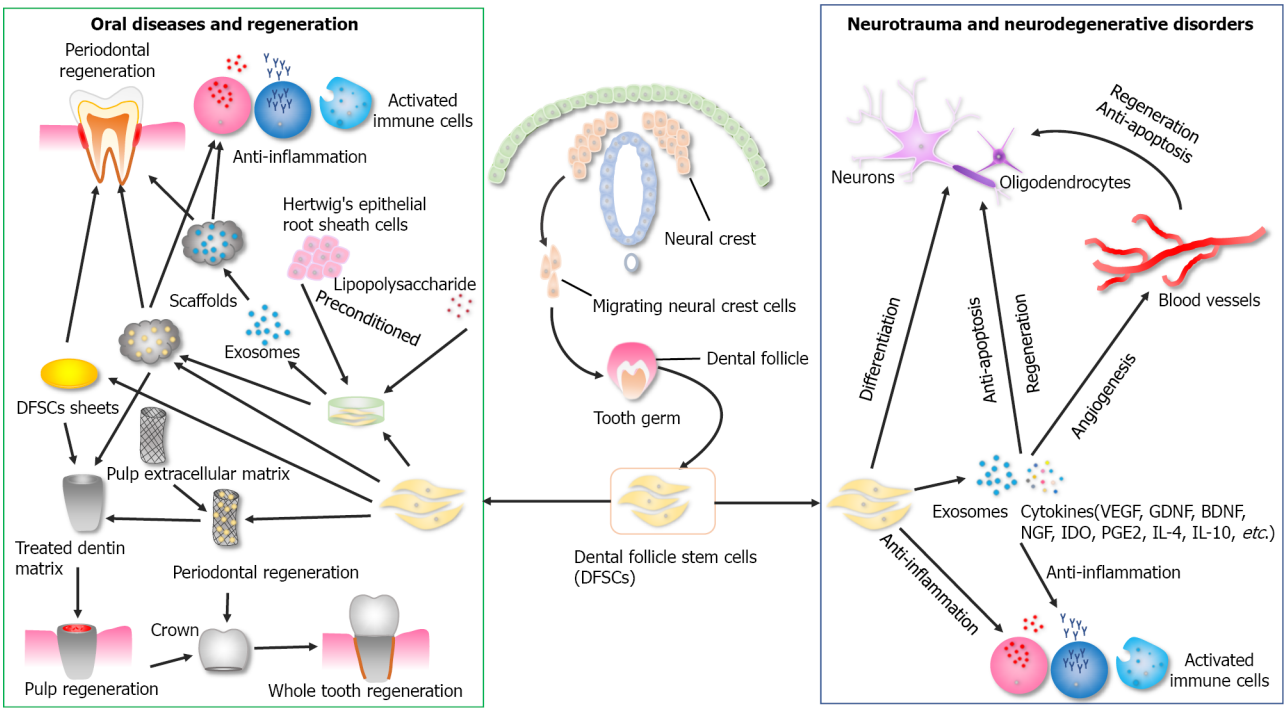

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Figure 2 The origin of dental follicle stem cells and the main strategies/mechanisms of dental follicle stem cell-based therapies in oral and neurological diseases. Dental follicle stem cells (DFSCs) originating from the craniofacial neural crest exhibit dental-like tissue differentiation potential and neuro-ectodermal features. For the regeneration of periodontium and pulp, the main strategy is the combination of DFSCs or their accessory products (exosomes) with various scaffolds (such as treated dentin matrix, native dental pulp extracellular matrix, and hydroxyapatite). The cell sheet technique prevents the degradation of the extracellular matrix and makes it a natural scaffold. In addition, appropriate stimulation may enhance the therapeutic effect, such as preconditioning with Hertwig's epithelial root sheath cells or lipopolysaccharides. For the treatment of neurotrauma and neurodegenerative disorders, DFSCs enable tissue regeneration primarily by reducing inflammatory response and apoptosis, promoting angiogenesis, and differentiating into mature functional neurons and oligodendrocytes.

Currently, SHEDs, DPSCs, PDLSCs, and GMSCs have been used in clinics[54,65,112,113], and clinical trials of DFSCs are also forthcoming. For future clinical applications of DFSCs, several key points need further investigation. First, as one of the three essential elements in tissue engineering, the selection of stem cells is vital. Therefore, potency assessment and screening criteria should be established, including donor screening and culture system optimization. In addition to sterility, safety, activity, homogeneity, purity, and stability, the levels of released cytokines or markers associated with immunomodulation (*e.g.*, IL-4 and IL-10), dental tissue (*e.g.*, VEGF, dentin sialophosphoprotein), or neural regeneration (*e.g.*, Nestin, GDNF, and brain-derived neurotrophic factor, nerve growth factor) should be detected[17]. Furthermore, the potency evaluation system should contain the inhibition of proinflammatory immune cells (*e.g.*, Th1, Th17, and macrophage M1) and cytokines (*e.g.*, TNF-α, interferon-γ, IL-1, and IL-6), the promotion of anti-inflammatory immune cells (*e.g.*, Tregs, macrophage M2) and cytokines (*e.g.*, IL-4 and IL-10) after PBMCs co-culture with DFSCs *in vitro*, and the promotion of neuron and oligodendrocyte or multi-differentiation capacity that includes neural/osteogenic differentiation[79,103,105,114-120]. Then, *in vivo* experiments should be used to verify the correctness of the potency assessment system. Second, in the field of tissue engineering, appropriate scaffold materials have a synergetic effect on the promotion of regeneration with stem cells. Some of these materials include beta-tricalcium phosphate, collagen sponge, xenogeneic bone substitute, and TDM, some of which have been used in the clinic[54,55,74,113,121,122]. Without a doubt, the quest for more suitable scaffold materials remains a long-term process. Third, a wide range of bioactive factors and RNAs, including proteins, cytokines, proinflammatory components, extracts from biological materials, long non-coding RNAs, and microRNAs, have been used to enhance differentiation and immunomodulation capacities[53,123-127], and these strategies may further improve the therapeutic potential of DFSCs in clinical applications in the future. Collectively, with the development of materials and preconditioning strategies, and in combination with the natural superiority exhibited by DFSCs in terms of medicinal properties, DFSC-based therapeutics are a promising strategy for the future treatment of oral and neurological diseases.

FOOTNOTES

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REVIEW

Current status and prospects of basic research and clinical application of mesenchymal stem cells in acute respiratory distress syndrome

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Abstract

Acute respiratory distress syndrome (ARDS) is a common and clinically devastating disease that causes respiratory failure. Morbidity and mortality of patients in intensive care units are stubbornly high, and various complications severely affect the quality of life of survivors. The pathophysiology of ARDS includes increased alveolar–capillary membrane permeability, an influx of protein-rich pulmonary edema fluid, and surfactant dysfunction leading to severe hypoxemia. At present, the main treatment for ARDS is mechanical treatment combined with diuretics to reduce pulmonary edema, which primarily improves symptoms, but the prognosis of patients with ARDS is still very poor. Mesenchymal stem cells (MSCs) are stromal cells that possess the capacity to self-renew and also exhibit multilineage differentiation. MSCs can be isolated from a variety of tissues, such as the umbilical cord, endometrial polyps, menstrual blood, bone marrow, and adipose tissues. Studies have confirmed the critical healing and immunomodulatory properties of MSCs in the treatment of a variety of diseases. Recently, the potential of stem cells in treating ARDS has been explored *via* basic research and clinical trials. The efficacy of MSCs has been shown in a variety of *in vivo* models of ARDS, reducing bacterial pneumonia and ischemia-reperfusion

injury while promoting the repair of ventilator-induced lung injury. This article reviews the current basic research findings and clinical applications of MSCs in the treatment of ARDS in order to emphasize the clinical prospects of MSCs.

Key Words: Acute respiratory distress syndrome; Mesenchymal stem cells; Pulmonary edema; Inflammatory response; Tissue repair; Pulmonary fibrosis

Core Tip: Acute respiratory disease syndrome (ARDS) is a common disease with high morbidity and mortality. ARDS is characterized by increased alveolar-capillary membrane permeability, influx of protein-rich pulmonary edema fluid, and surfactant dysfunction, resulting in severe hypoxemia. Mesenchymal stem cells (MSCs) have the self-renewal and multilineage differentiation properties, and their immunomodulatory abilities have been implicated in the treatment of disease. Herein, we discuss the pathophysiology of ARDS and recent research surrounding the clinical application of MSCs in the treatment of ARDS.

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INTRODUCTION

Acute respiratory distress syndrome

Acute respiratory distress syndrome (ARDS) is a clinicopathological condition characterized by increased lung fluid, decreased lung compliance, and severe hypoxemia[1,2]. ARDS was defined in 1994 by the American-European Consensus Conference[3]. After several decades of research and discussions, the current internationally recognized definition of ARDS is the Berlin definition which proposes three categories of ARDS based on the severity of hypoxemia: Mild [200 mmHg < arterial oxygen pressure (PaO_2)/fraction of inspired oxygen (FiO_2) < 300 mmHg], moderate (100 mmHg < PaO_2/FiO_2 < 200 mmHg), and severe (PaO_2/FiO_2 < 100 mmHg), along with explicit criteria related to the timing of the syndrome's onset, the origin of edema, and chest radiograph findings[4-6]. The pathogenesis of ARDS is characterized by an unregulated inflammatory cascade with increased pulmonary endothelial and epithelial permeability[7]. Endogenous chemicals and microbial products linked to cell injury are hypothesized to attach to receptors on epithelial cells and alveolar macrophages, triggering an immunological response. The unrestricted synthesis of reactive oxygen species, leukocyte proteases, chemokines, and inflammatory substances that results to gradual lung damage. The immune-mediated reaction is known as a "cytokine storm"[8,9]. The pathophysiological changes that occur during the development of ARDS are shown in Figure 1. Currently, the clinical treatment of ARDS is rather limited and is mainly based on organ function support, such as lung protective ventilation, liver and kidney function protection, gastrointestinal function protection, venous thrombosis prevention, and nutritional support [10]. Despite the profound understanding of the molecular mechanism of ARDS, improvement of pulmonary ventilation strategies, and strengthening of supportive care for critically ill patients, the prognosis of patients with ARDS is still unsatisfactory. Currently, the global mortality rate of ARDS exceeds 40%, whilst 6%–10% of patients with respiratory failure may develop ARDS in the emergency room[11]. The long-term sequelae of ARDS include long-term cognitive impairment, psychological disease, neuromuscular weakness, pulmonary dysfunction, and decline in quality of life because of long-term medical expenses[12]. Therefore, new and safer therapies are urgently needed for ARDS treatment.

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) were first described by Friedenstein *et al*[13], as an adherent, fibroblast-like cell population in the bone marrow (BM) that could regenerate rudiments of bone *in vivo*[13,14]. After decades of research, it has been found that MSCs are present in a variety of tissues and organs and can also differentiate into a variety of cells to play related roles (Figure 2)[15,16]. However, the understanding of MSCs is still inadequate. MSCs were officially defined by the International Society of Cell Therapy in 2006 as follows: (1) MSCs must display plastic-adherent capacities; (2) A simultaneous

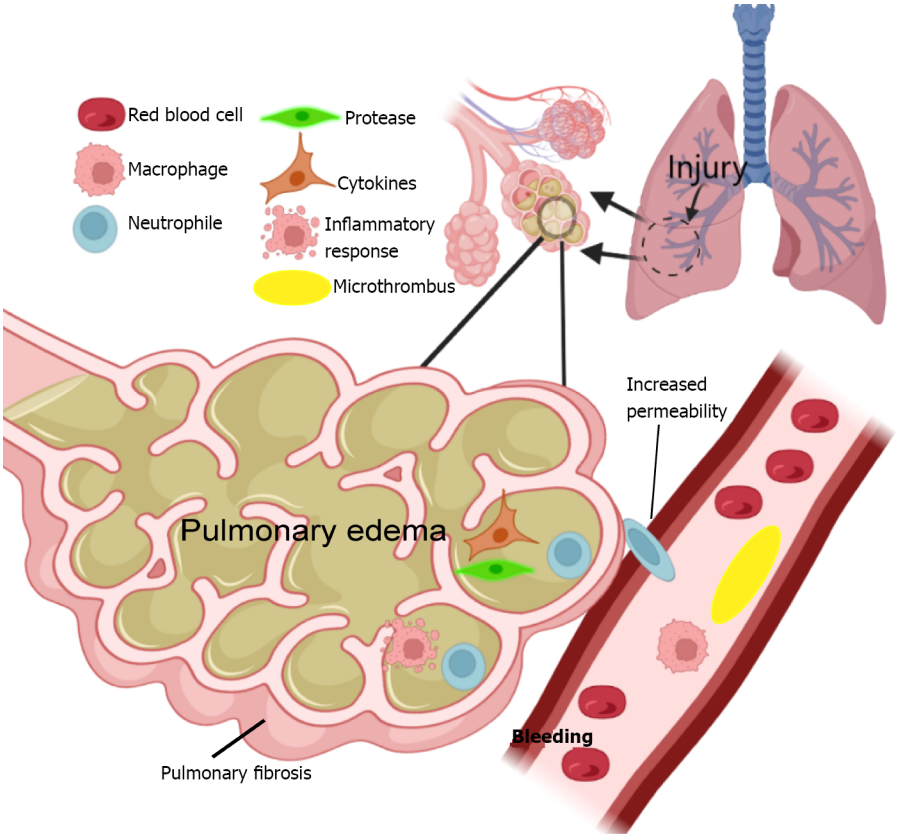

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Figure 1 Following acute lung injury, pulmonary vascular macrophages produce chemokines to increase vascular permeability, resulting in neutrophil aggregation. Neutrophils reach the pulmonary interstitium and alveolar space and release proteases, cytokines, and other harmful substances to destroy the alveolar microstructure, leading to cell dysfunction and eventually causing exudates and lung fluid to enter the alveolar space, resulting in the development of pulmonary fibrosis. Additionally, the disorder of pulmonary vascular coagulation and fibrinolysis caused by inflammation leads to pulmonary vascular microthrombosis, which is also one of the causes of pulmonary edema.

expression of stromal markers, an absence of hematopoietic or endothelial markers and human leukocyte antigen-DR surface molecules; and (3) An *in vitro* differentiation potential for osteoblasts, adipocytes, and chondroblasts[17,18]. The method of obtaining and culturing MSCs is simpler than other stem cells, and MSCs have broad application prospects in a variety of inflammatory-related diseases because of their unique immunomodulatory properties[19].

MSCs are considered a new approach for the treatment of ADRS[20]. The mechanism of MSCs in the treatment of ARDS is multifaceted, and the immunomodulatory effect of MSCs is a crucial aspect of it (Figure 3). At present, the collective view of the immune regulation ability of MSCs is based on the secretion of cytokines, such as tumor growth factor (TGF)-β and tumor necrosis factor-stimulated gene-6 [21]. By releasing a variety of cytokines and extracellular vesicles (EVs), MSCs play anti-inflammatory and anti-cell death roles and promote the generation of microcirculation, thereby promoting the clearance of bacteria and alveolar fluid, alleviating organ damage, thus alleviating ARDS-related symptoms[22-24].

CURRENT BASIC RESEARCH FOR THE USE OF MSCS IN ARDS TREATMENT

Immunoregulation ability of MSCs in ARDS

The hallmark of ARDS is a series of inflammatory responses. Uncontrollable inflammatory responses are known to cause catastrophic damage to various organs[25]. MSCs are pluripotent stem cells with immune properties that can secrete a variety of cytokines, such as anti-inflammatory factors, antiapoptotic factors, and antimicrobial peptides[26-28]. MSCs regulate immune activity *via* three different mechanisms: (1) Direct contact with tissue cells; (2) Production of a series of cytokines to regulate cell activities; and (3) Exerting immune effects by regulating the activity of T cells[29-31]. Currently, the MSC treatment of ARDS is mediated by controlling inflammatory responses. Therefore, the related mechanisms have become a hot research topic, and new research findings are constantly emerging, which introduce new views.

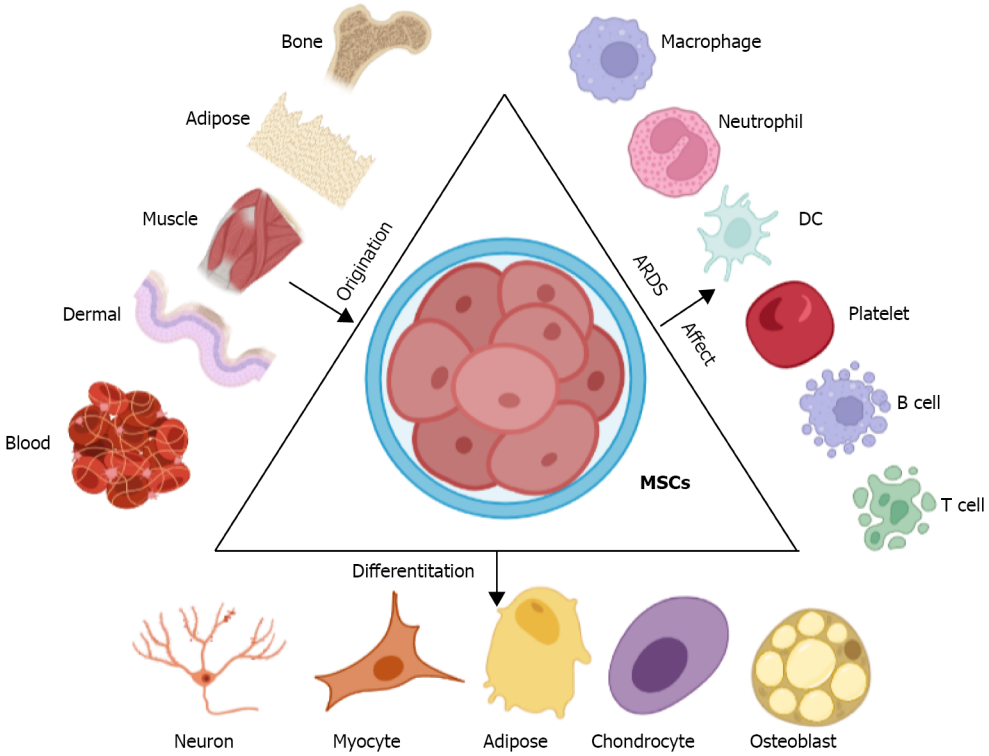

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Figure 2 Mesenchymal stem cells can be derived from a variety of cell types and can differentiate into different types of cells to play specific roles. In the pathological process of acute respiratory distress syndrome, mesenchymal stem cells can act on a variety of cells to play a protective role. MSC: Mesenchymal stem cell; DC: Dendritic cell.

MSCs regulate the immune activity of the dendritic cells: Conventional dendritic cells (cDCs) are unique antigen-presenting cells that bridge antigen immunity and innate immunity and can be activated by MSCs as regulatory DCs[32,33]. An existing study has shown that after lung injury induced by lipopolysaccharide, a large number of DCs accumulate in the lungs, which in turn aggravates lung inflammation and lung injury[34]. The underlying mechanism may involve the polarization of the T-helper cell (Th) 1 response and regulating neutrophil infiltration[35,36]. The aggregation of cDCs can lead to the activation of the Th1 pathway and aggravate the inflammatory response. At the same time, cDCs can also recruit neutrophils, prolong the life of neutrophils, upregulate innate immunity, and further intensify the inflammatory response[37,38]. MSCs also abolish the capacity of mDCs to migrate to chemokine (C–C motif) ligand 19, for DCs to display major histocompatibility complex class II peptide complexes recognized by specific antibodies, and for ovalbumin-pulsed DCs to support antigen-specific CD4+ T-cell proliferation[39].

Additionally, many studies have shown that MSC–EVs play key roles in the pathogenesis and progression of acute lung injury (ALI)/ARDS[40]. One of the underlying mechanisms may involve the potential impairment of antigen uptake, which may halt DC maturation[41]. MSC–EVs from the human BM may regulate the levels of maturation and activation markers (CD83, CD38, and CD80) and inflammatory cytokines [interleukin (IL)-6, IL-12p70, and TGF-β] *in vitro via* regulating the *CCR7* gene by carrying miR-21-5p[42]. Additionally, the emerging role of MSC–EVs in facilitating pulmonary epithelium repair, rescuing mitochondrial dysfunction, and restoring pulmonary vascular leakage has been shown[43,44]. Therefore, regulating the maturation of DC cells in the early stage of lung injury can effectively alleviate secondary lung injury[45] (Table 1).

MSCs induce the activation of macrophages: Alveolar macrophages are guardians of the alveoli and airways, and interstitial macrophages are guardians of blood vessels and the lung interstitium[46]. After lung injury, tissue monocyte-derived macrophages accumulate and have increased viability in the lungs, and persist at the lesion for a long time after lung injury[47]. Macrophages respond in a variety of ways, including modulation of function (activation), the release of inflammatory chemical mediators that control immune cell recruitment, and the modulation of epithelial responses[48]. Macrophages can be divided into two phenotypes based on their functions, the M1 type in resting states and the M2 type in the activated state[49]. M1-type macrophages can secrete a variety of cytokines and participate in many processes including pro-inflammatory, pro-apoptosis, free radical formation, and matrix degradation pathways, while M2-type macrophages play a role in anti-inflammatory and anti-cell death

Table 1 Recent studies on the mechanism of mesenchymal stem cell action in acute respiratory distress syndrome

Ref.	Time	Animal/cell line	Interference	Pathway	Conclusion/main effect
Zhang *et al* [112]	2022	C-mice	Human dermal fibroblasts or MSCs were intravenously	CAP	MSC treatment significantly protects mice against bacterial pneumonia or LPS-induced lung injury *via* the CAP pathway. When the CAP was inhibited through vagotomy (VGX) and pharmacological and genetic ablation experiments, the anti-inflammatory effects of MSCs were markedly reduced in lung injury models
Kakabadze *et al* [113]	2022	Wistar rats	HPMSCs	-	HPMSCs have the ability to migrate and attach to damaged lung tissue, contributing to the resolution of pathology, restoration of function, and tissue repair in the alveolar space
Wang *et al*[114]	2022	C-mice	Human placental MSCs	Macrophage polarization pathway	Human PMSC treatment preferentially rescued resident M2 AMΦs over recruited M1 BMMΦs with overall M2 polarization to improve KP-related ARDS survival
Wang *et al*[115]	2022	SD rats	LRMSC/HMSC-C/HMSC-BM	-	Three kinds of LRMSC, HMSC-C and HMSC-BM are protective against LPS-induced lung injury, HMSC-C was more effective than LRMSC and HMSC-BM to treat LPS-induced lung injury
Zhang *et al* [116]	2022	C-mice	MSC derived microvesicles	KEGG pathway and GO function	MSV microvesicles treatment was involved in alleviated lung injury and promoting lung tissue repair by dysregulated miRNAs
Xu *et al*[60]	2022	BALB/c mice	Umbilical cord-derived MSCs	-	Transplantation of UC-MSCs transfected with SP-B could potentiate M2 macrophage polarization and further relieve LPS-stimulated lung injury
Xue *et al*[117]	2022	C-mice	Bone marrow-derived MSC	-	TGF-β1 from MSCs restored skewed Treg/Th17 levels induced by hypoxic- and LPS-stimulated conditions and reduced inflammation
He *et al*[118]	2022	Hnsclc cell line A549 (ATCC, CCL-185)	MSCs	CXCL12/CXCR4 signal axis	*In vivo* transplantation of MSCs significantly attenuated lung injury in ARDS, inhibited serum pro-inflammatory factors in mice, and down-regulated expression of apoptotic and focal factors in lung tissues
Zhang *et al* [119]	2022	C-mice	Mouse bone marrow-derived MSCs	Wnt/β-catenin transition signaling	MVs released from MSCs exerted protective effects on early fibrosis by suppressing EMT in LPS-induced ARDS
Meng *et al*[120]	2021	-	MSCs derived from normal mouse bone marrow	Akt/Mtor signaling	MTORC2 like mTORC1 as an important signaling of regulation of MSC-secreted HGF protective against LPS-induced lung endothelial dysfunction
Ishii *et al*[121]	2021	Adult male Fischer 344 rats	Adipose-derived MSCs	-	AD-MSCs enhanced the barrier function between lung epithelial cells, suggesting that both direct adhesion and indirect paracrine effects strengthened the barrier function of lung alveolar epithelium *in vitro*
Wang *et al*[122]	2021	C-mice	Bone MSCs	Vimentin-Rab7a pathway	MSCs can reach the damaged lung tissue through migration, reduce inflammatory responses and alleviate lung injury
Liu *et al*[123]	2021	SD rats	Bone marrow mesenchymal stem cell	Beclin-1	BMSC-derived exosomes were taken up by the alveolar macrophages and attenuated LPS-induced alveolar macrophage viability loss and apoptosis. Exosomes effectively improved the survival rate of ALI rats, which was associated with alleviating lung pathological changes pulmonary vascular permeability and attenuating inflammatory response

C-mice: C57BL/6 mice; CAP: Cholinergic anti-inflammatory pathway; HPMSCs: Human placental mesenchymal stem cells; PMSCs: Placental MSCs; AMΦs: Alveolar macrophage; BMMΦ: Bone marrow–recruited macrophage; Hnsclc: Human non-small cell lung cancer; KP: Klebsiella pneumonia; LRMSC: Lung resident MSC; HMSC-C: Human chorion-derived MSC; HMSC-BM: Human bone marrow derived MSC; KEGG: Kyoto encyclopedia of genes and genomes; GO: Gene ontology; SP-B: Surfactant protein B; AD-MSC: Adipose tissue-derived MSC; EMT: Epithelial–mesenchymal transition; SD: Sprague-Dawley; mTOR: Mammalian TOR; ARDS: Acute respiratory distress syndrome; ALI: Acute lung injury; TGF-β1: Tumor growth factor-β1.

processes during the inflammatory response, and promote angiogenesis and tissue repair[50-52]. The transformation of the M1/M2 phenotype helps subside the inflammatory response and alleviate tissue

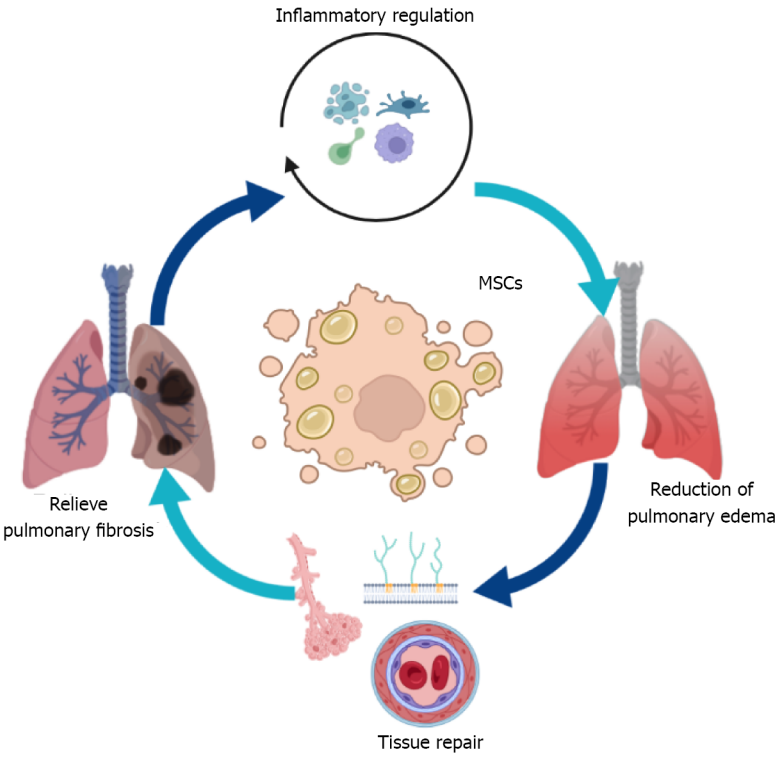

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Figure 3 The main mechanism of mesenchymal stem cell therapy for acute respiratory distress syndrome. Mesenchymal stem cell can treat acute respiratory distress syndrome by regulating inflammatory response, reducing pulmonary edema, alleviating pulmonary fibrosis, and promoting tissue repair. MSC: Mesenchymal stem cell.

damage. In ARDS, this balance can effectively remove harmful substances and pro-inflammatory factors from the body and promote lung tissue repair. Conversely, the destruction of this balance aggravates the pathological development of ARDS. Studies have shown that MSCs can control the pathological development of ARDS by regulating the polarization of macrophages and effectively promoting the repair process in ARDS[53]. Basic studies have shown that MSC treatment reduces the expression of CD86 on macrophages in the ALI models, indicating that MSCs can inhibit the transformation of macrophages to the M1 phenotype[54]. Several mechanisms of MSCs have been described, such as: (1) MSCS can promote the phenotypic transformation of macrophages through paracrine secretion of soluble cytokines[55]; (2) MSCS promotes macrophage polarization through exosomes[51]; (3) Metabolic Reprogramming[56]; (4) MSCS regulate mitochondrial transfer[57]; and (5) Apoptotic and efferocytosis effects[58]. The involved signaling pathways are as follows: (1) The nuclear factor erythroid 2-related factor 2/heme oxygenase-1 signaling pathway[59]; (2) The Notch signaling pathway; (3) The Janus kinase-signal transducer and activator of transcription signaling pathway[60]; and (4) The nuclear factor-kappa B signaling pathway[61].

MSCs regulate the T-cell balance: Imbalances between regulatory T cells (Tregs) and IL-17-producing Th17 are a sign of the development of inflammatory response in ARDS[62,63]. The main function of Th17 cells is to promote inflammation, release inflammatory factors, and play an important role in autoimmune diseases. Fortunately, they are precisely regulated by regulatory cells[64]. However, Treg cells can release anti-inflammatory factors (IL-4 and IL-10), control the inflammatory reaction process, and induce tissue damage repair[65,66]. Previous studies have shown that Tregs transferred into ALI animals can reduce the level of alveolar pro-inflammatory cytokines and inhibit neutrophil apoptosis and fibroblast recruitment[67,68]. A recent study also showed that a proportion of Th17 cells and Tregs > 0.79 was an independent predictor of 28-d mortality in patients with ARDS[62]. Therefore, maintaining the balance of Tregs and Th17 cells is crucial for patients with ARDS.

Existing studies indicated that, *in vitro*, MSCs repress the Th17 molecular program through the programmed cell death protein 1 pathway, prevent the differentiation of naive CD4+ T cells into Th17 cells, inhibit the production of inflammatory cytokines by Th17 cells, and induce Treg phenotype[69-71]. A study has shown that TGF-β1, as the main paracrine cytokine of MSCs, can significantly regulate the transformation of T cells into Tregs, disturb the Th17/Treg balance, and significantly contribute to the control of inflammatory response in ARDS[72]. It is also reported that MSCs can prevent the initial differentiation of CD4+ T cells into Th17 cells, inhibit the generation of inflammation, and induce the

generation of Tregs *in vitro*[73]. Several experiments have proved that controlling the level of Th17/Treg is the key to MSC-mediated control of the inflammatory response in ARDS. Therefore, it is extremely critical to find a method to regulate this balance, which may be the basis for a revolutionary breakthrough in the treatment of ARDS.

MSCs promote tissue repair

Lung epithelial cell and endothelial cell damage and the exudation of highly concentrated protein fluid are the basic pathological changes in ARDS. Therefore, the treatment of ARDS requires a combination of a lung-protective ventilation strategy and fluid manipulation[74]. The immediate effect of these strategies is that MSCs can directly participate in the reconstruction of lung injury by migrating to the site of lung injury, but this aspect has less impact on ARDS injury repair[75]. Evidence shows that MSCs can be directly transformed into type II alveolar epithelial cells to support the role of injured cells[74]. Meanwhile, cell-to-cell contact also provides a prerequisite for the control of inflammatory responses [76]. The formation and tissue damage of pulmonary edema is also related to the dysfunction of the pulmonary vascular system. The increased permeability of pulmonary capillaries leads to a series of serious consequences, such as the exudation of a variety of cells and cytokines and the formation of intravascular microthrombosis. Studies have shown that MSCs can also enhance the barrier system of the pulmonary vascular system, which is beneficial for promoting the repair of lung tissue[77]. Hepatocyte growth factor, angiopoietin-1, and keratinocyte growth factor secreted by MSCs can improve vascular endothelial barrier function[78-82]. Genetic engineering *in situ* has shown that MSCs could promote the potential of pulmonary angiogenesis[83]. Therefore, various results have shown that MSCs could inhibit pulmonary edema and also provide the basis for the regeneration of lung tissue.

Alleviation of pulmonary fibrosis

The cellular basis of the lung is composed of alveoli, various types of parenchymal cells, and BM-derived cells[84]. The interaction between various cells is crucial for maintaining the basic functions of the lungs[85]. Although lung protective ventilation strategies have been applied in clinical practice, ARDS survivors still have related health problems, and some patients develop fibroproliferative responses characterized by fibroblast accumulation and deposition of collagen and other extracellular matrix components in the lungs[86]. After ALI, vascular permeability increases, plasma exudates, and protein fluid aggregates, leading to pulmonary edema. Inflammatory factors from the coagulation/anticoagulation system and inflammatory system enter the lungs and damage the alveolar–capillary membrane barrier[87]. In the early inflammatory phase of ARDS, various immune cells continuously release a variety of harmful substances, including reactive oxygen species and nitrogen, as well as proteolytic enzymes such as elastase and matrix metalloproteinases, leading to lung endothelial and epithelial cell damage[86,88]. Persistent damage and failure to quickly repair this damage are the main factors that induce a pathological fibroproliferative response[89]. Late in the inflammatory response, massive and persistent accumulation of macrophages, fibroblasts, fibroblasts, and myofibroblasts in the alveolar space results in excessive deposition of fibronectin, collagen types I and III, and other components of the extracellular matrix[90,91]. The pro-fibrotic/anti-fibrotic balance is disrupted, and the fibrogenic effect increases dramatically, leading to irreversible pulmonary fibrosis. MSCs have shown gratifying advantages in anti-fibrotic effects. In preclinical models of lung fibrosis produced by bleomycin, silica, paraquat, and radiation, MSCs obviously show the ability to prolong life time[92-95]. However, MSC control of pulmonary fibrosis is also a double-edged sword. Studies have shown that MSCS can differentiate into ATII cells *in vitro*, inhibit the production of degradation enzymes, and thereby inhibit the secretion of pro-fibrotic factors by various immune cells[96]. There is also evidence that abnormally activated Wnt/β-catenin and TGF-β signaling pathways can induce the differentiation of pulmonary intrinsic MSCs into myofibroblasts and promote the development of pulmonary fibrosis [97].

CLINICAL EXPERIENCE USING MSCS FOR ARDS

To date, experience with the application of MSCs in patients is limited. The data of the available clinical evidence is summarized in Table 2. Wilson and his colleagues reported the results of the phase I stem cell research for ARDS treatment (START) in 2015[98]. Patients with moderate to severe ARDS received a single intravenous dose of low [1 × 10^6 MSCs/kg predicted body weight (PBW)], medium (5 × 10^6 MSCs/kg PBW), or high (1 × 10^7 MSCs/kg PBW) (n = 3/dose). All patients tolerated MSC infusion without prespecified infusion-related adverse events. High-dose MSCs improved daily SOFA scores compared with low-dose MSCs. Based on these promising results, the knowledge of the safety of administering MSCs to critically ill patients with ARDS is improving. A phase 2a clinical trial to evaluate the safety of BM-MSCs administered to patients with moderate to severe ARDS has also been conducted[99]. The primary outcome was safety, and secondary outcomes included respiratory, systemic, and serum biomarker endpoints. The study included 60 patients with ARDS, and intravenous Adipose-MSCs, 1 × 10^6/kg predicted body weight, *vs* the placebo was administered; however, there was

Table 2 Clinical study characteristics

Ref.	Cell type	Patient number	Outcome	Study design/evidence level	Publish time
Wilson *et al* [98]	MSC	9	No serious adverse events	Phase 1 clinical trial: A multicenter, open-label phase	2015
Matthay *et al* [99]	BM-MSCs	60	(1) No patients had any adverse events; (2) Mortality at 28 and 60 d was not significantly increased; and (3) ↑ Oxygenation index	Phase 2a safety trial: Prospective, double-blind, multicenter, randomized trial	2019
Yip *et al*[100]	UC-MSCs	9	(1) In-hospital mortality was 33.3% (3/9); (2) No serious prespecified cell infusion-associated or treatment-related adverse events; (3) ↓Circulating inflammatory biomarkers; (4) ↓Mesenchymal stem cell markers; and (5) ↑Immune cell markers	Phase I clinical trial: Prospective	2020
Lanzoni *et al* [107]	UC-MSCs	24	(1) No serious adverse events; (2) ↑Survival; and (3) ↓Inflammatory cytokines at day 6	Phase 1/2a clinical trial: A double-blind, randomized controlled trial	2021
Dilogo *et al* [108]	UC-MSCs	20	(1) ↑Survival; and (2) ↓Interleukin 6	Clinical trial: A multicentered, double-blind, randomized clinical trial	2021
Monsel *et al* [109]	UC-MSCs	45	(1) PaO_2/FiO_2 changes between D0 and D7 did not differ significantly; and (2) Clinical improvement	Clinical trial: A multicentered, double-blind, randomized clinical trial	2022
Grégoire *et al* [110]	BM-MSCs	8	(1) ↑Survival; (2) Clinical improvement; and (3) ↓Day-7 D-dimer value	A phase I/II Clinical Trial	2022
Kaffash Farkhad *et al* [111]	UC-MSCs	10	(1) ↑PaO_2/FiO_2; (2) ↓Serum CRP; (3) ↓IL-6, IFN-γ, TNF-α and IL-17 A; and (4) ↑TGF-β, IL-1B and IL-10	Phase 1 clinical trial: A single-center, open-label	2022

UC-MSC: Umbilical cord-derived mesenchymal stem cells; CDC: Cardiosphere-derived cells; BM-MCs: Bone marrow-derived mesenchymal stem cells; PaO_2/FiO_2: Arterial oxygen partial pressure/fractional inspired oxygen; CRP: C-reactive protein; CT: Computed tomography; ICU: Intensive care unit; ARDS: Acute respiratory distress syndrome; COVID-19: Coronavirus disease 2019; TNF-α: Tumor necrosis factor-alpha; IFN-γ: Interferon-gamma; TGF-β: Tumor growth factor-β; IL: Interleukin.

no difference in the outcome in patients treated with Adipose-MSCs *vs* the placebo. In another phase I study, nine consecutive patients were enrolled, between December 2017 and August 2019, the first three patients got low-dose human umbilical cord-derived MSCs, the following three patients received an intermediate dosage, and the last three patients received a high dose[100]. The results of the first phase of clinical trials demonstrated that a single dose of human umbilical cord-derived MSCs was safe and showed good results in all nine patients with ARDS. Swedish researchers tested the systemic administration of allogeneic BM-derived MSCs (2×10^6 cells/kg) in two patients with severe refractory ARDS, both of whom recovered from multiple organ failure and showed reduced markers of systemic and pulmonary inflammation[101]. In summary, clinical studies report that MSC administration is safe for patients with ARDS, with few adverse reactions. However, due to the relatively small number of patients in these studies, further research is needed to test the curative effect.

MSCS FOR CORONAVIRUS DISEASE 2019-INDUCED ARDS

Coronavirus disease 2019 (COVID-19) is an infectious disease responsible for the COVID-19 pandemic, caused by a novel coronavirus called severe acute respiratory syndrome-coronavirus 2[102,103]. COVID-19 has various respiratory and non-respiratory clinical manifestations, including mild or severe influenza-like syndrome, pneumonia, or respiratory failure, which may eventually lead to sepsis with multiple organ failure. The most common reason for being admitted to intensive care units is a respiratory failure caused by ARDS[104,105]. In a case series, Hashemian *et al*[106] found that multiple infusions of high-dose allogeneic prenatal MSCs are safe and can relieve the respiratory distress of severe patients with COVID-19 and inhibit the inflammatory response[106]. 24 participants were randomly assigned to either the umbilical cord-derived mesenchymal stem cell (UC-MSC) therapy or the control group in a double-blind, phase ½a randomized controlled trial. The UC-MSC treatment group got two intravenous infusions of $100 \pm 20 \times 10^6$ UC-MSCs, while the control group received two infusions of vehicle solution[107]. The primary endpoint was safety (adverse events) after 6 h; cardiac arrest or death within 24 h post-infusion) and secondary endpoints included patient survival at 31 d after the first infusion and time to recovery. Serious adverse events related to UC-MSC infusion were

not observed. Thus, UC-MSC is safe to inject into patients with COVID-19-induced ARDS. In subjects who received UC-MSC treatment, inflammatory cytokines decreased significantly on the sixth day. In a recent clinical research, 40 COVID-19 patients who were critically unwell got either saline or intravenous UC-MSCs[108]. The findings revealed that the survival rate of patients in the UC-MSCs group was 2.5 times greater than that of the control group. Among patients with complications, the UC-MSCs group had a fourfold greater survival rate than the control group. A multicenter, double-blind, randomized, placebo-controlled trial (STROMA–CoV-2) in France, with 45 enrolled patients, has also been conducted[109]. Patients were randomly assigned to receive three intravenous infusions of 1×10^6 UC-MSCs/kg or placebo (0.9% NaCl) over 5 d after recruitment. PaO_2/FiO_2 changes between D0 and D7 did not differ significantly between the UC-MSCs and placebo groups. Six (28.6%) of the 21 UC-MSCs patients and six (25%) of the 24 (25%) placebo patients had serious adverse events not related to UC-MSCs treatment. A phase I/II clinical study was also done in patients with severe COVID-19 to assess the safety and effectiveness of three intravenous infusions of BM-derived MSCs at 3-d intervals[110]. Eight intensive care unit patients requiring supplemental oxygen were treated with BM-MSCs. Survival was significantly higher in the MSC group at 28 and 60 d, but there was no significant difference in the number of invasive ventilation-free days, high flow nasal oxygenation-free days, oxygen support-free days, or intensive care unit-free days. MSC infusion was well tolerated, and no adverse effects associated with MSC infusion were reported. Furthermore, a single-center, open-label, phase 1 clinical trial enrolled 20 confirmed COVID-19 patients with mild-to-moderate degree ARDS, who were divided into two groups: The control and the intervention group (UC-MSCs)[111]. The patients received three intravenous infusions of UC-MSCs (1×10^6 cells/kg BW per injection) every other day. There were no adverse effects to cell infusion throughout the clinical study, oxygenation was greatly enhanced, anti-inflammatory factor levels were significantly increased, and pro-inflammatory factor levels were dramatically lowered. This intervention may reduce cytokine storms and restore respiratory function.

To summarize, MSCs from different tissues, such as BM, adipose, UC, and placental tissues, have entered the clinical trial stage. Some studies have used MSCs to treat COVID-19-induced ARDS.

PROSPECT

With the progress of scientific research, our understanding of the physiological and pathological processes of ARDS has gradually deepened, and the relevant treatment methods are also improving year by year. However, the final prognosis of patients has not improved much; therefore, it is particularly important to find a method to treat ARDS. MSCs have a variety of characteristics that are striking. At present, as a potential therapeutic method, MSCs have gradually entered the international arena of research and have been unanimously recognized by scientists worldwide. Their application has achieved some effect in improving the survival rate of patients with ARDS. However, because of various reasons, only a few clinical trials are conducted. Although the achievements of basic research are emerging endlessly, there is a theoretical basis for MSC use to enter clinical treatment, and the side effects of MSCs are not clear. Moreover, their clinical application involves ethical issues. As a cell therapy, its safety needs a lot of control experiments to be proven. This has hindered the successful application of MSCs. Fortunately, the basic experimental research on their mechanism of action is becoming more and more in-depth, and the application value of MSC therapy is also much clearer. Their successful application for the treatment of ARDS is expected to improve the quality of life of patients.

CONCLUSION

Due to the impasse that has been reached in the treatment of ARDS, MSC therapy has gained increasing attention. MSCs are known for their anti-inflammatory, differentiation, paracrine, and microvesicle transport abilities, which could perfectly target the pathological mechanisms of ARDS, providing a theoretical basis for treatment and precision treatment. Despite the current evaluation of MSC treatment of ARDS, further research is needed to observe the specific response to MSC treatment in the long term.

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FOOTNOTES

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REVIEW

Extracellular vesicles: Emerged as a promising strategy for regenerative medicine

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Abstract

Cell transplantation therapy has certain limitations including immune rejection and limited cell viability, which seriously hinder the transformation of stem cell-based tissue regeneration into clinical practice. Extracellular vesicles (EVs) not only possess the advantages of its derived cells, but also can avoid the risks of cell transplantation. EVs are intelligent and controllable biomaterials that can participate in a variety of physiological and pathological activities, tissue repair and regeneration by transmitting a variety of biological signals, showing great potential in cell-free tissue regeneration. In this review, we summarized the origins and characteristics of EVs, introduced the pivotal role of EVs in diverse tissues regeneration, discussed the underlying mechanisms, prospects, and challenges of EVs. We also pointed out the problems that need to be solved, application directions, and prospects of EVs in the future and shed new light on the novel cell-free strategy for using EVs in the field of regenerative medicine.

Key Words: Mesenchymal stem cells; Extracellular vesicles; Regenerative medicine; Cell-free strategy

Core Tip: Extracellular vesicles (EVs) play a critical role in tissue repair and regeneration medicine *via* cell-to-cell communication. In this review, we elaborate and discuss both the recent research progress and the advancements in the therapeutic effects and limitations of EVs in tissue regeneration and engineering medicine. Moreover, we summarize the underlying molecular mechanisms related to EV repair effects and point out the problems that need to be solved, application directions, and prospects of EVs in the future.

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INTRODUCTION

Tissue and organ loss are challenging complications that are usually caused by severe diseases such as cancer and serious accidents and impose great burdens on patients' lives. Tissue regeneration and engineering medicine, which aims to repair lost cells and damaged organs caused by diseases or accidents, is achieving great progress sparked by numerous studies of stem cells, biomaterials and so on. Stem cells, especially mesenchymal stem cells (MSCs), play a critical role in regeneration medicine due to their strong self-renewal ability and diverse differentiation potential. However, there are several shortcomings of using MSCs in regeneration medicine, including cell source limitations, ethical controversies, low survival rates after cell transplantation, immune rejection, and risk of tumorigenesis after transplantation[1,2]. To solve the shortcomings of cell-based therapy, the paracrine action of cells has become a focus of research attention.

Recent research has focused on the secretions of cells and tissues. Extracellular vesicles (EVs) are heterogeneous lipid bilayer-surrounded vesicles secreted by various cell types, including immune cells, endothelial cells, epithelial cells, neuronal cells, cancerous cells, Schwann cells (SCs), and MSCs, that behave as crucial mediators of intercellular communication[3-6]. EVs participate in various types of physiological and pathological activities, including immune responses, homeostasis maintenance, inflammation, angiogenesis, and cancer progression, by transferring biological signals[7,8]. According to their size and origin, EVs can be classified in several ways. On the basis of their origin, EVs can be divided into three categories: Apoptotic bodies generated during cell apoptosis; microvesicles originating from budding cellular membranes; and exosomes derived from multivesicular bodies in fusion with the plasma membrane[4,9]. EVs also can be divided into small EVs, medium-sized EVs, and large EVs[3,10,11].

Increasing evidence indicates that EVs play a critical role in tissue repair and regeneration medicine *via* cell-to-cell communication. Moreover, several studies imply that the beneficial effects of MSCs on tissue regeneration may be attributed to their paracrine action by secreting EVs rather than MSC engraftment and proliferation[3,12,13]. Moreover, EVs themselves possess the ability to recruit endogenous cells and lead to their enrichment by releasing several chemokines, which may contribute to angiogenesis and tissue repair[14,15]. Therefore, EVs are an appropriate and hopeful new source for tissue repair and regeneration.

In this review, we elaborate and discuss both the recent research progress and the advancements in the therapeutic effects and limitations of EVs in tissue regeneration and engineering medicine (Figure 1). Moreover, we summarize the underlying molecular mechanisms related to EV repair effects (Table 1) and point out the problems that need to be solved, application directions (Table 2), and prospects of EVs in the future.

EVs

The discovery of EVs dates back to 1940, when a brand-new subcellular factor was identified in cell-free plasma by high-speed centrifugation. The subcellular fraction was identified and shown to consist of small vesicles by electron microscopy in the 1960s. The term "exosomes" was introduced when vesicles were isolated from cell supernatant in 1987, and these exosomes were found to be related to the removal of obsolete transmembrane proteins[10]. Currently, EVs are secreted by both normal cells and cancerous cells and act as a means of cell-to-cell communication. Signals are communicated through vesicle membrane proteins or by vesicle contents such as proteins, miRNAs, or long noncoding RNAs (lncRNAs)[16].

Table 1 The mechanism of extracellular vesicles in the regeneration medicine

	Source of EVs	Mechanism
Kidney	MSC-EVs	Inhibiting oxidation, apoptosis, and inflammation
	HLSC-EVs	Regulating angiogenesis, the cell cycle, regeneration, autophagy, proliferation[36-38,46]
	BMSC-EVs	
Liver disease	hiPSCs-EVs	Inhibiting hepatocyte apoptosis
	Hepatocyte-EVs	Supporting hepatocyte function
	MSC-EVs	Promoting angiogenesis
		Reducing inflammatory responses[48-53]
Cardiac muscle	ESCs-EVs	Vascularization
	iPSCs-EVs	Amelioration of apoptosis and hypertrophy
	MSCs-EVs	Promoting cell proliferation and migration[55-60]
	CDCs-EVs	
	BMSC-EVs	
Tendon	BMSC-EVs	Modulating macrophage phenotypes
	ADSC-EVs	Anti-inflammatory reaction
		Enhancing proliferation, migration, tenogenic differentiation of TSCs
		Regulating angiogenesis
		Modulating immune responses[62-68]
Wound healing	BMSC-EVs	Promoting re-epithelialization
	ADSC-EVs	Promoting collagen maturity and angiogenesis
	hENSC-EV	Enhancing cell proliferation and migration[69-74]
	HUVECs-EVs	
	iPSC-EVs	
Tooth and periodontal tissue	BMSC-EVs	Modulating the inflammatory immune response
	ADSC-EVs	Enhancing cell proliferation and migration
	DPSC-EVs	Promoting odontogenic differentiation
	DFC-EVs	Stem cell recruitment[76-83]
	PDLSC-EVs	
	HERS-EVs	
	SC-EVs	
Nerve	SKP-SC-EVs	Mediating axon regeneration
	BMSC-EVs	Regulating the phenotype of Schwann cells
	ADSC-EVs	Promoting angiogenesis
	GMSC-EVs	Regulating inflammatory reactions[84-94]
Bone	ADSC-EVs	Angiogenesis
	BMSC-EVs	Osteoblast proliferation
	SMSC-EVs	Intercellular communication
	PRP-EVs	Immune regulation[96-104]

EVs: Extracellular vesicles; MSCs: Mesenchymal stem cells; HLSC: Human liver stem cells; hiPSCs: Human-induced pluripotent stem cells; ESCs: Embryonic stem cells; iPSCs: Induced pluripotent stem cells; CDCs: Cardiosphere-derived cells; hENSC: Human Endometrial stem cells; DPSC: Dental pulp stem cells; DFC: Dental follicle cell; PDLSC: Periodontal ligament stem cells; PRP: Platelet-rich plasma; SMSC: Synovium mesenchymal stem cells;

HERS: Hertwig's epithelial root sheath; BMSC: Bone marrow mesenchymal stem cell; ADSC: Adipose stem cells; GMSC: Gingiva-derived mesenchymal stem cell; SKP-SC: Skin-derived precursor Schwann cells; TSC: Trophoblast stem cells.

Table 2 Extracellular vesicles investigated in clinical studies

Research title	Interventions	Status
Treatment of Patients with Bone Tissue Defects Using Mesenchymal Stem Cells Enriched by Extracellular Vesicles	MSCs enriched by extracellular vesicles	Not yet recruiting
Bone Marrow Mesenchymal Stem Cell Derived Extracellular Vesicles Infusion Treatment for ARDS	Bone Marrow MSC Derived Extracellular	Not yet recruiting
Efficacy of Platelet- and Extracellular Vesicle-rich Plasma in Chronic Postsurgical Temporal Bone Inflammations	Plateletand extracellular vesicle-rich plasma	Completed
Use of Autologous Plasma Rich in Platelets and Extracellular Vesicles in the Surgical Treatment of Chronic Middle Ear Infections	Plateletand EVs-rich plasma	Recruiting
Extracellular Vesicle Infusion Treatment for COVID-19 Associated ARDS	ExoFlo	Completed
Safety of Mesenchymal Stem Cell Extracellular Vesicles (BMMSC-EVs) for the Treatment of Burn Wounds	Drug: AGLE-102 (BMMSC-EVs)	Not yet recruiting
Treatment of Non-ischemic Cardiomyopathies by Intravenous ExtracellularVesicles of CardiovascularProgenitor Cells	Extracellular vesicle-enriched secretome of cardiovascular progenitor cells differentiated from induced pluripotent stem cells	Not yet recruiting
Bone Marrow Mesenchymal Stem Cell Derived Extracellular Vesicles Infusion Treatment for Mild-to-Moderate COVID-19: A Phase II Clinical Trial	Drug: ExoFlo. Bone Marrow Mesenchymal Stem Cell Derived Extracellular Vesicles	Not yet recruiting
A Safety Study of IV Stem Cellderived Extracellular Vesicles (UNEX-42) in Preterm Neonates at High Risk for BPD	UNEX-42 is a preparation of extracellular vesicles that are secreted from human bone marrow-derived mesenchymal stem cells suspended in phosphate-buffered saline	Terminated
ExoFlo™ Infusion for Post-Acute COVID-19 and Chronic Post COVID-19 Syndrome	Bone Marrow Mesenchymal Stem Cell Derived Extracellular Vesicles	Not yet recruiting
Study of ExoFlo for the Treatment of Medically Refractory Ulcerative Colitis	ExoFlo. Intravenous administration of bone marrow mesenchymal stem cell derived extracellular vesicles	Recruiting
Study of ExoFlo for the Treatment of Medically Refractory Crohn's Disease	ExoFlo. Intravenous administration of bone marrow mesenchymal stem cell derived extracellular vesicles	Recruiting
Bone Marrow Mesenchymal Stem Cell Derived EVs for COVID-19 Moderate-to-Severe ARDS: A Phase III Clinical Trial	EXOFLO. Bone Marrow Mesenchymal Stem Cell Derived EVs	Recruiting
Pilot Study of Human Adipose Tissue Derived Exosomes Promoting Wound Healing	Adipose tissue derived exosomes	Not yet recruiting
Exosome Effect on Prevention of Hairloss	Placental Mesenchymal Stem Cells-derived Exosome	Recruiting
Expanded Access for Use of ExoFlo in Abdominal Solid Organ Transplant Patients	Bone Marrow Mesenchymal Stem Cell Derived Extracellular Vesicles Infusion Treatment	Not yet recruiting
Safety of Injection of Placental Mesenchymal Stem Cell Derived Exosomes for Treatment of Resistant Perianal Fistula in Crohn's Patients	Placenal MSC derived exosomes	Active, not recruiting
Safety and Efficacy of Injection of Human Placenta Mesenchymal Stem Cells Derived Exosomes for Treatment of Complex Anal Fistula	Placenta MSCs derived exosomes	Recruiting
Safety and Tolerability Study of MSC Exosome Ointment	Exosome ointment	Completed
The Pilot Experimental Study of the Neuroprotective Effects of Exosomes in Extremely Low Birth Weight Infants	Exosomes derived from MSCs	Not yet recruiting

ARDS: Acute Respiratory Distress Syndrome; MSC: Mesenchymal stromal cells; COVID-19: Coronavirus disease 2019; EVs: Extracellular vesicles.

Biogenesis and classification of EVs

EVs are classified by several traits, including their density, dimensions, and origin. Three subclasses of EVs have been reported recently. Although the sizes of these three main types of EVs may overlap, their biogenesis is different. Exosomes, whose dimensions range from 30-150 nm, are formed within the endosomal network, where endosomes target some protein/lipids for recycling or exocytosis. During early endosome transformation into late endosomes, proteins that are fated to be degraded or exported

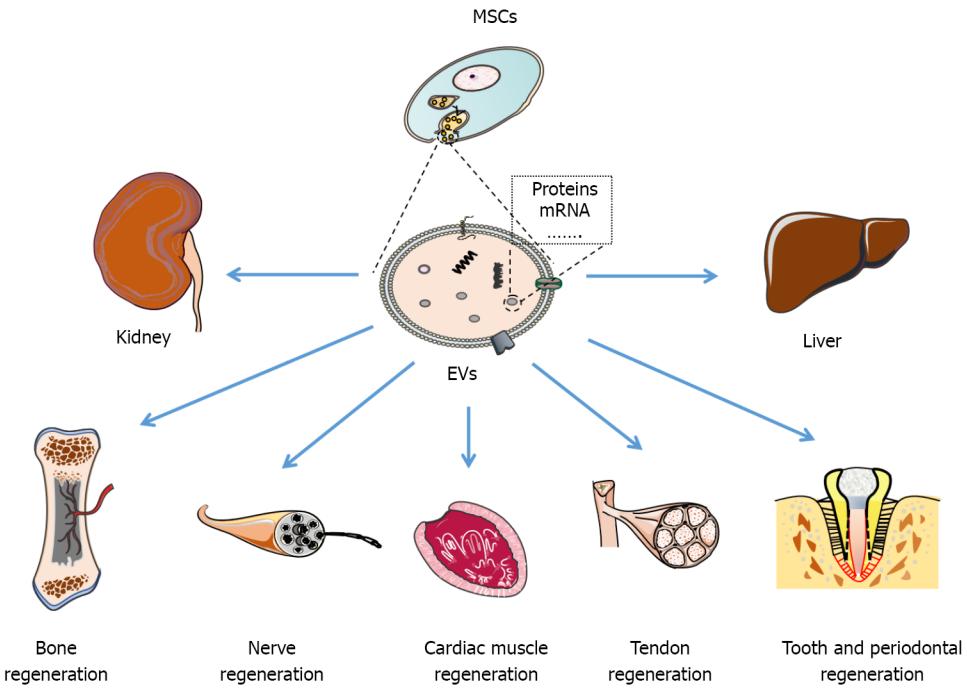

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Figure 1 The repair and regeneration effects of extracellular vesicles in different tissue and organs. EVs: Extracellular vesicles; MSCs: Mesenchymal stem cells.

are packaged into vesicles. Late endosomes, which contain small vesicles, fuse with the plasma membrane and finally lead to the secretion of small vesicles, named exosomes, into the extracellular space[16]. Microvesicles/Ectosomes originate from vesicles budding outward and fission of the plasma membrane directly, which is a dynamic interplay between redistribution and cytoskeletal protein contraction. The size of microvesicles/ectosomes is larger than that of exosomes, ranging from 50-2000 nm. Apoptotic bodies, ranging from 500-40000 nm, are formed during programmed cell death and contain organelles.

Isolation of EVs

Differential ultracentrifugation (UC), the gold standard EVs isolation approach, is the most reported and classical way to isolate EVs. Successive centrifugation was applied to eliminate large dead cells and cell debris. The supernatant acquired after successive centrifugation was used for another UC step at 100000 g to obtain the pellet related to EVs. The pellet was washed with PBS to obtain purified EVs[17]. UC has several advantages including low cost and low contamination risk with extra isolation reagents. UC is suitable for large volume preparation for its products is of high purity. However, UC is complicated, time-consuming and labor intensive. High speed centrifugation also may lead to potential mechanical damage. There are several isolation methods that can extract EVs in a more efficient way, including polymer-based precipitation[18], size exclusion chromatography (SEC)[19], ultrafiltration (UF)[20], flow field-flow fractionation[21], immunoaffinity capture[22], and microchip-based techniques [23]. Polymer-based precipitation is a commonly used strategy for EVs isolation. The principle of polymer-based precipitation is high hydrophilic water-excluding polymers can alternate the solubility of EVs. Highly hydrophilic polymers interact with water molecules surrounding the EVs to create a hydrophobic micro-environment, resulting in EVs precipitation[24]. Based on above principle, polyethylene glycol is well used in several popular commercial EVs isolation kits[25]. Polymer-based precipitation has high efficiency, but its products is easy to be contaminated by protein aggregates. SEC is according to the size of particle to realize the isolation. After adding to porous materials, substances eluted out in accordance with their particle size, with big particles eluted earlier[26]. Compared to UC, SEC is realized by the performance of passive gravity flow, which highly protect the structure and integrity of EVs[27]. UF uses filter membrane to isolate EVs from cell culture medium. Compared to traditional UC method, UF-based EVs isolation shortens time and presents relatively low requirements on experimental facilities. However, UF also has several shortages including the EVs production maybe limited due to clogging and membrane trapping[28,29]. Immunoaffinity capture is based on specific binding between EVs markers and immobilized antibodies such as Rab5, CD81, CD63, CD9, CD82, annexin, and Alix[30]. Immunoaffinity capture can harvest high-purity EVs with no chemical contamination. However, it is waste of antibodies and low yields[26]. The combination of the above methods is

also applied in the isolation of EVs. Moreover, there are several kits that are available for EV isolation, including ExoQuick and Total Exosome Isolation kits. However, the purity and quality of isolated EVs are extremely important because some soluble proteins or lipoproteins may be coisolated with EVs, leading to inaccurate experimental results. Therefore, isolation methods are of critical importance. Among all the methods, UC and UF followed by SEC are reported to be the most appropriate isolation methods because they can isolate high-purity and high-quality EVs[31,32].

Characteristics of EVs

EVs carry several specific surface markers, including proteins related to the cell membrane, annexin, flotillin and auxiliary proteins. Annexins and tetraspanins such as CD9, CD63 and CD81, which are often used for the identification of EVs, are located in the membrane of EVs. Moreover, EVs express ALIX, tumor susceptibility gene 101, VPS4 and heat shock proteins (HSP70 and HSP 90), which are associated with the biogenesis of EVs. EVs are encapsulated in a bilayer membrane, which can help in the safe transfer of their contents to secondary cells. Once released into the extracellular environment, EVs interact with recipient cells in three ways: (1) Endocytic uptake; (2) direct fusion with cells; and (3) adhesion to the cell surface and transmission of contents[4]. Among their contents, EVs are reported to contain large-scale genetic materials, such as mRNA, that play critical roles in cell-to-cell communication. miRNAs that are transferred by EVs also have an impact on biological functions, including cell proliferation, migration, and differentiation of recipient cells. EVs also carry several types of lipids that are related to EV structure, function and biogenesis[3]. In conclusion, EVs are heterogeneous and are composed of a bilayer membrane surrounding cargos that are indispensable for cell interactions.

REPAIR AND REGENERATION EFFECTS OF EVS

Kidney

Acute kidney injury (AKI) and chronic kidney disease (CKD) are two major causes of renal failure and exert great pressure on public health. AKI, the most common features of which are the rapid loss of renal tubular cells and a decline in renal function, usually leads to hospitalization[33]. As research has progressed, evidence has shown that MSC-EVs play a major role in treating AKI. EVs were first proven to be effective against AKI in 2009[34]. The intravenous administration of EVs not only alleviated or even reversed the detrimental effect on renal function caused by glycerol injection in an AKI model but also improved renal function and morphology by stimulating the proliferation of tubular epithelial cells (TECs)[35]. In a renal ischemia-reperfusion (I/R) injury AKI model, MSC-EVs accumulated in the renal tubules and facilitated with the recovery of kidney function through the Keap1-Nrf2 signaling pathway as well as the mitochondrial function of TECs[36]. Studies have also shown that EVs can reduce the presence of luminal cell debris, tubular hyaline casts and necrosis of tubular cells in an AKI model induced by toxins[37]. Moreover, EVs released by cells that were cultured under hypoxia could stimulate angiogenesis and help the formation of the peritubular microvasculature[38]. Furthermore, MSC-EVs attenuated mtDNA damage and inflammation after AKI through the mitochondrial transcription factor A (TFAM) pathway. MSC-EVs could attenuate renal lesion formation, mitochondrial damage, and inflammation in mice with AKI. TFAM overexpression (TFAM-OE) improved the rescue effect of MSC-EVs on mitochondrial damage and inflammation to some extent[39]. In general, MSC-EVs can relieve AKI not only by inhibiting oxidation, apoptosis, and inflammation, but also through regulating angiogenesis, cell cycle, autophagy, and cell proliferation[38]. There are multiple underlying mechanisms. Currently, it is believed that the repair effects of EVs on AKI are largely related to their transfer of genetic material and proteins[40].

CKD is a complex and long-term disease. The main trigger that causes CKD is diabetes. Hyperglycemia leads to glomerular and tubulointerstitial fibrosis, and the progression of fibrosis is the main reason for renal dysfunction[41]. Renal glomerulosclerosis and tubulointerstitial fibrosis are hallmarks of all types of CKD, including diabetic nephropathy (DN). Recent studies have shown that EVs are effective for the prevention of DN. For example, EVs can protect podocytes and TECs from apoptosis by secreting protective proteins, including transforming growth factor (TGF)-β1 and angiogenin[42]. Another study found that EVs derived from human liver stem cells (HLSCs) and MSCs could reduce or even revert the progression of profibrotic processes and finally ameliorate renal dysfunction and attenuate renal histopathological changes[43]. Moreover, studies have shown that EVs can ameliorate DN by inducing autophagy induction through the mTOR pathway. Rat bone marrow-derived EVs present nephroprotective and antifibrotic effects by upregulating autophagy through suppressing the mTOR pathway in a DN model[44]. Furthermore, HLSC-derived EVs could also prevent the development of CKD. HLSC-EVs not only present a regenerative and anti-inflammatory role but also downregulate profibrotic genes, including alpha smooth muscle actin, Col1a1 and TGF-β1, and modulate miRNAs that are related to the fibrotic pathway in the kidney of an aristolochic acid-induced CKD model[45]. In conclusion, MSC-EVs can eliminate the pathogenic damage of CKD by targeting renal fibrosis, reducing tubular atrophy and inflammation, and promoting angiogenesis to

facilitate with tissue regeneration[46]. EVs present a promising approach for renal repair and regeneration. Some evidence based on clinical trials has shown that EVs are safe and effective for CKD patients[47].

Liver disease

Liver dysfunction is classified into acute and chronic diseases, including hepatitis, alcoholic liver disease, fatty liver disease, cirrhosis, and hepatocellular carcinoma. Liver failure can manifest with several symptoms, such as jaundice, encephalopathy, cerebral edema, sepsis, and gastrointestinal bleeding, and its prognosis is relatively limited. Currently, liver transplantation is still the gold-standard therapy for liver diseases; however, it has many limitations, such as nonspecific treatment approaches, donor organ shortages and lifelong immunosuppressive therapy[48]. Although the liver possesses a great capacity for regeneration through the proliferation of mature liver cells, it can lose its function and lead to severe results when the injury progresses into a state of functional impairment, which may finally lead to liver failure or even death[49]. EVs can prevent further damage to injured liver cells. Studies have reported that EVs can reduce liver injury based on alanine aminotransferase and aspartate aminotransferase levels after CCl_4-induced liver impairment *in vivo*. Moreover, EVs promoted hepatocyte regeneration gene expression and $PCNA^+$ expression and induced quiescent hepatocytes to re-enter the cell cycle, ultimately assisting with hepatocyte proliferation. Another report showed that EVs derived from human-induced pluripotent stem cells (hiPSCs) could alleviate hepatic I/R injury by suppressing inflammatory responses, attenuating the oxidative stress response and inhibiting cell apoptosis[50]. Furthermore, Du *et al*[51] found that hiPSC-derived EVs could alleviate hepatic I/R injury by activating sphingosine kinase and the sphingosine-1-phosphate pathway in hepatocytes and ultimately promote cell proliferation[51]. EVs can not only be therapeutic but can also serve as diagnostic tools for liver disease and regeneration in the near future. A report outlined that hepatocyte-derived EVs played a key role in hepatocyte-to-hepatocyte communication and provided a new method for liver disease diagnosis, and progenitor cell-derived EVs offered a new opportunity for the treatment of liver diseases[52]. In conclusion, recent evidence supports that MSC-derived EVs inhibit hepatocyte apoptosis, support hepatocyte function, promote angiogenesis and hepatocyte proliferation, and reduce inflammatory responses by preventing immunocyte infiltration and inflammatory cytokine secretion [48]. In addition, animal model-based studies suggest that EVs may represent a novel and effective cell-free therapeutic agent as an alternative to cell-based therapies for patients with liver diseases[53].

Cardiac muscle regeneration

Myocardial repair and regeneration are important in the context of the increasing occurrence of heart failure and cardiac-related diseases. Key mechanisms related to cardiac repair and regeneration include survival and protection, inflammation reduction, angiogenesis, cardiomyogenesis and cell–cell communication. All these mechanisms work collectively and contribute to cardiac regeneration[54]. However, myocardial repair is slow and limited. Stem cell–based therapies have acted as an effective method for cardiac repair. Moreover, EVs, a key component of stem cell secretion, bring new hope to cell-free therapies for cardiac repair and regeneration. EVs of embryonic stem cells, iPSCs, MSCs, and cardiosphere-derived cells (CDCs) have been proven to be effective in cardiac repair[55]. Recent research has shown that EVs derived from murine iPSCs impart cytoprotective properties to cardiac cells *in vitro* and induce cardiac repair *in vivo* through vascularization, amelioration of apoptosis and hypertrophy and improvement in left ventricular function[56]. Moreover, iPSC-EVs contained numerous miRNAs (miR-17-92 cluster) and proteins [BMP-4, teratocarcinoma-derived growth factor 1 and vascular endothelial growth factor (VEGF)-C] that are related to cellular proliferation and differentiation, enhanced angiogenesis, and the prevention of apoptosis. Therefore, iPSC-EVs could induce angiogenesis, migration and anti-apoptosis of cardiac endothelial cells and finally induce superior infarct repair[56]. MSC-derived EVs also play a critical role in MSC-based therapy in cardiac diseases. Bian *et al*[57] found that intramyocardial injection of MSC-EVs could markedly enhance blood flow recovery in an acute myocardial infarction (MI) rat model. MSC-EVs could protect cardiac tissue from ischemic injury by promoting blood vessel formation. Receptors for growth factors, cytokines and signaling molecules are contained in EVs, among which Sonic hedgehog and platelet-derived growth factors have been proven to be effective for proangiogenic activities. The results of direct comparisons between MSCs and MSC-EVs have highlighted the beneficial effects of EVs. EVs also present a safer profile than their cells of origin because EV injection does not produce tumors[56]. Moreover, combinatorial treatment with both MSCs and their derived EVs exhibits advantages in MI treatment. Rat bone marrow mesenchymal stem cells (BMSCs) and their derived EVs improved cardiac function and reduced infarct size when compared to groups treated with BMSCs or EVs alone[57]. CDC exosomes were proven to enhance angiogenesis and promote cardiomyocyte survival and proliferation. Moreover, CDC exosomes improved cardiac function and imparted structural benefits after MI. miR-146a was the most highly enriched in CDC exosomes and mediated some of the therapeutic benefits of CDC exosomes[58]. EVs combined with biomaterials present better effects for the treatment of cardiac disease. Studies have shown that MSC-derived EVs incorporated into alginate hydrogels are a sustained delivery system that allows for better retention of EVs in the heart *in vivo* than EV single injection. EVs-Gel has a good effect on promoting angiogenesis, reducing the apoptosis and fibrosis of cardiac tissue,

enhancing scar thickness and improving cardiac function[59]. Another study showed that self-assembling peptide hydrogel-encapsulated exosomes could also promote cardiac repair due to the better retention and stability of EVs[60]. In general, EVs combined with biomaterials provide a novel approach for cell-free therapy and optimization of the therapeutic effects of EVs in MI.

Tendon regeneration

Tendons are soft tissues that connect muscles to bones. Tendon diseases and injuries have high morbidity owing to various sports and exercises. The effects of treatments for tendon diseases are limited due to the limited regenerative capacity of tendons. Current treatments for tendon injuries, including surgery and conservative treatments, always have side effects, including secondary injury and scar formation. Therefore, the ideal treatment is to promote tendon regeneration[61]. There are three important stages of tendon healing: Inflammation, proliferation, and remodeling. Suppression of the inflammatory response is critical for tendon repair[62,63]. Research has found that BMSC-EVs may help improving tendon healing by regulating macrophage phenotypes, creating anti-inflammatory environment, promoting apoptotic cell accumulation, and increasing the ratio of tendon resident stem/progenitor cells[64]. Moreover, EV-educated macrophages (EEMs), a kind of M2-like macrophage treated by EVs, present a more functional and regenerative ability to heal tendons. EVs isolated from MSCs were able to reduce the M1/M2 macrophage ratio and increase the number of endothelial cells 14 days after tendon injury. Injured tendons treated with exogenous EEMs presented improved mechanical properties, reduced inflammation and earlier angiogenesis[62]. Furthermore, Yu *et al*[29] found that BMSC-EVs could promote the proliferation, migration and tenogenic differentiation ability of tendon stem/progenitor cells (TSPCs). BMSC-derived EVs could activate the regenerative potential of endogenous TSPCs in tendon injury sites. EVs embedded in fibrin glue may allow the sustained release of EVs in injured regions and promote the regeneration of patellar tendon tissue in rats[15]. In addition to BMSCs, ADSC-EVs can also promote the proliferation and migration of tendon cells, reduce fatty infiltration, promote tendon-bone healing, improve the biomechanical properties of the tendon-bone junction and improve the mechanical strength of the repaired tendon[65,66]. The overexpression of H19 can enhance tendon regeneration potential. As an ideal drug carrier, EVs are a reliable delivery method to transmit lncRNAs. Reports have shown that engineered EVs with overload of H19 can regulate tendon regeneration by activating YAP through the H9-pp1-YAP axis[67]. In conclusion, MSC-EVs facilitate tendon regeneration by inhibiting apoptosis as well as enhancing the proliferation, migration and tenogenic differentiation of tendon stem cells and tenocytes. In addition, they can also regulate angiogenesis and modulate immune responses and extracellular matrix (ECM) remodeling of tendon tissue[68]. These results indicate that EVs have broad prospects in tendon repair and regeneration.

Skin and wound healing

EVs are currently widely used in wound healing and skin regeneration. EVs can participate in four stages of wound healing, namely, hemostasis, inflammatory response, cell proliferation and remodeling [69]. Both ADSC-EVs and BMSC-EVs exert benefits on cells related to skin wound healing, including fibroblasts, keratinocytes and endothelial cells, in different ways. BMSC-EVs mainly promote proliferation, whereas ADSC-EVs have a major effect on angiogenesis. BMSC-EVs and ADSC-EVs presented synergistic effects on wound healing[70]. Great progress has been made in the field of wound healing with the rapid development of the combination of EVs and biomaterials. EVs and glycerol hydrogels have synergistic effects on the proliferation of human skin fibroblasts. The full-thickness excisional wound model in mice showed that the fibrosis, vascularization, and epithelial thickness of wounds reached a maximum level after treatment with EV-loaded hydrogels. Moreover, research has found that EVs derived from human endometrial stem cells (hENSCs) contain several growth factors, including VEGF, basic fibroblast growth factor and TGF-1, which benefit angiogenesis. hENSC-EV-loaded chitosan hydrogel has positive impacts on wound healing by promoting angiogenesis and tissue granulation formation. hENSC-EV-loaded chitosan hydrogel could be an ideal scaffold for skin wound dressing and skin tissue regeneration[71]. EVs derived from HUVECs (HUVECs-EVs) could promote the proliferation and migration activities of keratinocytes and fibroblasts, which are two critical cells for skin regeneration. Gelatin methacryloyl (GelMA) hydrogel scaffolds combined with HUVEC-EVs could not only repair wound defects but could also achieve sustained release of EVs, which promoted re-epithelialization, collagen maturity and angiogenesis that ultimately contributed to wound healing[72]. In addition to wound healing and skin regeneration, EVs also play critical roles in skin aging and several skin diseases. For example, ADSC-EVs can relieve atopic dermatitis. ADSC-EVs can also promote cuticle hydration and ceramide synthesis and significantly reduce the secretion of inflammatory cytokines [interleukin (IL)-4, IL-5, IL-13, and IL-17] when applied *in vivo*[73]. Moreover, ADSCs could protect against oxidative stress by promoting the proliferation of human dermal fibroblasts (HDFs) and inhibiting reactive oxygen species and MMP production *via* the secretion of various cytokines. ADSC-EVs might be potential therapeutic tools for addressing the problem of photoaging [74]. Studies have also shown that EVs derived from human iPSCs could regulate the genotypic and phenotypic changes of HDFs induced by UV photoaging and natural aging. EVs derived from human iPSCs affected cellular responses related to skin aging, including the expression levels of MMP-1 and type I collagen and the proliferation and migratory ability of HDFs. iPSC-EVs can mediate intracellular

transportation and reconstruct the matrix in aging skin by enhancing the expression of structural proteins and regulating the expression of age-related proteins[75]. In general, EVs serve as efficient carriers of molecular cargos for wound healing and skin regenerative medicine.

Tooth and periodontal regeneration

EVs have been widely used in the repair of dental tissue, including dental pulp, dentin and periodontal tissue. Dental pulp stem cells (DPSCs)-EVs can bind to matrix proteins such as type I collagen and fibronectin, enabling them to be tethered to biomaterials. EVs are endocytosed by both DPSCs and human MSCs in a dose-dependent and saturable manner *via* the caveolar endocytic mechanism and trigger the P38 mitogen-activated protein kinase (MAPK) pathway. In addition, EVs can trigger the increased expression of genes required for odontogenic differentiation. In the generated pulp tissue, DPSC-EVs can also promote the expression of several growth-promoting factors, including TBG-β and BMP-2, which ultimately promote the repair of dental pulp tissue[76]. Moreover, DPSC-EVs can promote the proliferation and angiogenesis of vascular endothelial cells by activating the P38/MAPK pathway, which provides the possibility for the regeneration of vascular pulp tissue[77]. SC-EVs could promote DPSC proliferation and enhance neurite outgrowth, neuron migration, and vessel formation *in vitro*. SC-EVs facilitate dental pulp regeneration through endogenous stem cell recruitment *via* the SDF-1/CXCR4 axis without exogenous cell transplantation[78]. In addition to SC-EVs, EVs derived from Hertwig's epithelial root sheath cells could trigger regeneration of dental pulp-dentin-like tissue composed of hard (regenerative dentin-like tissue) and soft (blood vessel and neuron) tissue in an *in vivo* tooth root slice model by activating the Wnt pathway[79]. Periodontitis is the primary cause of tooth loss, but there is no effective treatment to repair inflammatory bone loss in periodontitis. In terms of periodontal tissue regeneration, studies have shown that EVs secreted by periodontal ligament stem cells (PDLSCs) are therapeutics for bone defects in periodontitis. EVs derived from healthy PDLSCs could rescue the osteogenesis capacity of endogenous stem cells under an inflammatory environment and promote the regeneration of alveolar bone by recovering the osteogenic differentiation ability of inflammatory PDLSCs through the inhinbitation of canonical Wnt signaling[80]. BMSC-EVs are also an ideal cell-free strategy for periodontal regeneration. BMSC-EVs could promote the regeneration of periodontal tissues through the OPG-RANKL-RANK signaling pathway to regulate the function of osteoclasts and affect macrophage polarization and TGF-β1 expression to modulate the inflammatory immune response, thereby inhibiting the development of periodontitis and immune damage in periodontal tissue[81]. Furthermore, the therapeutic effect of ADSC-EVs was the same as that of periodontal surgery in a rat periodontitis model[82]. Lipopolysaccharide (LPS)-induced dental follicle cell-derived EVs (L-D-EVs) could promote the proliferation of periodontal ligament cells. L-D-EV-loaded hydrogel applied in the treatment of periodontitis was beneficial to repairing lost alveolar bone in the early stage of treatment and maintaining the level of alveolar bone in the late stage of treatment in experimental periodontitis rats by decreasing the expression of the RANKL/OPG ratio *in vivo*[83]. EVs are presented as a novel cell-free therapeutic strategy for both dental pulp and periodontal regeneration.

Nerve regeneration

Neurological dysfunction usually causes great physical and psychological distress for patients. Autologous nerve transplantation is widely accepted as the gold standard for peripheral nerve repair, but its inherent defects greatly reduce its availability. Regeneration of peripheral nerves after injury remains a great challenge for researchers. EVs play a fundamental role in the physiological and pathological processes of the nervous system. There is growing evidence that EVs can play a neurotherapeutic role by mediating axon regeneration, activating SCs, promoting angiogenesis, and regulating inflammatory reactions. EVs from skin-derived precursor SCs (SKP-SC-EVs) could promote neurite outgrowth of sensory and motor neurons *via* the AKT/mTOR/p70S6K pathway *in vitro*[84,85]. SKP-SC-EV-incorporating silicone conduit nerve grafts could significantly accelerate the recovery of motor, sensory, and electrophysiological functions of rats; facilitate outgrowth and myelination of regenerated axons; and alleviate denervation-induced atrophy of target muscles, which raises the possibility of cell-free therapy in nerve regeneration[86]. Moreover, repair SCs (rSCs) could also release pro-regenerative EVs. Neuronal activity enhances the release of rSC-derived EVs and their transfer to neurons *via* the ATP-P2Y signaling pathway[87]. Mechanical stimuli could control the intercellular communication between neurons and SCs by changing the composition of miRNA in SC-EVs. MS-SC-EVs transferd miR-23b-3p from mechanically stimulated SCs to neurons, and lead the inhibition of neuronal Nrp1 expression, which was indicated the beneficial effect of MS-SC-EVs on axonal regeneration, and provided evidence for the role of miR-23b-3p-enriched EVs in peripheral nerve injury repair[88]. BMSC-EVs could promote the functional recovery of sciatic nerve injury and increase the expression of GAP-43, a marker of axon regeneration[89]. BMSC-EVs can also promote nerve regeneration by regulating the miRNA mediated genes which related to regeneration, such as vascular endothelial growth factor A and S100b[90]. In addition to BMSC-EVs, ADSC-EVs also benefited nerve regeneration. ADSC-EVs could increase neurite outgrowth *in vitro* and enhance regeneration after sciatic nerve injury *in vivo*[91]. Furthermore, ADSC-EVs contained mRNAs of neurotrophic factors, including NGR, brain-derived neurotrophic factor, ciliary neurotrophic factor and glial cell-derived neurotrophic factor. ADSC-EVs could deliver mRNAs as well as microRNAs that facilitate with neurotrophic factor

secretion and proliferation, support the SC repair phenotype, and provide a solid therapeutic evidence for nerve regeneration[92]. Gingiva-derived mesenchymal stem cell (GMSC)-derived EVs could obviously promote axonal regeneration and functional recovery of injured mouse sciatic nerves. GMSC-derived EVs promoted the expression of SC dedifferentiation/repair phenotype-related genes *in vitro*, particularly c-JUN, a key transcription factor that drives the activation of the repair phenotype of SCs during PNI and regeneration[93]. Revascularization treatment is a critical measure for nerve repair. A report revealed that EVs derived from hypoxic preconditioned HUVECs could facilitate MSC angiogenesis activity and the anti-inflammatory impacts of MSCs, which contribute to *in vivo* effective nerve tissue repair after rat spinal cord transection and provide inspiration for therapies based on stem cells and EVs[94]. In general, EVs are an effective therapeutic tool in nerve regeneration by mediating axon regeneration, regulating the phenotype of SCs, promoting angiogenesis, and regulating inflammatory reactions.

Bone regeneration

EVs play four potential roles in bone regeneration, namely, angiogenesis, osteoblast proliferation, intercellular communication, and immune regulation. EV-mediated intercellular communication between osteoblasts and osteoclasts may represent a novel mechanism of bone modeling and remodeling. EVs transmit signals between osteoblasts and osteoclasts to regulate bone remodeling. Osteoblasts release RANKL-containing EVs, which are transferred to precursor osteoclasts and promote osteoclast formation by stimulating RANKL-RANK signaling[95]. MSC-derived EVs (MSC-exos), with their inherent capacity to modulate cellular behavior, are emerging as a novel cell-free therapy for bone regeneration. Reports have revealed that EVs derived from osteoinductive BMSCs (BMSC-OL-EVs) contribute to bone regeneration *via* multicomponent exosomal miRNAs (let-7a-5p, let-7c-5p, miR-328a-5p and miR-31a-5p), which target Acvr2b/Acvr1 and regulate the competitive balance of Bmpr2/Acvr2b toward Bmpr-elicited Smad1/5/9 phosphorylation[96]. Moreover, lyophilized delivery of BMSC-OI-EVs on hierarchical mesoporous bioactive glass scaffolds showed the possibility of bone regeneration in a rat cranial defect model[96]. Umbilical MSC-derived exosomes (uMSCEXOs) also showed great promise in bone regeneration. uMSCEXOs encapsulated in hyaluronic acid hydrogel and combined with customized nanohydroxyapatite/poly-ε-caprolactone (nHP) scaffolds could repair cranial defects in rats by promoting the proliferation, migration, and angiogenic differentiation of endothelial progenitor cells *via* the miR-21/NOTCH1/DLL4 signaling axis[97]. Compared to EVs derived from uMSCs exposed to normoxia, EVs derived from uMSCs treated with hypoxia promoted angiogenesis, proliferation, and migration to a greater extent. Hypo-Exos facilitated with the recovery of bone fracture by exosomal miR-126 and the SPRED1/Ras/Erk signaling pathway. In addtion, hypoxia preconditioning promote the transferring of exosomal miR-126 through the activation of hypoxia-inducible factor-1α. Hypoxia preconditioning is an effective and promising approach for optimizing the therapeutic actions of MSC-derived exosomes for bone fracture healing[98]. Moreover, the comparison between adipose, bone marrow, and synovium-derived EVs (ADSC-EVs, BMSC-EVs, and SMSC-EVs, respectively) showed that ADSC-EVs, BMSC-EVs, and SMSC-EVs could facilitate the viability and migration of MSCs and possessed favorable capacities for chondrogenesis and osteogenesis. Among these three types of EVs, ADSC-EVs presented the best performance. The different effects between ADSC-EVs, BMSC-EVs, and SMSC-EVs could be attributed to the different factors associated with the focal adhesion, ECM-receptor interaction, actin cytoskeleton regulation, cAMP, and PI3K-Akt signaling pathways[99]. EVs constitutively expressing BMP2 could facilitate the effects of bone regeneration. BMP-engineered EVs potentiate the BMP2 signaling cascade, possibly due to an altered miRNA composition. EV functionality may be engineered by genetic modification of the parental MSCs to induce osteoinduction and bone regeneration[100]. EVs combined with biomaterials present a better effect on bone repair. Three-dimensional engineered scaffolds (PLAs) complexed with human gingival MSCs have therapeutic effects that can improve bone tissue regeneration[101]. EVs combined with tricalcium phosphate-modified scaffolds can promote osteogenic differentiation of cells and promote the recovery of cranial defects *in vivo* by activating the PI3K/Akt signaling pathway[102]. In addition to bone regeneration, MSC-EVs or platelet-rich plasma-derived EVs also have high therapeutic value for treating osteoarthritis by suppressing the inflammatory immune microenvironment. BMSC-derived exosomes can effectively promote cartilage repair and extracellular matrix synthesis and alleviate knee pain in OA rats[103]. In addition, the modification of EVs and the combination of EVs with biomaterials can enhance targeting effects and extend retention which contribute to an effectively treatment of OA [104].

PROSPECTS AND CHALLENGES OF EVS IN TRANSLATIONAL MEDICINE

Previous studies based on EVs have shed new light on the application of EVs in tissue regeneration, but there are some major problems that still need to be solved in the clinical translation of EVs. First, the storage of EVs is unstable. EVs can be stored at 80 °C for several months; however, the pH value of the storage solution and freeze-thaw cycles can affect EV activity. The transport and storage conditions of

EVs need to be further studied. Second, there is no effective method to isolate purified EVs in large quantities. At present, EVs are extracted mainly by UC, immunoadsorption, precipitation or microfluidic separation and are easily contaminated by proteins. Nowadays, there are several strategies to enhance the purity and quality of harvested EVs. From the perspective of stimulation of EVs secretion, many factors including protein regulations, thermal and oxidative stress, oxygen concentration, low pH (< 6.0), radiation, starvation can enhance the secretion of EVs from cells[105]. From the perspective of the methods of EVs isolation, how to simplify the isolation procedure and improve the EVs yield are the main two obstructions in the research field of EVs. Recent report reveals that the isolation procedure of EVs may be simplified with the improvement of newly EVs separation techniques including immunoaffinity, chromatography and polymer precipitation[106]. Moreover, with the development of commercial EVs isolation kits, EVs also can be extracted from limited sample in a short period of time. Artificial EVs is another way to realize large scale EVs generation. Artificial EVs generation technologies use the physical forces or chemicals (nitrogen cavitation[107], extrusion *via* porous membrane[108], sonication[109]) to break cells and release the cellular components. With reconstitution of the released lipids, proteins, and nucleic acids, artificial EVs can be generated in large quantity[105].

Moreover, improving the therapeutic efficiency of EVs is also a great challenge for applying EVs to clinical use. EV surface engineering can realize the EVs selective enrichment in specific cells and potentially tissues by introduction of targeting moieties[110]. To improve the therapeutic effects of EVs, a variety of methods (active drug loading, passive modification, electroporation, acoustic degradation, chemical transfection, *etc.*) can be used to modify EVs. Moreover, Cytochalasin B treatment and osmotic pressure can enhance the production of EVs with improved drug loading capacity[111]. Engineered EVs are a new strategy to enhance the expression of targeted proteins of EV-related RNA. These engineered EVs with specific targeted biomolecules can be used specifically for different therapeutic purposes, including as *in vivo* tracers or in targeted cell tracking, which could improve the efficacy of disease therapy and tissue regeneration.

CONCLUSION

In conclusion, EVs are intelligent and controllable biomaterials that can participate in a variety of physiological and pathological activities, tissue repair and regeneration by transmitting a variety of biological signals, showing great potential in cell-free tissue regeneration. Engineered EVs, which represent a clean, highly purified, and highly controllable means of achieving the sustained release of drugs, have broad prospects in future tissue regeneration engineering.

FOOTNOTES

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REVIEW

Human pluripotent stem cell-derived β cells: Truly immature islet β cells for type 1 diabetes therapy?

Helen Jiang, Fang-Xu Jiang

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Abstract

A century has passed since the Nobel Prize winning discovery of insulin, which still remains the mainstay treatment for type 1 diabetes mellitus (T1DM) to this day. True to the words of its discoverer Sir Frederick Banting, "insulin is not a cure for diabetes, it is a treatment", millions of people with T1DM are dependent on daily insulin medications for life. Clinical donor islet transplantation has proven that T1DM is curable, however due to profound shortages of donor islets, it is not a mainstream treatment option for T1DM. Human pluripotent stem cell derived insulin-secreting cells, pervasively known as stem cell-derived β cells (SC-β cells), are a promising alternative source and have the potential to become a T1DM treatment through cell replacement therapy. Here we briefly review how islet β cells develop and mature *in vivo* and several types of reported SC-β cells produced using different *ex vivo* protocols in the last decade. Although some markers of maturation were expressed and glucose stimulated insulin secretion was shown, the SC-β cells have not been directly compared to their *in vivo* counterparts, generally have limited glucose response, and are not yet fully matured. Due to the presence of extra-pancreatic insulin-expressing cells, and ethical and technological issues, further clarification of the true nature of these SC-β cells is required.

Key Words: Human pluripotent stem cells; Stem cell-derived β cells; Islet β cells; Type 1 diabetes mellitus; Cell replacement therapy

Core Tip: Diabetes mellitus (DM) is a metabolic condition of absolute or relative deficiency in insulin. Since the discovery of insulin 100 years ago, there has been slow progress in the treatment of type 1 DM (T1DM) in clinical practice. In the scientific community however, there is much interest and progression in the research of human pluripotent stem cell derived insulin producing β-like cells, pervasively known as stem cell-derived β cells (SC-β cells). If they are determined to be genuine, scalable and functionally matured, SC-β cells have the potential to cure T1DM through cell replacement therapy.

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INTRODUCTION

In this coronavirus disease 2019 pandemic era, there is a silent growing epidemic of significant public health burden with tremendous social and economic costs. This growing epidemic is not an infectious disease, but a chronic non-communicating metabolic disease - it is the epidemic of diabetes mellitus (DM). There was an estimated 537 million adults with DM globally in 2021[1], with the prevalence increasing each year due to the rising incidence of type 2 DM (T2DM) worldwide[2]. DM is a metabolic disorder characterised by a disruption in glucose homeostasis leading to hyperglycaemia, and broadly consists of 2 main types: T1DM and T2DM. T1DM is the absolute deficiency of insulin due to the autoimmune destruction of insulin-secreting β cells in the islets of Langerhans of the pancreas, and is usually first diagnosed in children and young adults. T2DM is the relative deficiency of insulin function due to insulin resistance in peripheral tissues, and sometimes with reduced insulin secretion due to dysfunctional or dedifferentiated β cells, usually occurring in adults.

Hyperglycaemia in T1DM and T2DM can cause various microvascular complications such as diabetic retinopathy and blindness, nephropathy and kidney failure requiring dialysis, as well as peripheral neuropathy and infected foot ulcers that lead to amputations. It can also cause macrovascular complications such as peripheral artery disease, coronary artery disease and stroke. These complications lead to significant morbidity and mortality, as well as substantial associated health and social costs[3,4].

INSULIN IS NOT A CURE FOR DIABETES

These were Sir Frederick Banting's words to the world during his Nobel Lecture for his 1923 Nobel Prize winning discovery of insulin. Subsequent discoveries on primary insulin sequences and radioimmune assay for insulin and other peptide hormones were also awarded the Nobel Prizes (Figure 1)[5]. A century later, unfortunately there is still no cure for DM, and life-long insulin replacement remains the mainstay of treatment for T1DM and controlling high blood sugar levels with antihyperglycaemic agents in most T2DM individuals. The continuous blood glucose monitoring and insulin pump known as artificial pancreas or bionic pancreas still presents the risk of developing complications, though reduced, because this and other current treatments cannot achieve physiological glucose homeostasis in patients[6,7]. These treatments themselves are also not without risks. Insulin as well as some oral anti-hyperglycaemics, such as sulfonylureas and glinides, are associated with the risk of hypoglycaemia which can lead to seizures, coma and even death[8,9]. Thus, there is a critical need for more effective and curative treatments to reduce the global burden of this disease.

The landmark proof-of-concept has demonstrated over the last 2 decades that clinical transplantation of donated human islets are able to restore β-cell function and achieve insulin independence immediately with improvement in glycaemic control and avoid the risk of hypoglycaemia episodes[10-13]. However, a large amount of approximately 340-750 million islet cells are required for successful transplantation in a patient of 68 kg weight[11,14]. Thus, the widespread application of donor islet transplantation is severely limited by the insufficient supply of human organ donor pancreases[15,16]. In addition to supply issues, another challenge to this treatment option is the prevention of transplant rejection, immune destruction and cell death of the transplanted islet cells[17]. To address the donor shortage issue, alternative scalable insulin-secreting tissues must be identified and developed. Due to their ability for theoretically infinite self-renewal and differentiation into all cell types in the body, human pluripotent stem cells (hPSCs) hold great promise for generating surrogate insulin-secreting cells *ex vivo*, pervasively known as stem cell-derived β cells (SC-β cells)[18-20] or SC-islets[21] in the literature. In order to help understand the true nature of these SC-β cells, we briefly introduce how islet β cells develop *in vivo*.

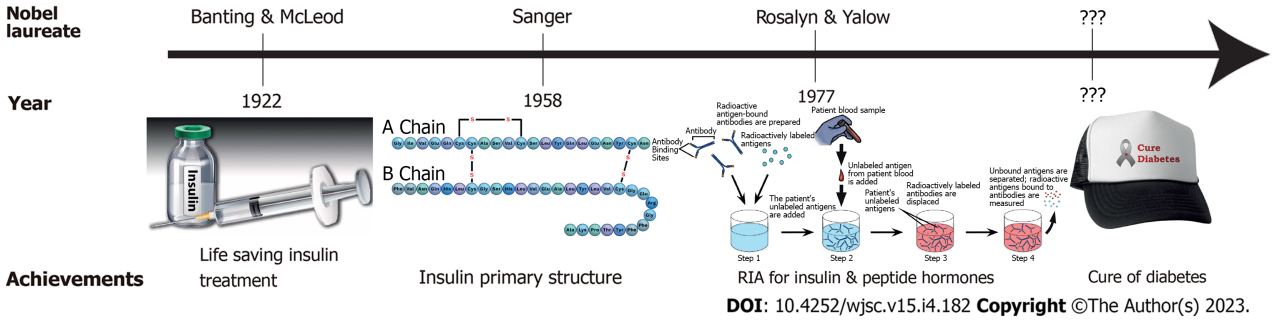

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Figure 1 Nobel prizes awarded for the endeavour towards curing diabetes. Insulin is not a cure for diabetes. Three Nobel prizes have been awarded in this endeavour[5].

IN VIVO DEVELOPMENT OF ISLET BETA CELLS

The pancreas is derived from the embryonic endoderm, one of the three germ layers, which is formed during gastrulation of embryogenesis. In addition to the pancreas, the definitive endoderm also gives rise to the liver, lung, thymus and other organs of the respiratory and digestive tracts[22]. The endoderm located in the foregut region gives rise to the dorsal and ventral buds of the pancreas which rotate to form one organ, then pancreatic epithelium is induced and expands, from which endocrine progenitors arise. The endocrine progenitors then differentiate into the β cells that secrete the hormone insulin, α cells that secrete the hormone glucagon, δ cells that secrete the hormone somatostatin, ε cells that secrete the hormone ghrelin, and PP cells that secrete the hormone pancreatic polypeptide. The pancreatic endocrine cells start to organize into clusters forming islets before birth, and the Islets of Langerhans become fully formed at around 2-3 wk after birth[22]. Human islets are made up of 40%-60% β cells and 30% α cells[22]. The adult pancreas is made up of exocrine cells that secrete digestive tract enzymes, duct cells that make up the ductal tree to transport digestive enzymes and islet cells that secrete hormones into the bloodstream for glucose homeostasis[23].

Mechanistically, the pancreatic islets are initiated by the transient expression of a high level of the transcription factor neurogenin-3 (NGN3)[24]. NGN3 is important in committing all pancreatic endocrine cell types, the deficiency of which leads to the absence of pancreatic endocrine cells[25,26]. The molecular mechanisms for the development of each pancreatic endocrine cells are not completely defined, however it is suggested that insulin-producing β cells are differentiated from the pancreatic progenitors that express transcription factor genes pancreatic and duodenal homeobox 1 (PDX1) and NK6 homeobox 1 (NKX6-1), and then turn on NGN3[27,28]. There are several β cell transcription factors, including PDX1, NKX6-1 and MAF BZIP transcription factor A (MAFA), which play a critical role in activating insulin transcription and regulating insulin secretion[29-31].

PDX1 is a homeodomain transcription factor homogenously expressed in the early pancreatic bud and its expression persists into mature β cells; the absence of PDX1 leads to agenesis of the pancreas [32]. NKX6-1 and the helix-loop-helix transcription factor Beta2/NeuroD determine islet cell differentiation during embryogenesis, and maintain specific islet cell hormone expression in adults[32]. Knockout of mouse *Nkx6-1* gene leads to a significant inhibition in the formation of β cells[33]. NeuroD is initially expressed in pancreatic epithelium during development, before being expressed in $NGN3^+$ endocrine progenitors, and finally exclusively expressed in β cells after birth. The absence of Beta2/NeuroD leads to reduced mouse endocrine cells, in particular β cells, increased apoptosis and arrestment in islet morphology[25,34]. Beta2/NeuroD is also a critical transcriptional activator of the insulin gene[35,36].

β-cell maturation including maturation of other clinically important cell types is a postnatal development process. For example maturation of mouse and human β cells takes place approximately 3 wk[37] and 26-44 wk after birth[38,39] respectively. The maturation process is controlled by transcription factors and exhibited in maturing at the gene, protein, subcellular, intercellular and metabolic levels.

ISLET BETA CELL MATURATION REGULATED BY TRANSCRIPTION FACTORS

Following β-cell specific NeuroD deletion, the mice developed glucose intolerance and the islets displayed features of foetal/neonatal β cells such as overexpression of glycolytic genes, lactate dehydrogenase (LDHA), Neuropeptide Y, and higher basal insulin secretion and oxygen consumption due to the reliance on oxidative metabolism of glucose[40-46]. That is, the glucose metabolic profile of mouse β cells without NeuroD was equivalent to immature β cells. The mutations of NeuroD cause maturity onset diabetes of the young[22,33]. NeuroD is also critical for maintaining a matured functional state of

islet β cells[40]. These data suggest that NeuroD regulates islet β-cell maturation, though its postnatal dynamic expression profile is not available. Thus, identification of Beta2/NeuroD activators may help mature hPSC-derived insulin-secreting cells *ex vivo*.

MafA is another transcription factor being demonstrated to regulate the maturation of islet cell organisation, β cell mass and β cell function from 3 wk of age in mice using the gene targeting strategy [47]. MafA expression reaches their adult levels at 3 mo in rats[48] coinciding with the obtaining of mature glucose stimulated insulin secretion (GSIS). Aguayo-Mazzucato *et al*[49] were the first to demonstrate that MAFA overexpression and the thyroid hormone triiodothyronine (T3) treatment are able to increase human foetal islet-like clusters, insulin secretion at 16.8 mmol/L glucose and proinsulin-to-insulin processing. Chromatin immunoprecipitation experiment showed binding of thyroid receptors to MafA promoter, thereby confirming that T3 directly regulates the expression of MafA[50]. The thyroid hormone receptor is also demonstrated to be expressed on human mature islets [51], though its postnatal development profile is unknown.

Furthermore, a recent study shows that the expression of the orphan nuclear transcription factor estrogen-related receptor gamma (ERRγ) is a hallmark of mature β cells[52]. ERRs consist of three paralogs in mammals, namely ERRα (NR3B1 or Esrrα), ERRβ (NR3B2 or Essrβ) and ERRγ (NR3B3 or Essrγ). ERRγ is progressively upregulated in mouse islets from 2 to 6 wk of age (5-fold higher in adults compared to neonatal β cells) and ERRγ transcriptional network promotes mitochondrial oxidative metabolism in mouse β cells, required for functional maturation of β cells and glucose homeostasis[52]. Mice with β cell-specific ERRγ deletion failed to develop a mature GSIS. With the developmentally deleted β cell-specific ERRγ knockout mouse islets, RNA sequencing (RNA-seq) revealed that the expression of 4189 genes were altered, with almost equal numbers of genes down- and up-regulated (2008 and 2182 genes respectively). Gene ontology analysis revealed that ERRγ-regulated genes are associated with processes critical for β cell function including ATP biosynthesis, cation transport, oxidative phosphorylation, electron transport and secretion[52]. However, data is not available on postnatal developmental expression of ERRs in human islet cells, which will have to be addressed in the near future. Identification of ERRγ activators may help mature hPSC-derived insulin-secreting cells *ex vivo*.

Expression of the *Sine Oculis* family of homeodomain transcription factors SIX2 and SIX3 increased with age in the human pancreatic islet β cells[53]. SIX2 and SIX3 are localised to the nucleus of adult human β cells but not detected in juvenile (under 9 years of age) β cells[53]. Using gain-of-function experiments in human β cell line, the EndoC-bH1 cells or primary juvenile human islets, evidence has demonstrated that expression of SIX2 or SIX3 were sufficient to enhance cardinal functions of human β cells[53]. Identification of SIX2 and SIX3 activators may therefore help generate matured hPSC-derived insulin-secreting cells *ex vivo*.

METABOLIC MATURATION OF ISLET BETA CELLS

Although rat islets acquired GSIS by postnatal day 21, a mature GSIS was only achieved by 3 mo[54], coinciding with the time when insulin dynamics reaches their adult levels[48]. The metabolic maturation is underscored by genes of important metabolic players in β cells such as glucose transporter 2, glucokinase, glucagon-like peptide-1 receptor and prehormone convertase 1 (encoded by *Pcsk1*) that were expressed from very low levels at postnatal day 2 to higher levels with increased age[48]. Similarly, the metabolic maturation is also underscored by genes transcribing for malate dehydrogenase, glycerol-3-phosphate dehydrogenase, glutamate oxaloacetate transaminase, pyruvate carboxylase and carnitine palmitoyl transferase 2 from much lower levels at neonatal postnatal day 2 to high levels at day 28[55]. In the same period, genes encoding proliferation regulators in β cell genes encoding platelet-derived growth factor receptor A, platelet-derived growth factor receptor B, platelet-derived growth factor B and fibroblast growth factor (FGF) receptor 1 are progressively downregulated. Mature β cells tend to have lower levels of LDHA and glycolytic genes, as it is downregulated by NeuroD from embryonic to adult islets, which then appears to possess the ability to correspond glucose metabolism with insulin secretion[56-58].

The molecular mechanism of mature β-cell secretion is well understood. The higher blood glucose levels stimulate its active transportation into the β cell cytoplasm, increasing the ATP/ADP ratio through glycolysis and oxidative metabolism and triggering the depolarisation of the β cell membrane and opening the voltage-gated Ca^{2+} channel. The AMP-activated protein kinase (AMPK) is a highly conserved sensor of intracellular adenosine nucleotide levels that is activated even with modest decreases in ATP production resulting in relative increases in AMP or ADP. In response, AMPK promotes catabolic pathways to generate more ATP, and inhibits anabolic pathways. The increase in cytoplasmic Ca^{2+} triggers the fusion of insulin granules with the plasma membrane for exocytosis of insulin contents. Coordinating with other islet cells, mature β cells produce insulin in adequate amounts and timeliness to maintain plasma glucose within a narrow concentration range[39,59]. Thus, β cell function is critical for mature GSIS. Destruction and failure of islet β cells will lead to T1DM and T2DM, respectively (Figure 2).

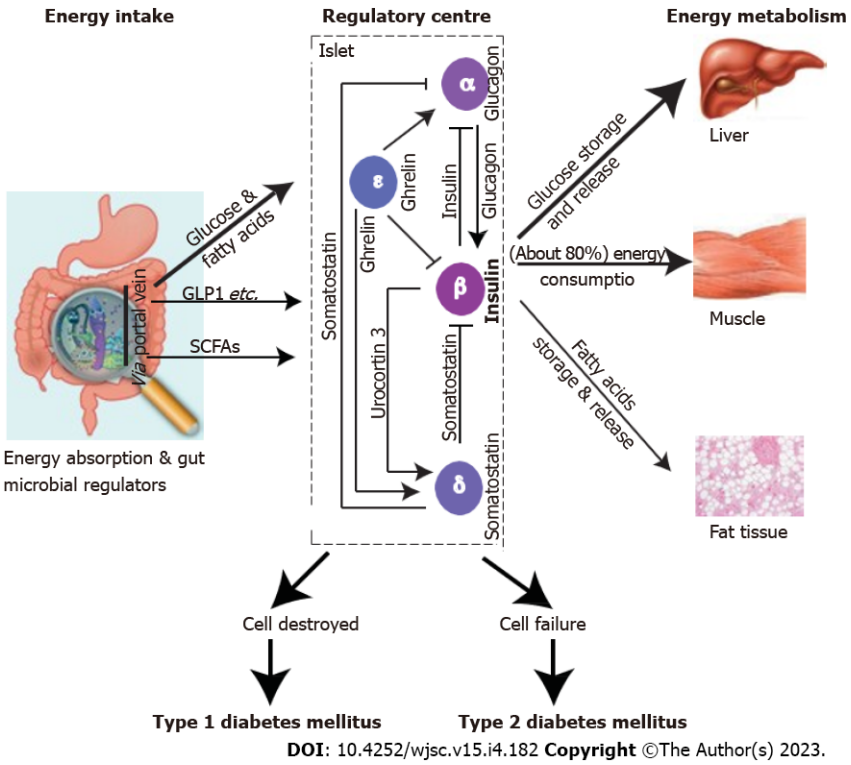

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Figure 2 Mature islets are a regulatory centre for glucose homeostasis. A simplified graphic representation of how mature pancreatic islets regulates glucose homeostasis. Skeletal muscle is the largest organ in the body (45%-55% body mass) and consumes about 80% insulin. GLP1: Glucagon-like peptide-1; SCFAs: Short-chain fatty acids.

MATURATION MARKERS OF ISLET BETA CELLS

To help with the characterization of whether hPSC-derived insulin-secreting cells *ex vivo* are matured, we briefly summarize maturation markers for *in vivo* islet β cells. Over the last decade, several potential markers for maturation of immature islet β cells were discovered. Blum *et al*[60] were the first to demonstrate that functional islet β cell maturation is marked by expression of the corticotropin-releasing factor family peptide urocortin 3 (UCN3), along with an increased glucose threshold.

Mature rat β cells expressed significantly higher levels of the gap junction connexion 36 gene (*Cx36*, also known as *Gjd2*) compared to neonatal immature counterparts, corresponding to a significantly higher membrane density of gap junctions and greater intercellular exchange of ethidium bromide[61]. Human mature islets predominantly express CX36 at mRNA and protein levels with β cell membrane harboring detectable levels of CX36 gap junction proteins[62]. Though the developmental profile of human islet CX36 is unknown, we speculate that the dynamic pattern of CX36 expression from human neonatal to mature β cells is similar to that in rats and CX36 is a potential maturation marker for matured hPSC-derived insulin-secreting cells *ex vivo*.

Our group recently showed that claudin 4 is the only tight junction molecule family member highly upregulated in the postnatal mouse islets and global deletion of this gene affects mature GSIS in a sex difference manner[63]. Thus, claudin 4 may also be a maturation marker for matured hPSC-derived insulin-secreting cells *ex vivo*.

THE DIFFERENTIATION OF SC-BETA CELLS *EX VIVO*

The advent of hPSC provided an important opportunity to overcome major challenges of clinical islet transplantation therapy through its accessibility, theoretically unlimited self-renewability and the boundless potential to generate an alternative source of donor insulin-secreting cells *ex vivo*[64,65]. The generated insulin-secreting cells can also be used for disease modelling and pharmaceutical drug testing to help establish therapeutics that improve cell function, survival and proliferation. Insulin-secreting cells differentiated from hPSCs that include human embryonic stem cells (hESC) and induced hPSCs (ihPSC) are ubiquitously termed as SC-β cells[18-20] in the literature. hESCs are generated from the inner cell mass of human blastocysts and have the infinite ability to proliferate as undifferentiated cells or differentiate into cells of all ectoderm, mesoderm or endoderm lineages[66,67].

Over recent years, there have been various protocols developed of *ex vivo* differentiation of SC-β cells [18-20] and SC-islets[18,21]. Thus far, hPSCs have been differentiated towards SC-β cells through a step-wise manner emulating *in vivo* pancreatic embryonic development[68-72]. The differentiation of hPSCs towards SC-β cells have been achieved with the application of growth factors, proteins or molecules to modulate signaling pathways to progress through each stage of pancreatic development, and is usually measured by expression of a couple of key transcription factors or C-peptide[22]. hPSCs (characterised by expression of Oct4) are first differentiated into definite endoderm cells expressing FOXA2 and SOX17 through application of a mix containing Wnt, activin A, inducer of definite endoderm, wortmannin, and sodium butyrate[22]. Then application of FGF10 and FGF7 differentiates the definite endoderm into gut tube endoderm expressing HNF1B and HNF4A[22]. The differentiation mixture containing retinoic acid, noggin KAAD-cyclopamine, FGF, and indolactum V leads to differentiation into pancreatic progenitors expressing PDX1 and HNF6, which further differentiates into endocrine progenitors (NKX6-1, NGN3, NKX2-2, PTF1A), and finally into β-cells (characterised by presence of C-peptide and insulin)[22]. We here summarize several representative protocols used to generate SC-β cells and SC-islets (Table 1).

Pagliuca *et al*[73]'s differentiation protocol was the first using specific and cocktail of inducing factors to differentiate hPSCs sequentially through 6 stages into SC-β cells (Table 1). At stages 5 and 6, there is however significant heterogeneity in the final population containing SC-β and SC-α cells, as well as SC-endocrine cells (resembling enterochromaffin cells) and non-endocrine cells (*e.g.*, exocrine cells such as pancreatic acinar, mesenchymal and ductal cells)[18]. Nevertheless, these cells appear to be stable, maintaining their identity as evidenced by their global transcriptional profiles during stage 6 cultures. At this stage, they also express the maturation marker SIX2 but several other β cell markers of maturity are not expressed such as UCN3, MAFA and SIX3[18].

The Velazco-Cruz *et al*[19]'s protocol was built upon and modified Pagliuca *et al*[73]'s protocol, and demonstrated that the SC-β cells had improved insulin secretion and greater gene expression of β cell markers compared to the cells generated with Pagliuca *et al*[73]'s protocol, but still much less than the average human islet (Table 1). Follow-up studies with the addition of differentiation factors or changes to the differentiation processes were unfortunately unsuccessful in producing more functional SC-β cells equal to human islet β cells[74-76].

In Balboa *et al*[20]'s protocol, the SC-islets had similar cytoarchitecture and functional insulin secretion pattern to islet β cells, though with immature glucose-induced mitochondrial respiration and instead retained pyruvate sensitivity - thus the SC-islets were not completely similar to functional adult islets (Table 1). Balboa *et al*[20]'s SC-β cells showed heterogeneous mature β cell marker expression, required further maturation *in vivo* after transplantation, showed upregulated expression of CHGB and MAFA after 6 mo, and did not express adult β cell factors RBP4 and SIX3[20].

Nevertheless, studies indicate that several current pancreatic progenitor differentiation protocols promote precocious endocrine commitment; ultimately resulting in the generation of non-functional polyhormonal cells[74]. The efficiency of differentiation decreases with each step, and at the final step there are very small amounts of SC-β cells that have a low insulin content, co-express insulin and glucagon, and usually respond poorly to glucose stimulation[22,70]. It was also found that these SC-β cells have little to no expression of maturation genes including MAFA and G6PC2[18,73,77]. Following transplantation, the amount of insulin secreted by SC-β cells rises[73,77,78] and the previously low or non-expressed genes of islet β cells such as MAFA, G6PC2, MNX1 and INS increases[79].

Cell purification steps will increase the safety of, and ability to upscale the manufacture of β cells. However, there are difficulties in including this step in large-scale manufacturing processes for production of reproducible PSC-derived cellular products with less variability in composition and function[80]. Several cell surface markers have been used to purify different developmental stages of PSC-derived cells[80]. Markers used include CD177 for anterior definitive endoderm cells[81], CD142, CD24 and glycoprotein 2 for pancreatic progenitors[82-85], CD49a for SC-β cells[18], and CD9 for negative selection of SC-β cells[86]. Monoclonal antibody against extracellular domain of claudin 4 might help enrich matured SC-β cells differentiated *ex vivo*.

Finally, a few maturation factors have proven useful in maturing SC-β cells *ex vivo*. For example, T3 enhanced the MAFA expression in the SC-β cells, and increased insulin content and insulin secretion at 16.8 mmol/L glucose[49]. Using an adenoviral ERRγ vector, overexpression of ERRγ increased glucose-stimulated C-peptide secretion in hPSC-derived insulin-secreting cells, thus may promote their functional maturation[52]. Therefore, identification of molecules that activate NEUROD, ERRγ, SIX2 and SIX3 will be important. Application of the activators individually or in combination may indeed promote functional maturation of genuine SC-β cells.

CURRENT *EX VIVO* SYSTEMS ARE DISTINCT FROM *IN VIVO* ISLET DEVELOPMENT NICHES

The current PSC differentiation protocols for insulin-secreting cells are mostly bulk cultures and consist of cocktails of inducing factors, which are generally based on accumulative knowledge generated from using the animal model systems. In these bulk cultures, there are cells types in the targeted lineage as

Table 1 A summary of several differentiation protocols for generating stem cell-derived β cells *ex vivo*

Differentiation protocol	Marker expression	Characterization	Ref.
The stem cell-derived β cell protocol is a 6-stage differentiation protocol using specific inducing factors for each stage (11 factors) to produce SC-islets from hPSCs, in a 3D suspension-based cell culture system (4-5 wk)	Pancreas progenitor marker: PDX1 (about 90% at stage 3). Endocrine markers: C-peptide, CHGA and NKX6-1. SC-β cell markers: INS, NKX6-1, ISL1, and others	Immunofluorescence. Ultrastructure. Insulin packaging into secretory granules. qRT-PCR. Static and dynamic GSIS. Functional test. Glucose-responsive Ca^{2+} flux, KCl depolarisation, ameliorate hyperglycaemia in diabetic mice. scRNA-seq. Purified SC-β cells with anti-CD49a	Pagliuca *et al*[73], 2014; Veres *et al* [18], 2019
Optimised 7 stage *in vitro* differentiation protocol of serial culture steps using factors such as vitamin C, ALK5 inhibitor, TGF-β receptor inhibitors, thyroid hormone (T3), R428 (AXL inhibitor), N-acetyl cysteine, Notch and BMP signalling inhibitors	Pancreas progenitor marker: PDX1 (about 90% at stage 3), NKX6-1. Endocrine markers: C-peptide, CHGA, NEUROD1, NKX2-2 and NKX6-1. β cell markers: INS, NKX6-1, ISL1, MAFB. Mature β cell markers: MAFA, ABCC8, IAPP, HOPX, NEFM, SIX2, G6PC2	Immunofluorescence. Transmission electron microscopy. qRT-PCR. Static and dynamic GSIS. Functional test in diabetic mice. Flow cytometry. Immunohistochemistry. Diabetes reversal within 40 d in mice. Metabolic analysis. Perifusion assay. Calcium imaging.	Rezania *et al*[78], 2014
Scalable 3D suspension culture system based on previous methods [101,102], with the addition of retinoic acid, cyclopamine (SHH inhibitor), Noggin (BMP inhibitor), then treatment with epidermal growth factor, KGF and Noggin (EKN). Followed by exposure to a cocktail of factors *e.g.*, TBP, ALK inhibitor, Noggin, TANK	Pancreas progenitor marker: PDX1 and NKX6-1 (90%). Endocrine markers: C-peptide, NEUROG3, NKX2-2 and NKX6-1. β cell markers: INS, NKX6-1, NKX2-2, PDX1. Mature β cell markers: MAFA, MAFB, PDX1, NKX6.1, NXK2.2, ISL1, PAX6, NEUROD1, and CHGA	Immunofluorescence. Transmission electron microscopy. qRT-PCR. Static and dynamic GSIS. Insulin biosynthesis and glucose metabolism, blood glucose reduction but not reversal of diabetes in mice. Flow cytometry. Western blot analysis. Statistical analysis	Russ *et al*[74], 2015
Six-stage differentiation strategy modulating TGF-β signaling by modulating Alk5i exposure, combined with controlling cell cluster size and use of enriched serum-free media culture	Pancreas progenitor marker: PDX1. Endocrine markers: CHGA (96%), C-peptide (73%), NKX6-1. SC-β cell markers: INS, CHGA, NKX2-2, PDX1, NKX6-1, MAFB, GCK, and GLUT1	Immunofluorescence. qRT-PCR. Static and dynamic GSIS. Flow cytometry. Light microscopy. Glucose responsive, first- and second-phase insulin release, improved glucose tolerance in mice. Western blot. Perifusion assay. Glucose tolerance test	Velazco-Cruz *et al* [19], 2019
Optimised differentiation protocol combining previous protocols. Changes made: Differentiation of hPSCs in adherent conditions until pancreatic progenitor stage. Then optimised with nicotinamide, epidermal growth factor, activin A and a ROCK inhibitor; a microwell aggregation step; and a final maturation step in suspension culture	Pancreas progenitor marker: PDX1. Endocrine markers: C-peptide. SC-β cell markers: INS. Mature β cell markers: INS, G6PC2, SIX2, GLIS3, RBP4, SIX3. HOPX, UCN3, IAPP, CPE and FXYD2 upregulated post engraftment. CHGB and MAFA upregulated 6 mo post-engraftment. B cell differentiation: SIX2, HOPX, ZBTB20. Insulin secretion genes: PCSK1, CPE, CHGB, ABCC8, FXYD2, GABRA2	Immunohistochemistry. Flow cytometry. Perifusion assay. Respirometry. Transmission electron microscopy. Electrophysiology. Exocytosis imaging. $[Ca^{2+}]_i$ imaging. $[cAMP]_m$ imaging. Metabolite tracing analysis. Ratiometric analysis. Transplantation study. scRNA-seq transcriptomic profiling. Glucose responsive biphasic insulin secretion. Glucose tolerance test	Balboa *et al*[20], 2022
Differentiation protocol using hCiPSC-islets by optimising pancreatic progenitor to β cells fate commitment by modulating signaling pathways and reconstructing islet spatial structure through 3D cell aggregates of posterior foregut-committed cells and combination of ISX9 and Wnt-C59 at stage 5	Pancreas progenitor marker: PDX1. Endocrine markers: C-peptide, CHGA and NKX6-1. β cell markers: PDX1, NKX6.1 and NKX2.2. Mature β cell markers: MAFA, UCN3	Immunofluorescence. qRT-PCR. Transmission electron microscopy. Static and dynamic GSIS. Glucose-stimulated calcium flux assay. Flow cytometry. scRNAseq. Glucose responsive biphasic insulin secretion, decrease HbA1c, restore endogenous C-peptide secretion. Glucose tolerance tests. Preclinical diabetic non-human primate transplantation study. Fasting blood glucose levels. Glycated HbA1c. scRNA-seq. Teratoma assay. Karyotype analysis. Calcium imaging. Cryo-electron microscopy. ELISA	Du *et al*[21], 2022

PDX1: Pancreatic and duodenal homeobox 1; SC-islets: Stem cell-derived islets; hPSC: Human pluripotent stem cells; C-peptide: Connecting peptide; CHGA: Chromogranin A; NKX6-1: NK6 homeobox 1; qRT-PCR: Quantitative real time-polymerase chain reaction; GSIS: Glucose-stimulated insulin secretion; SC-β cell: Stem cell-derived β cell; INS: Insulin gene; ISL1: ISL LIM homeobox 1; Ca^{2+}: Calcium; KCl: Potassium chloride; scRNA-seq: Single-cell RNA sequencing; anti-CD49a: CD49a (Integrin alpha 1) antibody; TGF-β receptor inhibitor/ALK5i: Transforming growth factor β-receptor I/activin receptor-like kinase 5 inhibitor; R428 (AXL inhibitor): A selective small-molecule inhibitor of AXL (bemcentinib, BGB324); BMP: Bone morphogenetic protein; NEUROD1: Neurogenic differentiation 1; NKX2-2: NK2 homeobox 2; MAFA: MAF BZIP transcription factor A; MAFB: MAF BZIP transcription factor B; ABCC8: ATP Binding Cassette Subfamily C Member 8; IAPP: Islet amyloid polypeptide; HOPX: HOP homeobox; NEFM: Neurofilament medium chain; SIX2: SIX homeobox 2; G6PC2: Glucose-6-phosphatase catalytic subunit 2; KGF: (FGF7) Keratinocyte growth factor; TBP: TATA-binding protein; TANK: TRAF family member associated NFKB activator; PAX6: Paired box 6; GCK: Glucokinase; GLUT1: Glucose transporter 1; ROCK inhibitor: Rho-kinase inhibitor; GLIS3: GLI-similar 3/zinc finger 3; RBP4: Retinol binding protein 4; SIX3: SIX homeobox 3; UCN3: Urocortin 3; CPE: Carboxypeptidase E; FXYD2: FXYD domain containing ion transport regulator 2; cAMP: Cyclic adenosine monophosphate; ZBTB20: Zinc finger and BTB domain containing 20; PCSK1: Proprotein convertase subtilisin/kexin type 1; GABRA2: Gamma-aminobutyric acid type A receptor subunit alpha-2; hCiPSC: Human chemically induced pluripotent stem cells; ISX9: Isoxazole-9; Wnt-C59: Nanomolar inhibitor of mammalian PORCN acyltransferase activity and blocks activation of all evaluated human Wnts; HbA1c: Hemoglobin A1c; ELISA: Enzyme-linked immunosorbent assay.

well as unwanted lineages. One or two *in vivo* biomarkers are selected based on *in vivo* islet lineage development to characterise targeted cells at different differentiated stages *ex vivo*. However, these *in vivo* biomarkers should not be extrapolated as biomarkers for the *ex vivo* differentiation conditions because of clear differences in spatiotemporal and microenvironment niches between the *in vivo* development and *ex vivo* differentiation (Figure 3). In other words, we do not yet fully understand the full regulatory program, or the molecular details of the 3D microenvironment niche for specific islet lineage development *in vivo* to guide the specific differentiation of hPSCs into insulin-secreting cells *ex vivo*.

EXTRAPANCREAS INSULIN-SECRETING CELLS

Perhaps the research community have also forgotten the fact that in our body, extra-pancreas insulin-secreting cells exist, which may complicate the efforts of generating genuine SC-β cells. Subverted to general knowledge, approximately a quarter of human foetal enteroendocrine K/L cells were recently shown to express high levels of insulin and other β cell genes including the transcription factor PDX1, by using samples of foetal and neonatal human small intestines derived from the endoderm during development[87]. Notably, the expression of UCN3 in the human foetal enteroendocrine K/L cells was higher than in foetal human pancreatic β cells[87]. These results were confirmed with single molecule fluorescence *in-situ* hybridisation of insulin mRNA combined with immunofluorescent antibody staining of the insulin protein[87]. Secondly, thymocytes that are derived from the foregut, adjacent to which gives rise to the pancreas, normally produce insulin to induce self-tolerance and protect the body from the autoimmune destruction of pancreatic insulin-secreting β cells[88]. Lastly, though the central nervous system is an ectoderm-derived organ, the neuronal progenitors derived from adult hippocampus and the olfactory bulb were demonstrated to undergo insulin biosynthesis[89]. Human *INS* mRNA expression is also detected in the hippocampus, amygdala and temporal lobe in addition to the olfactory bulb, cerebellar and pontine regions[90]. A historical account of the extrapancreas insulin-secreting cells is referred to in a recent review article[88]. These data suggest that it is possible that the current reported SC-β cells contain a varied percentage of non-pancreatic insulin-secreting cells. Future studies are required to increase the percentage of genuine insulin-secreting β-like cells in the *ex vivo* systems.

SC-BETA CELLS TRANSPLANTED INTO NON-HUMAN PRIMATES

To further test their functions, the chemically induced SC-islets were recently intraportally transplanted into immunosuppressed adult diabetic rhesus macaques[21]. Three months after the SC-islet transplantation, all four macaques reportedly had improvements in diabetic symptoms, glycaemic control, fasting blood sugar levels, hemoglobin A1c (HbA1c), and reduced exogenous insulin requirements[21]. However, after 5-6 mo, two of the macaques developed graft failure (the other two macaques died of immunosuppression-related complications)[21]. Autopsy conducted on the macaques found no evidence of teratoma or tumorigenesis, but levels of β cells had fallen. The authors concluded that the immunosuppression regimen used was not appropriate in preventing immune attack against the grafts[21]. Whether the short-term improvements in diabetic rhesus macaques are related to the immaturity of grafted SC-islets and/or the presence of non-pancreatic insulin-secreting cells needs to be determined in the future.

SC-BETA CELLS IN CLINICAL TRIALS AS A T1DM THERAPY

The first hPSC-derived, differentiated cell replacement T1DM therapy product named VX-880 was approved by the United States Food and Drug Administration for phase 1/2 clinical trials in March 2021. The VX-880 are SC-islets for T1DM patients with certain indications; that is, impaired hypoglycaemic awareness and severe hypoglycaemia[91]. The preliminary outcomes of the clinical trials were presented in June 2022 at the American Diabetes Association 82nd Scientific Sessions by Vertex, a United States Pharmaceutics company[91]. A half-dose of VX-880 in two patients was able to achieve glucose-responsive insulin secretion, significantly improve time-in-range (the amount of time that blood glucose level is measured to be within target blood sugar range 70-180 mg/dL or 3.9-10 mmol/L), reduce exogenous insulin requirements and improved HbA1c[91]. VX-880 was also well tolerated although with some largely mild or moderate adverse reactions[91]. For example, patient 1 showed blood glucose time-in-range change from 40.1% on 34.0 units per day of exogenous insulin at baseline to 99.9% and insulin independence at day 270 onwards. Patient 2 showed blood glucose time-in-range change from 35.9% on 25.9 units per day of exogenous insulin at baseline to 51.9% with a 30% reduction in exogenous insulin use at day 150[91]. Whereas these results are very promising, VX-880 requires a

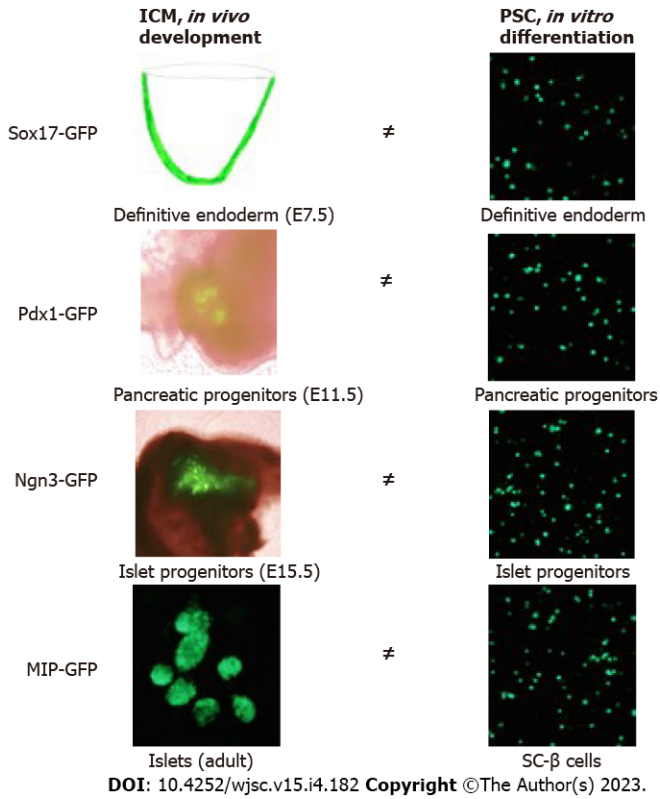

Figure 3 There are clear differences in spatiotemporal and microenvironment niches between the *in vivo* development and *ex vivo* differentiation of islet lineages. Details of lineage tracing mouse lines can be found in articles[103,104]. ICM: Inner cell mass; PSC: Pluripotent stem cell; Sox17: SRY-box transcription factor 17; GFP: Green fluorescent protein; PDX1: Pancreatic and duodenal homeobox 1, also known as insulin promoter factor 1; NGN3: Neurogenin-3; MIP: Mouse insulin 1 promoter; SC-β cells: Stem cell-derived β cells.

lengthy *in vivo* maturation period for blood sugar control (in patient 1) in contrast to donated islets retrieved from deceased persons, which achieved immediate insulin independence after transplantation into recipients[10-13]. The lengthy *in vivo* maturation period of grafted VX880 is a strong independent indicator that these SC-β cells are immature. It is also premature to claim the VX-880 SC-β cells are all genuine immature counterparts of islet β cells, as the duration and longevity of insulin independence was not yet available at the time of writing this article.

CONCLUSION

Immaturity of PSC-derived cells is a general obstacle, not only in the case of SC-β cells and SC-islets, but also other clinically important cell types[92]. Maturation biology is the final frontier in stem cell biology, of which our knowledge is still in its infancy. As summarised in Table 1, multiple hPSC differentiation protocols have been used in different laboratories. Consequently, off-target differentiation and aberrant differentiation from these protocols are more likely unavoidable, resulting in only a low frequency of genuine SC-β cells. Furthermore, the stage-specific differentiation factors selected may direct non-specific spatiotemporal differentiation, thus resulting in multiple cell types of the endodermal germ layer and even neuronal lineage origins. This may result in some differentiating cells along unwanted pathways and give rise to extrapancreas insulin-secreting cells. On the other hand, unwanted or off-target differentiated cellular products have accumulated in the bulk culture protocols and not been excluded for subsequent differentiation steps, which further increases the possibility of compromising the characterization through use of one or two developmental markers of *in vivo* cellular lineages. Finally, in addition to the above, there are still other challenges in this exciting field of research, such as ensuring SC-β cell survival post-transplantation given the highly vascularised islets are susceptible to ischaemic injury and loss of cell mass[93,94]. Developing methods that evade autoimmune attack in T1DM patients without the use of lifelong immunosuppression would be valuable[95].

Stage-specific *in vivo* pancreatic and islet lineage cell types would provide ideal positive controls for the *ex vivo* hPSC-derived insulin-secreting cells. Nevertheless, the human ethics issues and lack of human embryonic and foetal pancreatic tissues available prevent such reliable and precise comparison to be made between the islet lineage cells and the PSC-derived cells. However, future efforts should be made to resolve these issues. Similarly, it would be wise not to solely concentrate on undertaking

human β cell differentiation and maturation studies from hPSCs. Instead, investigating β cell differentiation and maturation from model animals will be invaluable and will facilitate the realisation of a curative stem cell therapy for people with T1DM.

In order to minimise confusion between the *ex vivo* differentiated insulin-secreting cells and islet β cells, our laboratory proposed a 4-criterial post-genomic concept for naming "β cells" a few years ago [96]. Recently, Kaestner *et al*[97] described many islet biologists/scientists much like the "Parable of the Blind Men and the Elephant" in terming "β cells". This appears to be the case in respect to claims made about SC-β cells without proper positive controls of corresponding *in vivo* islet lineage cells. Kaestner *et al*[97] further proposed six salient features of normal, fully functional mature β cells and made a recommendation to not name PSC-derived insulin-producing cells as "β cells", but conservatively as insulin-producing cells, insulin$^+$ cells or β-like cells, when there is no clear evidence that the six features of *in vivo* β cells are met.

The degree of single-cell RNA-seq (scRNA-seq) data similarity between the SC-β cells and donated islet β cells remains largely unclear. First, all scRNA-seq datasets of SC-β cells lacked a direct positive control from *in vivo* pancreatic and islet lineage cells. Second, most current scRNA-seq methods provide a high throughput but sacrifice full transcript coverage and sensitivity[98]. Third, as barcodes/inducers are introduced by the template switching of reverse transcriptase, strand invasion becomes problematic through systematic bias, namely biases from the introduction of artefacts. Fourth, loss of cDNA synthesis and bias in cDNA amplification leads to severe quantitative errors of these scRNA-seq methods[99]. Fifth, the current scRNA-seq methods suffer from impaired mRNA accounting. However, molecular spikes have significantly improved single cell mRNA accounting[100], adoption of the molecular spike method and further improvements may help address the above issues. As such, genuine SC-β cells will eventually become available as donor cells for establishing curative therapies for people suffering from T1DM in the not too distant future.

FOOTNOTES

Author contributions: Jiang H wrote the first draft of the manuscript; Jiang FX conceived and designed the research; and all authors have edited, revised, reviewed and approved submission of the manuscript.

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REVIEW

Mechanisms of analgesic effect of mesenchymal stem cells in osteoarthritis pain

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Abstract

Osteoarthritis (OA) is the most common musculoskeletal disease, and it is a major cause of pain, disability and health burden. Pain is the most common and bothersome presentation of OA, but its treatment is still suboptimal, due to the short-term action of employed analgesics and their poor adverse effect profile. Due to their regenerative and anti-inflammatory properties, mesenchymal stem cells (MSCs) have been extensively investigated as a potential therapy for OA, and numerous preclinical and clinical studies found a significant improvement in joint pathology and function, pain scores and/or quality of life after administration of MSCs. Only a limited number of studies, however, addressed pain control as the primary end-point or investigated the potential mechanisms of analgesia induced by MSCs. In this paper, we review the evidence reported in literature that support the analgesic action of MSCs in OA, and we summarize the potential mechanisms of these antinociceptive effects.

Key Words: Osteoarthritis; Pain; Inflammation; Mesenchymal stem cells; Regeneration; Analgesic mechanisms

Core Tip: Osteoarthritis (OA) is the most common musculoskeletal disease, and it is a major cause of pain, disability and economic burden. Pain is the most common and bothersome presentation of OA, but its treatment is still suboptimal, which highlights the need for new analgesic agents for OA. Mesenchymal stem cells (MSCs) have been extensively investigated as a potential therapy for OA due to their regenerative and anti-inflammatory properties. The administration of MSCs resulted in significant improvement in joint pathology and function, pain scores and/or quality of life in numerous preclinical and clinical studies. Only a limited number of studies, however, addressed pain control as the primary end-point or investigated the potential mechanisms of analgesia induced by MSCs. So, this paper reviews literature for evidence of analgesic actions of MSCs in OA, and summarizes the potential mechanisms of these anti-nociceptive effects

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INTRODUCTION

Osteoarthritis (OA) is a disease of movable joints characterized by anatomic and/or physiologic derangements including cartilage degradation, bone remodeling, osteophyte formation, joint inflammation and loss of normal joint function. It is initiated by micro- and macro-injury of the joint, which activates maladaptive repair responses producing abnormal tissue metabolism[1].

Clinical manifestations of OA include joint pain, tenderness, limitation of movement, coarse crepitus and, occasionally, effusion and mild local inflammation[2]. Diagnosis depends on the observation of signs and symptoms through clinical examination[3]. X-ray can be performed to help with differential diagnosis and in case of atypical features[4]. However, radiologic findings do not always complement the clinical presentation of pain[5].

Pathophysiology of OA is complex and is based on cartilage degeneration preceded by subchondral bone lesions. Historically, OA had been considered a disease of joint wear and tear, but recently, low-grade chronic inflammation has been found to play a key role in OA pathology. Both innate and adaptive central immunological mechanisms are involved in inflammation. Formation of ectopic bone and osteophytes are a characteristic pathologic features of OA[6]. Neuroinflammation and central sensitization mechanisms contribute to the development of chronic pain[7].

OA is the most common musculoskeletal disorder worldwide, and it poses a huge health and economic burden. It is considered a major cause of chronic pain, disability -due to diminished joint mobility and function-, and decreased quality of life[8,9]. Thus, finding effective and safe therapies for the treatment of OA is a significant clinical need.

Despite the recent progresses in understanding the pathophysiology of OA[7], the treatment of this condition is still suboptimal[10]. For low-grade OA, pain management and lifestyle changes are the only available therapeutic options, with total joint replacement considered the end-stage therapy. However, no effective therapeutic options are available that can stop the progress of the disease[10]. Analgesia may be achieved using nonsteroidal anti-inflammatory drugs (NSAIDs) (topical or systemic) as a first line, followed by paracetamol or tramadol. Less used medications include duloxetine and topical capsaicin. Intra-articular steroids are effective and recommended if other agents do not provide sufficient pain control, although their effect is short-termed[3]. Pharmacological therapies should be always accompanied by physical and psychosocial interventions, such as exercise, weight management and manual therapy. Patients whose joint symptoms are substantially impacting their quality of life and in which non-surgical management is ineffective or unsuitable should be referred for joint replacement surgery[3]. Table 1 summarizes the treatment guidelines for OA as recommended by the American College of Rheumatology and Arthritis Foundation[11].

PAIN IN OA

Pain is the major manifestation of OA and it significantly affects the function and quality of life of patients[12]. Both peripheral and central mechanisms contribute to OA pain. Peripherally, nociceptive signals may arise from the synovium, bone marrow, soft tissues and even cartilage in the advanced stages of the disease[13]. These inputs are modulated at the central level through mechanisms involving spinal and cortical sensitization, and the activity of discrete areas of the brain. Central sensitization may explain the poor correlation between pain severity in OA and the extent of cartilage damage, and the

Table 1 Recommended therapies for the management of osteoarthritis by the American College of Rheumatology and Arthritis Foundation

	Hand	Knee	Hip
Physical and psychosocial approaches			
Exercise[1]	√	√	√
Self-Efficacy and Self-Management Programs[1]	√	√	√
Weight loss[1]		√	√
Tai Chi[1]		√	√
Heat, therapeutic cooling[2]	√	√	√
Cognitive Behavioral Therapy[2]	√	√	√
Acupuncture[2]	√	√	√
Paraffin[2]	√		
Yoga[2]		√	
Pharmacological approach			
Oral NSAIDs[1]	√	√	√
Topical NSAIDs	√[2]	√[1]	
IA corticosteroids	√[2]	√[1]	√[1] (image-guided)
Acetaminophen[2]	√	√	√
Tramadol[2]	√	√	√
Duloxetine[2]	√	√	√
Chondroitin[2]	√		
Topical capsaicin[2]		√	

[1]Strongly recommended.
[2]Conditionally recommended.
Strongly and conditionally recommended approaches for the management of hand, knee, and hip osteoarthritis are shown. NSAIDs: Nonsteroidal anti-inflammatory drugs; IA: Intra-articular.

persistence of pain after joint replacement in some patients[14].

In the periphery, sensitization of afferent neurons is mediated by cytokines, chemokines and neuropeptides and is associated with low-grade inflammation and innate immunity[15], immune cell infiltration and activation, and damage-associated molecular patterns. These mechanisms involve early post-translational changes to receptor ion channels, followed by late transcription-dependent mechanisms, which produce changes to the chemical phenotype of the cell[16]. Animal models of OA demonstrated the role of nerve growth factor (NGF) in nociceptor sensitization after tissue injury, mainly through the tropomyosin receptor kinase A (TrkA) receptor. In addition, increased NGF levels were found in the synovial fluid of OA patients and were associated with pain[17,18]. Other molecules associated with the peripheral component of OA pain include the neuropeptide calcitonin gene-related peptide (CGRP)[19], interleukin (IL)-1β, IL-6 and tumor-necrosis factor α (TNF-α)[20].

Central sensitization occurs both at the spinal and supraspinal levels. In the spinal cord, acute pain is accompanied with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor activation by glutamate, followed by an early phase of sensitization, mediated by substance P (SP) acting on neurokinin 1 receptors, in which N-methyl-D-aspartate (NMDA) receptors are activated. The late phase sensitization witnesses gene induction with enhanced synthesis of prostaglandins and other local inflammatory mediators[14]. In models of OA, spinal cord exhibits enhanced transient receptor potential cation channel subfamily V member 1 (TRPV1) activity and increased levels of SP, CGRP, IL-1 α/β, IL-13, IL-17, TNF-α, L-selectin, tissue inhibitor of metallopeptidase inhibitor 1 and vascular endothelial growth factors[21]. In addition, activation of glial cells was found to contribute to spinal sensitization, indicating a strong neuropathic component of the OA associated pain[22]. Supraspinal sensitization occurs in several areas of the brain, including the rostral ventral medulla and the periaqueductal gray and it involves inflammatory mediators, such as prostaglandins, as well as serotoninergic and noradrenergic facilitation[23].

TREATMENT OF OA PAIN

Pain management in OA is still considered suboptimal. The use of paracetamol, once recommended as the first-line analgesic for OA pain, has now been reviewed after meta-analysis suggested that monotherapy with paracetamol may be ineffective[24] and that long-term treatment provides no more pain relief than placebo for most patients[11]. NSAIDs are effective on the short term to control OA pain and are thus strongly recommended. Their use, however, is limited by the adverse effect profile[11]. Intra-articular injections of steroids also showed short-term effectiveness (2-10 wk)[3], but they are limited in frequency of administration and may cause cartilage damage if used repeatedly[25]. The use of other analgesics, such as duloxetine, tramadol, and non-tramadol opioids is not supported by strong evidence of efficacy, as well as other supplements, such as hyaluronic acid, glucosamine, chondroitin, fish oil and vitamin D[11]. These factors highlight the need for the development of novel therapies that are both effective and safe on the long term.

MSCS AND THEIR THERAPEUTIC USES

Mesenchymal stem cells (MSCs) are multipotent adult stem cells likely derived from diverse embryonic lineages and isolated from different sources. They were discovered by Friedenstein in 1970[26]. The International Society for Cellular Therapy has proposed a set of standards to define MSCs: (1) Expression of a certain set of cluster of differentiation (CD) markers (CD105, CD73, and CD90); (2) Lack of expression of hematopoietic lineage CD markers (CD45, CD34, CD14 or CD11b, CD79α or CD19 and histocompatibility complex (HLA)-DR surface molecules); (3) Differentiation into osteoblasts (bone), adipocytes (fat) and chondroblasts (cartilage) *in vitro*; and (4) Plastic adherence and ability to form colony-forming unit fibroblasts[27]. MSCs from different sources showed differences in differentiation potential, immunophenotype, immunomodulatory activity, proteome, and transcriptome, producing their specific characteristics and features in their application.

MSCs are an attractive option and among the most frequently used stem cell types for clinical application and regenerative medicine due to numerous advantages, including self-renewal and differentiation, mostly due to their secreted trophic factors that mediate cell-to-cell communication. Moreover, immune rejection is an important concern with allogeneic cell-based therapy, but the lack of cell surface HLA class II molecules and T cell costimulatory molecules and their paracrine-mediated immunomodulatory activity, and secretion of immunomodulatory factors indicate that the MSCs have broad anti-inflammatory properties and active in tissue repair[28-31].

A large number of studies demonstrated the beneficial effects of MSC-based therapies to treat different pathologies, including neurological disorders, cardiac ischemia, diabetes, and bone and cartilage diseases[32,33], which indicate that MSCs holds great promise for cell therapies and tissue engineering.

Studies showed the ability of MSCs to migrate toward damaged tissues, which functionally influence the repairing of these tissues[34]. MSCs act to accelerate healing and reduce inflammation, which is essential to remove dead cells, and facilitate cell migration and proliferation in the injury site[35,36]. For instance, a study using a rat model of cardiomyopathy showed that MSC transplantation significantly improved cardiac function by induction of myogenesis and angiogenesis, resulting in decreased left ventricular end-diastolic pressure and increased left ventricular maximum[37]. Another study showed that basic fibroblast growth factor (bFGF) can promote the migration and survival of bone marrow MSCs *in vitro*, as the perfusion of the coronary vein with retrograde bFGF can enhance the graft transplantation of MSCs, promote the differentiation of MSCs to cardiomyocytes, and restore cardiac function[38]. Some studies used a laboratory-grown cell sheet patches of MSCs to repair large damaged areas instead of intravenous infusion of MSCs. Kim *et al*[39] transplanted adipose-derived stem cells (AD-MSCs) sheet to treat myocardial infarction in a rat model and showed that the stem cell sheet promoted cellular engraftment and upregulated growth factor and cytokine expression. An *in vivo* study of neonatal stroke rat model proved that intranasal delivery of MSC reduced ischemic brain damage and reduced white and gray matters loss[40]. The study attributed the healing in the injury site to an increase in cell proliferation after transplantation of MSCs.

In skeletal disorders, MSCs could be helpful in tissue repairing and regeneration through several mechanisms, including homing, angiogenesis, differentiation, and response to inflammatory condition [41]. Liu *et al*[42] used umbilical cord-MSCs (UC-MSCs) to treat rheumatoid arthritis in collagen-induced arthritis mice through suppression of T follicular helper cell differentiation partly *via* the production of indoleamine 2,3-dioxygenase. In addition, MSCs prevented arthritis progression by inhibiting both the number and function of follicular helper cell *in vivo*. Another study showed that transplantation of MSCs from allogeneic related donors treated severe progressive systemic sclerosis [43]. A third *in vitro* study treated bone marrow derived-MSCs (BM-MSCs) with all-trans retinoic acid, then co-cultured them with CD3/28-activated peripheral blood mononuclear cells derived from ankylosing spondylitis (AS) patients. The results showed that BM-MSCs treated with all-trans retinoic acid significantly decreased pathogenic cytokine, TNF-α, IL-17A and interferon-γ (IFN-γ) in AS[44].

MSCS IN OA

MSCs are the most studied stem cells for treating bone related diseases and the associated inflammation. The effects of treatment by MSCs on OA pathology and presentation was widely investigated *in vitro* and in different preclinical models of OA using different routes of administration of multiple types and amounts of MSCs. Numerous clinical trials also addressed the potential therapeutic role of MSCs in OA.

MSCs have the ability to differentiate into mesoderm-derived cells, including osteoblasts and chondrocytes. More importantly, MSCs are considered powerful immunomodulators and inflammation combatants rendering them suitable for many immunological and bone diseases where inflammation is a prominent component. OA is the most common form of arthritis and clinically variable in its severity. Patients with symptomatic form of OA suffer from intermittent attacks which are usually associated with joint pain. OA pathology encompasses the inflammation and degeneration of articular cartilage that defects its integrity and leads to subsequent changes in the subchondral bone. Many preclinical and clinical studies have corroborated the therapeutic potential of MSCs in alleviating the inflammation associated with OA and initiating the regeneration of the defective articular cartilage. A pilot study conducted by Song *et al*[45] reported positive outcomes of injecting autologous human adipose-derived MSCs (haMSCs) intra-articularly in patients with OA. Song *et al*[45] reported that a dosage of 5×10^7 haMSCs produced the desired improvement in the pain scale and restored the volume and the function of knee cartilage. Similarly, Lee *et al*[46] reported that single intra-articular injection of 1×10^8 AD-MSCs for patients with knee OA produced a significant reduction in the Western Ontario and McMaster Universities OA (WOMAC) index total score and the test related sub-scores for pain, stiffness and physical function at 6 mo post-injection. The range of articular motion remarkably enhanced after MSCs transplantation with no change in the joint space width of medial and lateral compartment and size of the cartilage damage[46]. On the other hand, Matas *et al*[47] found that intra-articular injection of two repeated doses of umbilical cord-derived MSCs (UC-MSCs) (20×10^6 *per* dose) at baseline and 6 mo was superior than using a single dose in lowering the WOMAC pain scores. Patients with knee OA who were recruited in Matas *et al* study experienced 86% reduction in pain and 89% reduction in disability with no progression in the chondral damage and intra-articular calcifications examined by magnetic resonance imaging at 12 mo[47]. Bastos *et al*[48] compared the effect of injecting autologous bone marrow-derived culture-expanded MSCs (hBM-MSCs) intra-articularly with or without the addition of platelet-rich plasma to intra-articular corticosteroid injections in patients with symptomatic knee OA. MSCs alone or in combination with platelet rich plasma were superior to corticosteroids in improving the Knee Injury and OA Outcome Score, increasing the range of motion and reducing the expression of IL-10, which is usually increased in OA knees, at 12 mo follow up[48].

The extracellular vesicles (EVs) derived from different MSCs have currently gained a wide interest due to their high therapeutic efficacy and safety profile. Li *et al*[49] injected UC-MSCs-EVs in the articular lumen of a rat model of OA created by the surgical transection of the anterior cruciate ligament (ACLT), and reported that the UC-MSCs derived EVs can deliver therapeutically effective miRNAs, including has-miR-122-5p, has-miR-148a-3p, has-miR-486-5p, has-miR-let-7a-5p, and has-miR-100-5p, which were able to promote the reprogramming of macrophages into anti-inflammatory M2-macrophages and increasing the level of inflammation inhibitory cytokine IL-10. Moreover, these miRNAs were effective in inducing the phosphoinositide-3-kinase-Akt signaling pathway, which is integral to prevent further degeneration of the knee cartilage and seize the progression of OA[49]. Duan *et al*[50] conducted an interesting study by isolating synovial MSCs and chondrocytes from the knee cartilage of patients getting total knee arthroplasty. After culturing these cells, they primed the MSCs with lipopolysaccharide (LPS) and isolated EVs from these preconditioned synovial MSCs. The EVs were injected in the right knees of mice model of OA created by surgical removal of anterior cruciate ligament and medial meniscus. It was found that the presence of Let-7b miRNA in the EVs isolated from LPS primed synovial MSCs were effective in reducing the cartilage damage, increasing the thickness of cartilage layer, and decreasing the level of the inflammatory and matrix lysis protein disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) while increasing the levels of aggrecan and type II collagen alpha 1 chain, which are essential for the interaction with hyaluronan and enhancing the load-bearing characteristics of the knee[50]. Likewise, Jin *et al*[51] reported that lncRNA MEG-3 existed in the exosomes derived from bone marrow MSCs (BM-MSCs) and prevents the apoptosis and senescence of chondrocytes through lowering the level of IL-1β and decreasing the inflammatory damage of knee cartilage, which helps in restoring the trabecular bone volume and density of the knee joint in OA rat model. Mao *et al*[52] observed that miR-92a-3p was significantly less in the chondrocytes isolated from the cartilage of OA patients who underwent total knee replacement. BM-MSCs derived exosomes can deliver miR-92a-3p following their injection in the knees of collagenase-induced OA mouse model, which enhances the differentiation potential of resident stem cells to chondrocytes and increases the synthesis of cartilage matrix. These protective and regenerative effects are mediated by the inhibition of Wnt Family Member 5A[52]. Huang *et al*[53] demonstrated that miR-206 was downregulated and E74-like factor 3 (Elf3) was upregulated in the femoral tissues of OA mouse model. The administration of BM-MSCs derived exosomes that have sufficient amount of miR-206 was effective in downregulating Elf3 and ameliorating the inflammation and apoptosis of resident osteoblasts besides increasing the expression of osteocalcin and bone morphogenetic protein 2 and

enhancing the deposition of calcium and the activity of alkaline phosphatase[53]. In the rat model of temporomandibular joint OA, Zhang *et al*[54] injected exosomes derived from MSCs. They reported a significant suppression of inflammation mediated by decreasing the level of IL-1β and increasing the levels of nitric oxide and matrix metalloproteinase 13 (MMP13), which promoted the synthesis of glycosaminoglycans required for matrix restoration and regeneration[54].

Genetic modification of MSCs is one of the most studied approaches to enhance their therapeutic and regenerative potential. It has been found that the transplantation of MSCs overexpressing platelet-derived growth factor (PDGF) or heme oxygenase-1 in the surgery-induced canine OA model can considerably suppress the destructive inflammation and increase the levels of aggrecan and collagen type 2 in chondrocytes. PDGF-MSCs were more effective in improving the limb function and reducing pain[55]. The accumulative evidence supported by the above mentioned studies highlights the importance of MSCs in mitigating OA inflammation and restoring the integrity and density of volume of articular cartilage and its associated matrix, which indicates that MSCs can be a possible new therapeutic tool for OA.

EFFECTS OF MSCS ON OA PAIN

Despite the large number of preclinical studies investigating the effects of MSC administration on OA pathophysiology, only a small part of them included the effects on pain, and even less had pain control as the main outcome. This may be due to the difficulty of pain assessment in animal models of OA[56] and the complexity of the pain phenomenon[57]. In addition, reported results were contrasting, probably due to the variability in animal models and the methods of pain evaluation, as well as types of MSCs used and routes of their administration[56].

A number of *in vivo* studies, however, found an improvement in OA associated pain following treatment with MSCs. Transforming growth factor β1 (TGF-β1)-modified MSC-derived exosome was found to attenuate cartilage damage and pain behaviors in the ACLT model of OA by inhibiting angiogenesis, suppressing calcification of the cartilage zone and osteoclastogenesis[58]. Intra-articular injection of adipose derived MSCs (ADSCs) in rats diminished monoiodoacetate (MIA)-induced OA cartilage lesions by paracrine-based mechanisms and restored the OA associated mechanical allodynia and thermal hyperalgesia. In patients, ADSCs also reduced OA pain, as measured by the visual analog scale and WOMAC[59].

In a study by Zeng *et al*[60], bone marrow-derived MSCs (BMSCs) were enhanced by kartogenin (KGN) nanoparticles and administered to osteoarthritic rats. In addition to articular cartilage repair and enhanced chondrogenesis, KGN-enhanced BMSCs also ameliorated OA pain, as shown by increased weight bearing on the injured leg and decreased latency period after hot plate exposure. Administration of different concentrations of MSCs into OA rat knees improved both the histological damage and weight bearing distribution in the ACLT model[61].

Sakamoto *et al*[62] found that early intra-articular injection of adipose-derived MSCs resulted in significant suppression of inflammation and pain and prevented degenerative OA changes, but did not promote cartilage repair. In another study involving the MIA model of OA, STAT3 signaling pathway was suppressed by treatment of MSCs with STA21. Both intravenous (IV) and intra-articular (IA) administration of STA21-treated MSCs decreased expression of proinflammatory cytokines in the joint, which improved pain severity and cartilage damage[63].

A trial of adult human bone marrow-derived mesenchymal stromal cells in the MIA model of OA caused significant pain reduction, along with articular damage repair. In patients, the same cells did not produce the significant improvement in pain scores[64].

Intra-articular injection of a large variety of MSCs in patients with knee OA was found to significantly improve joint pathology, disease symptoms, pain score and quality of life[65]. Human umbilical cord MSCs (hUC-MSCs) was found to improve pain scores and quality of life in OA patients. hUC-MSCs increase the expression of chondrocytes and activate anti-inflammatory mechanisms, preventing degradation of cartilage and bone[66]. A clinical trial of autologous bone marrow stem cells (BM-SC) concluded that a single IA injection of these stem cells significant reduced knee pain and improved quality of life[67]. Other clinical trials reviewed by Hwang *et al*[68] also resulted in improved joint function, reduction of pain severity and improved life satisfaction.

PROPOSED MECHANISMS OF ANALGESIA OF MSCS IN OA

Data from literature suggest that the analgesic action of MSCs in OA involves a peripheral component, originating in the joint tissues, and a central component related to central hypersensitization. This reflects the mechanisms of nociception reported in OA[69].

It can be speculated that a major antinociceptive mechanism of MSCs in OA is through inhibition of inflammation. MSCs secrete anti-inflammatory and growth factors that support their immunomodulatory, immunosuppressive and trophic capacities. These properties contribute to the regeneration of

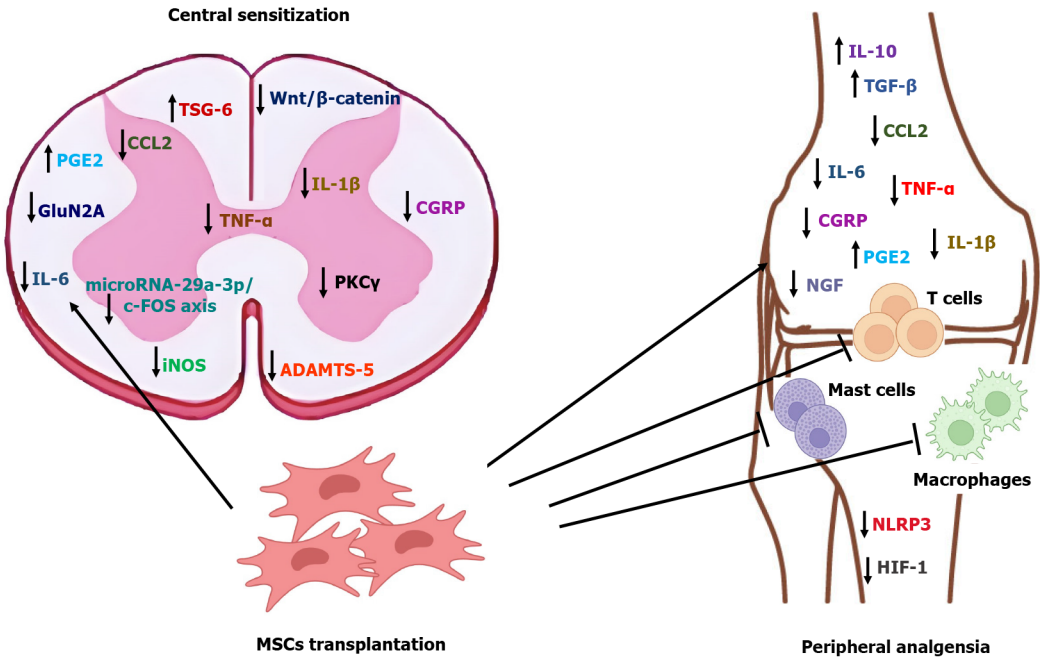

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Figure 1 Graphical summary of proved and anticipated analgesic mechanisms of mesenchymal stem cells in osteoarthritis. Mesenchymal stem cell (MSCs) transplantation reverts central sensitization and induces peripheral analgesia due to their anti-inflammatory and immunomodulatory properties and by attenuation of specific pain pathways *via* exosomes-derived microRNAs and MSCs paracrine factors. TSG-6: TNF-stimulated gene 6 protein; CCL2: Chemokine (C-C motif) ligand 2; PGE2: Prostaglandin E2; GluN2A: N-methyl-D-aspartate receptor subunit 2A; IL-6: Interleukin 6; iNOS: Inducible nitric oxide synthase; TNF α: Tumor necrosis factor α; IL-1β: Interleukin-1β; CGRP: Calcitonin gene-related peptide; PKCγ: Protein kinase C γ; ADAMTS-5: A disintegrin and metalloproteinase with thrombospondin motifs 5; IL-10: Interleukin 10; TGF-β: Transforming growth factor β; NGF: Nerve growth factor; NLRP3: NLR family pyrin domain containing 3; HIF-1: Hypoxia inducible factor 1.

damaged cartilage and joint homeostasis, improving inflammatory and catabolic aspects of OA[70,71]. Initially, the bioactive substances secreted by MSCs change the inflammatory milieu in the joint from pro-inflammatory to anti-inflammatory, producing analgesia[65]. On the longer term, MSCs are inserted in the joint tissues and trigger the repair and regeneration of damaged tissues, including cartilage[72]. Numerous molecules have been reported to contribute to the antiinflammatory effects of MSCs in OA, including nitric oxide (NO), inducible nitric oxide synthase (iNOS)-27, IL-10, TGF-β, IL-6, IFN-γ, CCL2, hepatocyte growth factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and TNF-α[73-75]. Cyclooxygenase 2/prostaglandin E2 (PGE2) pathway plays a key role in the anti-inflammatory effect of MSCs in OA[76], where MSCs increase the levels of the antiinflammatory mediator PGE2[75]. Similar antiinflammatory results were also produced by MSC EVs[77]. Since these molecules and their related pathways are well established contributors to OA pain, their involvement in the analgesic mechanisms of MSCs in OA can be hypothesized.

Chemokine (C-C motif) ligand 2 (CCL2)/C-C chemokine receptor type 2 (CCR2) signaling is crucial in the development of knee OA pain. Neuronal CCL2 and CCR2 from dorsal root ganglion (DRG) mediate macrophage infiltration, while local CCL2/CCR2 signaling in the joint directly stimulates intra-articular CCR2 positive sensory nerves, producing knee hyperalgesia[78]. Interestingly, the analgesic effect of MSCs in different models of pain was associated with decreased levels of CCL2, produced by the downregulation of the NF-κB and c-Jun N-terminal kinase/mitogen activated protein kinase pathways[79,80], which suggests that a similar mechanism could be involved in the analgesic effect of MSC in OA.

Other molecules and pathways relevant to OA pain include: (1) NGF / TrkA; (2) CGRP; (3) IL-1β; (4) Pyrin domain-containing protein 3 (NLRP3) inflammasome; and (5) Wnt/β-Catenin[69]. NLRP3 inflammasome is a component of the innate immune system and is involved in the regulation of active IL-1β. NF-κB increases the expression of inactive NLRP3 and pro-IL-1 mRNA, followed by the assembly of the inflammasome, which results in the activation of caspase 1 and release of IL-1, IL-18, MMP13 and ADAMTS5. In OA, dysregulation of NLRP3 inflammasome in OA contributes to chronic pain[69]. MSCs were found to inhibit the NLRP3 inflammasome in macrophages[81], inflammatory cardiomyopathy[82] and inflammatory renal disease[83], which could indicate inhibition of NLRP3 inflammasome as a potential mechanism of MSCs induced analgesia in OA.

In OA, high levels of Wnt are associated with progressive joint damage[84] and hyperalgesia[69]. Since MSCs were found to inhibit the Wnt/β-Catenin pathway in a number of disorders[85,86], this pathway could be involved in MSC analgesic mechanism and should be investigated further. In the

study by Lee *et al*[63], administration of signal transducer and activator of transcription 3-inhibited MSCs reduced the levels of inflammatory mediators and chemokines in the OA joint, and this was associated with improvement in pain behavior and decreased TRPV1 expression in the dorsal root ganglion. This effect was more pronounced with IV administration, indicating a systemic immunomodulatory effect on inflammation.

The interaction of MSCs with immune cells, including macrophage, dendritic cells, T lymphocytes, and natural killer cells, also contributes to MSC antiinflammatory properties[87]. MSCs induce polarization of macrophages to an antiinflammatory M2 phenotype through: (1) Cellular interaction and paracrine factor-mediated mechanisms; and (2) exosome-mediated mechanisms. The first involves cytokines and hormones, and the latter depends on RNAs and other molecules[88]. Following an intra-articular injection of BM-MSCs in patients with knee OA, a decrease in synovial fluid levels of pro-inflammatory monocytes/macrophages and IL-12p40 was recorded[89].

Ai *et al*[90] investigated the effects of MSCs and derived EVs (MSC-EVs) on pain behaviors in the destabilization of the medial meniscus murine model of OA. It was found that treated OA mice did not display pain behaviors observed in untreated counterparts, and that did not result from reduced joint damage, but rather from a lack of knee-innervating sensory neuron hyperexcitability. MSC-EV treatment also prevented NGF-induced sensory neuron hyperexcitability. These results suggest that MSCs and MSC-EVs may reduce pain in OA by direct action on peripheral sensory neurons[90]. Another study[91] found that the intrathecal administration of umbilical cord blood MSCs (UCBMSCs) improved both pain behavior and inflammation in the MIA model of OA. This effect is regulated by LncRNA H19 and involves microRNA-29a-3p/ FOS axis. microRNA29a-3p, the target gene of LncRNA H19, and FOS mRNA were down-regulated after stem cell therapy, suggesting that microRNA-29a-3p and FOS might play a role in pain improvement. c-fos in spinal dorsal horn of rats was significantly down-regulated after UCBMSCs treatment, which may be the reason for pain improvement. Phosphorylation levels of NMDA receptor 1 (NR1), NR2B, protein kinase C γ (PKCγ), extracellular signal-regulated kinase in spinal dorsal horn of rats with OA pain decreased significantly after intervention of UCBMSCs, indicating that the central sensitization of rats with advanced OA pain decreased and the pain symptoms improved. Similar results were observed in astrocytes.

Intra-articular injection of human adipose tissue-derived MSCs was reported to improve pain behavior in a medial meniscal transection (MMT) rat model of OA[92]. These effects were attributed to the recruitment of endogenous cells through paracrine communication[93], and to a lesser extent, to direct engraftment coordinated with the local environment[94]. Paracrine factors excreted by MSCs help recruitment of stem and progenitor cells, repair of degraded tissue and, most importantly, counteracting inflammation. A similar result was reported after administration of bone marrow MSC (BMSC)-derived exosome in the MIA model of OA[95], which was found to inhibit CGRP and iNOS expression in the DRG, indicating relief of both inflammatory and neuropathic aspects of OA pain[95]. BMSC-derived exosome also attenuates the inhibitory effect of IL-1β on the upregulated inflammatory mediators.

In the meniscal transection (MNX) model of OA, different effects were reported on pain behavior and joint pathology with the use of early and late passage MSCs. Late passage MSCs attenuated established pain behavior, while early passage MSCs exacerbated it. Interestingly, none of them modified MNX-induced joint pathology, which suggests an analgesic mechanism not related to articular pathology[96]. SiMAG-labelled MSCs were detected within the synovial cavity at 29 d postinjection, indicating a peripheral site of analgesic action of the MSCs. The recorded decrease in serum TNFα indicated inhibition of systemic inflammation, which is the expected cause of pain relief[97]. Similarly, van Buul *et al*[56] reported that intra-articular injection of bone marrow mononuclear cells in the MIA model of OA significantly improved pain behavior – measured as weight bearing distribution - but did not affect cartilage damage, subchondral bone changes and synovial inflammation. Similar results were observed with the administration of MSCs and MSC-EVs in a mouse collagenase-induced OA model[98]. MSCs were also found to downregulate ADAMTS-5 expression, inhibit the expression of anti- CGRP and increase the expression of TNF-stimulated gene/protein-6 (TSG-6)[99]. These changes indicate the suppression of the central sensitization of pain.

Several pain pathways were found to be inhibited by MSCs in other types or models of pain. Small EVs from induced pluripotent stem cell-derived MSCs alleviated acute pain in tendinopathy by inhibition of mast cell degranulation and infiltration and the expression of proinflammatory cytokines and genes involved in the HIF-1 signaling pathway[100]. Human BMSCs relieved pain behavior in rodents by inhibition of neuronal hyperexcitability and primary afferent input, as well as suppression of GluN2A (N-methyl-D-aspartate receptor subunit 2A) tyrosine phosphorylation and PKC gamma (PKCg) immunoreactivity in the rostral ventromedial medulla[101]. In murine chronic constriction injury and spared nerve injury models, TGF-β1 was found to suppress spinal synaptic plasticity and DRG neuronal hyperexcitability *via* TGF-β receptor 1-mediated noncanonical signaling. This effect was mediated by paracrine mechanism by which BMSCs target C-X-C motif chemokine ligand 12-producing DRGs[102]. We thus anticipate that MSCs use in OA may exhibit their analgesic effects through the HIF-1, GluN2A tyrosine, PKCg and TGF-β signaling pathways. Figure 1 summarizes the established and proposed mechanisms of analgesia exerted by MSCs or MSC related EVs in OA.

CONCLUSION

Numerous *in vitro*, *in vivo* and clinical studies demonstrated the capability of MSCs in halting and/or reversing the progression of joint tissue damage in OA, as well as mitigating joint inflammation, improving pain sensation and enhancing overall patient quality of life. MSCs were able to produce an analgesic effect in models of OA through both peripheral mechanisms, mainly involving antiinflammatory processes, and on a central level, by preventing or reversing central sensitization. This evidence further reinforces the potential of MSCs as a safe and effective treatment for OA pain. Additional pathways which were observed in other types of pain may contribute to the analgesic mechanisms of MSCs in OA, and require further investigations.

FOOTNOTES

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REVIEW

Roles of cancer stem cells in gastrointestinal cancers

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Abstract

Cancer stem cells (CSCs) are the main cause of tumor growth, invasion, metastasis and recurrence. Recently, CSCs have been extensively studied to identify CSC-specific surface markers as well as signaling pathways that play key roles in CSCs self-renewal. The involvement of CSCs in the pathogenesis of gastrointestinal (GI) cancers also highlights these cells as a priority target for therapy. The diagnosis, prognosis and treatment of GI cancer have always been a focus of attention. Therefore, the potential application of CSCs in GI cancers is receiving increasing attention. This review summarizes the role of CSCs in GI cancers, focusing on esophageal cancer, gastric cancer, liver cancer, colorectal cancer, and pancreatic cancer. In addition, we propose CSCs as potential targets and therapeutic strategies for the effective treatment of GI cancers, which may provide better guidance for clinical treatment of GI cancers.

Key Words: Cancer stem cells; Gastrointestinal cancers; Promotion; Inhibition; Treatment

Core Tip: This review summarizes the role of cancer stem cells (CSCs) in gastrointestinal (GI) cancers, focusing on esophageal cancer, gastric cancer, liver cancer, colorectal cancer, and pancreatic cancer. In addition, we propose CSCs as potential targets and therapeutic strategies for the effective treatment of GI cancers, which may provide better guidance for clinical treatment of GI cancers.

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INTRODUCTION

Cancer stem cells (CSCs) are small subgroups of undifferentiated cancer cells. CSCs possess an infinite self-renewal capability and a set of unique surface biomarkers[1]. Gastrointestinal (GI) cancer is the most common major malignancy, and includes esophageal cancer (EC), gastric cancer (GC), liver cancer (LC), colorectal cancer (CRC), pancreatic cancer and other related diseases[2]. The incidence of GI cancers is high. Recently, a growing number of studies have been conducted on the important role of CSCs in GI cancers. Research shows that CSCs are mainly involved in the growth, initiation, maintenance, survival, metastasis and recurrence of GI cancer. There are certain limitations in the methods of treatment for currently accepted GI cancers, often leading to treatment failure. This is due to CSCs resistance to chemotherapy and radiation therapy. In current treatment, CSCs cannot be erased, causing metastasis and recurrence of the tumor. Recently, studies have been conducted to clarify the signaling pathway which plays a critical role in the specific surface markers of CSCs and the self-renewal of CSCs. These cell surface markers as well as signaling pathways are potential targets for the treatment of GI cancer that provide the environment necessary for tumor growth[3-5]. Thus, certain therapies for CSCs will potentially help to eliminate the tumor.

This review aims to summarize the mechanism and treatment of CSCs in GI cancer, and to propose a potential target and therapeutic strategy for the treatment of GI cancer.

ROLES OF CSCS IN EC

EC, a common tumor of the digestive tract, causes a majority of cancer deaths[6]. EC includes esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). EAC may be associated with obesity and ESCC may be associated with drinking and narcotics. Approximately 300000 people die of EC worldwide every year. The incidence and mortality rates of EC vary from country to country[7]. Recently, the relationship between CSCs and the occurrence and development of EC has attracted more and more attention. Chen *et al*[8] discovered that esophageal CSCs activate matrix metalloproteinase 9 in EC cells and promotes cancer metastasis by the expression of placental growth factor. Moreover, CSCs expressing placental growth factor can promote[9] or suppress tumor angiogenesis by stimulating vascular endothelial growth factor[10]. Wang *et al*[11] found that by acting on ATG7-dependent β-catenin, OV6 CSCs can stably promote the progression of ESCC. In addition, studies have shown that exosomal O-GlcNAc transferase derived from esophageal CSCs can promote the suppression of cancer immunity by increasing programmed death-1 (PD-1) in CD8 T cells[12].

CSCs play a critical role in the treatment of EC. CSCs markers could help to identify the functions of CSCs in EC and can be used as targets for the treatment of EC. It was found that CD44, a CSC surface marker, can isolate and detect ESCC[13]. In addition, Lu *et al*[14] found CD133 and CXCR4 markers on the surface of ESCC, and the high expression of CD133-CXCR4 may become a marker for forecasting poor prognosis in patients with ESCC. Liu *et al*[15] found that Cripto-1 is a functional marker of CSC-like cells (CSLCs) and can predict the prognosis of patients with ESCC. In addition, studies have shown that MYH9 is a novel esophageal CSC marker and prognostic indicator, which promotes tumorigenesis through the PI3K/AKT/mTOR axis[16]. Studies have demonstrated that esophageal CSLCs can resist ferroptosis induced cell death through the active HSP27-GPX4 pathway. Hence, targeting HSP27 or GPX4 blockade is a promising therapeutic strategy to eradicate CSCs in ESCC[17]. Li *et al*[18] found that exosomal FMR1-AS1 can promote CSLCs homeostasis through TLR7-NFκB-Myc signaling in female EC. Song *et al*[19] analyzed clinical specimens and found that BCL-2 inhibitor AT101 could overcome drug resistance by targeting the CSC pathway and had good antitumor activity. In addition, Zarei *et al*[20] found that salinomycin could destabilize the low-pathway sensor TAZ in CSLCS and reduce the viability of esophageal CSLCs. Studies have shown that TRPV2 can maintain the growth of CSCs, and its specific inhibitor tranilast may be used as a targeted therapeutic agent for ESCC[21]. STAT3 and miR-181b activate each other *via* the CYLD pathway, thereby regulating the proliferation of esophageal

CSLCs[22]. Metformin can inhibit EC cell growth and sensitize EC cells to the cytotoxic effects of 5-FU by targeting components of CSCs and mTOR[23].

CSCs play an important role in the development, progress and future treatment of EC, but the associated research is inadequate, and the underlying mechanisms have not been fully explored. Therefore, more studies are needed to determine the mechanism and support the corresponding conclusions.

ROLES OF CSCS IN GC

GC is a malignant tumor derived from gastric mucosal epithelium. Most GCs are early adenocarcinomas with no apparent symptoms and are easily overlooked[24]. GC is the main cause of global cancer deaths. The treatment of GC involves surgery in combination with chemotherapy and radiotherapy, but the prognosis of terminal GC is still poor[25]. Over the past decade there is increasing evidence to show that CSCs have a significant role in GC development.

The concept that CSCs cause GC and may lead to invasion, metastasis, and treatment resistance has profound implications for anticancer therapy. Takaishi *et al*[26] implanted gastric CSCs (GCSCs) in the skin of immunodeficient mice, and a few weeks later observed that these GCSCs showed strong tumorigenic ability. Yang screened out GCSCs with serum-free medium, and found that these GCSCs had high tumorigenesis ability in nude mice, and high expression of GCSCs surface markers OCT4 and SOX2[27]. E-cadherin is a vital adhesion molecule, and its expression is closely related to the degree of cell adhesion[28]. Studies have found that the expression of E-cadherin in GCSCs is down-regulated, resulting in a decrease in the adhesion of tumor cells; thus, GCSCs are highly aggressive and easily metastasize to local lymph nodes or distant metastasis[29]. CSC Lgr5+ can promote the growth and proliferation potential of GC[30]. In addition, CSCs may maintain their viability through autophagy[31].

Identifying and targeting CSCs play a vital role in the treatment of GC. Chemotoxicity-induced exosomal lncFERO can regulate ferroptosis and stemness in GCSCs. Therefore, chemotherapy targeting the lncFERO/hnRNPA1/SCD1 axis could be an implicit strategy for CSCs-based GC therapy[32]. In addition, microRNA (miR)-375 can trigger ferroptosis by targeting SLC7A11, thereby attenuating the stemness of GCSCs[33]. CBX7 was found to positively regulate the stem-like characteristics of GC cells by inhibiting p16 and activating the AKT-NF-κB-miR-21 pathway[34]. Sezer *et al*[35] found that lymphatic metastasis-associated TBL1XR1 promotes the metastasis of gastric CSLCs by sensitizing ERK1/2-SOX2 signaling. Huang *et al*[36] found that SIRT1 inhibits both chemoresistance and cancer stemness in GC by launching a positive feedback loop of AMPK/FOXO3. The m6A methyltransferase METTL3 was found to promote oxaliplatin resistance in CD133+ GCSCs by improving PARP1 mRNA stability to increase base excision repair pathway activity[37]. *Celastrus orbiculatus* ethyl acetate extract can prevent GC growth by limiting the stemness of GCSCs by changing PDCD4 and EIF3H expression [38]. Methionine has been found to inhibit autophagy in GCSCs by promoting RAB37 methylation and phosphorylation. Therefore, supplementation with methionine γ-lyase can induce autophagy in GCSCs and inhibit tumor growth[36].

The role of CSCs in growth, metastasis, treatment and prognosis of GC is increasingly important. In the past few years, an increasing number of studies have been conducted on the mechanism of action of CSCs against GC and to offer promising targets and therapeutic tactics for GC treatment. Many treatments targeting CSCs have been developed, but there are limits to CSCs targeting therapy. It affects normal stem cells and causes tissue renewal problems. For example, Lgr5 is a marker associated with common stem cells in gastric tissue. mTORC1 can maintain self-renewal of the Lgr5 population, preventing cell differentiation and causing gastric tumorigenesis. However, the use of mTORC1 inhibitors may cause gastric glandular atrophy due to tissue malfunction, limiting its therapeutic use [39]. Therefore, more efforts are needed to treat GC based on CSCs.

ROLES OF CSCS IN LC

LC is the third most common cause of cancer associated deaths worldwide. This is due to its high recurrence rate, which after normal treatment, can reach 70%. Hepatocellular carcinoma (HCC) is the main pathology and causes approximately 80% of LC cases[40]. Liver CSCs (LCSCs) are now known to be responsible for HCC growth, metastasis and recurrence, in addition to failure of chemotherapy and radiation therapy[41]. These findings indicate that LC therapy kills most of the tumor cells, but cannot eliminate LCSCs and treatment can eventually fail as LCSCs survive and generate new tumors. Therefore, CSCs theory has provided new findings in the diagnosis, treatment and prevention of LC (Table 1).

LCSCs play a vital role in the development, progression, recurrence and drug tolerance of HCC. Studies have shown that LCSCs can accelerate tumor growth in primary cancer cells and metastasis of secondary tumors, causing the recurrence of HCC[42]. Studies have found that EpCAM-high HCC stem cells can promote tumor growth by upregulating CEACAM1 to weaken the capacity of natural killer

Table 1 Methods for targeting liver cancer stem cells in the treatment of liver cancer

Ref.	Genes/transcription factors/protein	Inducing way	Role
Park *et al*[43]	EpCAM-high HCC stem cells	Upregulating CEACAM1	Promoting the growth of LC
Deng *et al*[44]	Histone demethylase JMJD2D	Enhancing Sox9 expression	Promoting the growth of LC
Yang *et al*[45]	lncARSR	Targeting STAT3 signaling	Promoting LCSCs expansion
Chen *et al*[47]	4-PBA	Activating β-catenin signaling pathway	Promoting the growth of LC
Galizia *et al*[49]	CD133	-	Indicator of tumor recurrence
Haraguchi *et al*[51]	CD13	-	Causing drug resistance of LCSCs
Yang *et al*[52]	OV6	-	Causing drug resistance of LCSCs
Wei *et al*[53]	CD44	Regulating PES1	Promoting the growth of LC
Chen *et al*[55]	FOXM1	Reducing the expression of ALDH2	Inducing the apoptosis of LCSCs
Dou *et al*[56]	BC-02	Inhibiting CD13	Eradicate LCSCs
Zhou *et al*[57]	CD133-apt-Dox	Targeting CD133-expressing cells	Inhibiting the growth of LC
Feng *et al*[58]	MiR-124	-	Inhibiting LCSCs self-renewal
Jiang *et al*[59]	MiR-365	Regulating RAC1 pathway	Inhibiting the proliferation and invasion of HCC cells
Li *et al*[60]	MicroRNA-21	-	Inhibiting highly invade LCSCs
Si *et al*[61]	MiR-219	E-cadherin pathway	regulates the expansion of LCSCs
Dou *et al*[62]	MicroRNA-6838-5p	Down-regulating CBX4 expression and Inactivating ERK signaling	Inhibiting self-renewal and metastasis of Human LCSCs
Zhang *et al*[63]	MiR-589-5p	Inhibiting MAP3K8	Inhibiting the growth of LC
Wang *et al*[64]	HAND2-AS1	-	Promoting the growth of LC
Li *et al*[66]	Neuropilin1	The loss of neuropilin1	Inhibiting LCSCs
Wang *et al*[67]	ZBP-89	Inhibiting Notch1 signaling pathway	Regulating self-renewal of LCSCs

HCC: Hepatocellular carcinoma; LC: Liver cancer; LCSCs: Liver cancer stem cells.

cells to recognize and kill cancer cells[43]. Histone demethylase JMJD2D can enhance EpCAM and Sox9 expression to promote the self-renewal of liver CSLCs, promoting tumor growth[44]. Yang *et al*[45] found that lncARSR promoted HCC cell dedifferentiation and LCSCs expansion by targeting STAT3 signaling. Moreover, LCSCs release exosomes in a RAB27A-dependent manner which can lead to resistance to regorafenib in LC cells[46]. Chen *et al*[47] found that activating the β-catenin signaling pathway leads to upregulation of PPAR-α by 4-PBA, which in turn initiates LCSCs and promotes early HCC. Cao *et al*[48] found that RACK1 advances self-renewal and chemoresistance of CSCs in HCC by stabilizing nanoparticles.

The biomarkers of LCSCs are important targets in the treatment of LC. CD133 is one of the common surface markers of LCSCs, and studies have found that CD133 isolated from HCC cell lines has high proliferative and tumorigenic potential; thus, enhanced CD133 expression can also serve as a prognostic indicator for survival and tumor recurrence in patients with LC[49]. Yin *et al*[50] found that aldehyde dehydrogenase was expressed in LCSCs and was positively correlated with CD133 expression. CD13 is a marker of LCSCs, which has the function of dormancy and slow growth, and is the main reason for drug resistance of LCSCs[51]. The LCSCs marker OV6 was found to be chemoresistant, but this was reversed when lentivirus-delivered miRNAs targeting β-catenin were stably expressed. Therefore, targeting Wnt/β-catenin signaling could be a potential strategy to reverse the drug resistance properties of OV6 LCSCs[52]. Wei *et al*[53] found that CD44 regulated PES1 in LCSCs through miR-105-5p to promote tumor growth. Twist2 advances self-renewal of liver CSLCs by changing CD24[54].

LCSCs play a vital role in the treatment of LC. It was found that the transcription factor FOXM1 inhibits the dryness of LCSCs by reducing the expression of ALDH2, and inhibits proliferation, migration, invasion and tumorigenesis, while inducing apoptosis of LCSCs[55]. The CD13 inhibitor BC-02 can target CD13 and upregulate intracellular reactive oxygen species (ROS) and ROS-induced DNA damage to damage LCSCs. Therefore, BC-02 may be a potential therapeutic strategy to eradicate LCSCs and overcome chemoresistance in LC[56]. Studies have found that aptamer-based drug delivery agents

(CD133-apt-Dox) targeting CD133-expressing cells can impair the self-renewal ability of liver CSLCs and inhibit the growth of LC[57]. MiRNAs are important regulators of CSCs therapy in LC. Feng *et al*[58] found that forced expression of miR-124 can inhibit LCSCs self-renewal and tumorigenesis. It was shown that miR-365 regulated LCSCs through the RAC1 pathway and prevented the proliferation and invasion of HCC cells[59]. In addition, miR-21 downregulation can inhibit cell proliferation and highly invade LCSCs[60]. Si *et al*[61] found that miR-219 regulates the expansion of LCSCs through the E-cadherin pathway. Dou *et al*[62] showed that miR-6838-5p inhibited self-renewal and metastasis of LCSCs by down-regulating CBX4 expression and inhibiting ERK signaling. In addition, miR-589-5p inhibited MAP3K8 in HCC and inhibited CD90 CSCs[63]. It was shown that HAND2-AS1 can promote self-renewal of LCSCs and drive liver tumorigenesis, providing a potential new target for HCC treatment[64]. In addition, tumor-associated macrophages produce interleukin-6 and signal through STAT3 to improve the expansion of LCSCs[65]. Li *et al*[66] found that the loss of neuropilin 1 inhibits the LCSC population and blocks metastasis in HCC through epithelial-mesenchymal transition. Wang *et al*[67] found that ZBP-89 negatively regulated self-renewal of LCSCs by inhibiting the Notch1 signaling pathway.

Traditional HCC treatment mainly targets fast growing and differentiated HCC cells. However, a part of the emerging CSCs concept explains the failure of these therapies. The research progress of LCSCs has provided a new viewpoint on the possible adhibition in the clinical treatment of LCSCs. Detection of LCSCs is useful for predicting postoperative survival of patients. The development of treatment strategies for LCSCs may greatly improve the treatment of LC[68].

ROLES OF CSCS IN CRC

CRC is one of the most common cancers and the fourth most frequent cause of cancer death worldwide [69]. Despite the great progress in surgery and chemotherapy over the past decade, the five year survival rate of CRC patients is 50%-65%[70]. In the past few years, an increasing number of studies have shown that CSCs are closely associated with the occurrence and development of CRC, which provides promising directions for the diagnosis and treatment of CRC (Table 2).

CSCs are the major cause of drug resistance and disease recurrence in CRC. Studies have found that Lgr5 CSCs have a vital role in primary and metastatic colon cancer, and can promote tumor growth and metastasis[71]. In addition, most colorectal CSCs (CCSCs) express Lgr5 and form distant metastasis, which is a major factor driving CRC metastasis[72]. Recent studies have found that human CCSCs can give rise to vascular endothelial cells and constitute the vasculature in cancer tissue, which provides a new mechanism for tumor angiogenesis in cancer[73]. CD26 CCSCs can lead to CRC metastasis[74]. Razi *et al*[75] found that DCLK1 is a promising CCSC marker that changes tumor progression and invasion in a miR-137- and miR-15a-dependent manner. The JAK2/STAT3/CCND2 axis promotes the persistence and radioresistance of CRCs, which is the drug resistance mechanism for the continuous growth of CSCs after radiotherapy[76]. Liu *et al*[77] found that Sec62 promotes stemness and chemoresistance in human CRC by sensitizing the Wnt/β-catenin pathway. Studies have shown that CCSCs acquire chemoresistance by the upregulation of F-Box/WD repeat-containing protein 7 and the consequent degradation of c-Myc[78]. In addition, PD-L1 can maintain CSCs self-renewal by activating the HMGA1-dependent signaling pathway[79]. 5-FU improves the stemness of CRC through p53-mediated WNT/β-catenin pathway activation[80].

Whereas conventional therapies target proliferating and mature cancer cells, CSCs are mostly quiescent and poorly differentiated, so they could easily survive the chemotherapy attack. Therefore, novel therapies targeting CSCs are necessary. Chen *et al*[81] found that phenethyl isothiocyanate inhibits CCSCs by suppressing the Wnt/β-catenin pathway. In addition, (-)-Epigallocatechin-3-Gallate can also inhibit CCSCs by suppressing the Wnt/β-catenin pathway[82]. Studies have shown that AGR2 is a new stem cell marker that is changed by the canonical Wnt/β-catenin pathway in CCSCs and is important for stemness maintenance of CCSCs[83]. Jang *et al*[84] reported that tankyrase inhibitors downregulated c-KIT tyrosine kinase and prevented the growth of CD44-positive CCSCs. Liu *et al*[85] found that PTK6 interacts with JAK2 and phosphorylates to activate JAK2/STAT3 signaling, which can improve stemness and chemoresistance of CRC cells and reverse chemoresistance in CRC. Studies have shown that mithramycin A inhibits CRC growth by targeting CSCs[86]. Studies have also found that disruption of endolysosome RAB5/7 effectively eliminates CCSCs[87]. PrPC inhibits CSCs properties by interacting with c-MET in CRC cells[88].

According to the data accumulated during cancer research, CSCs have become a fundamental cause of cancer progression and resistance to treatment. Therefore, it is important to understand the biological, functional and clinical significance of CSCs in CRC tolerance in order to develop an effective treatment model for CRC patients. However, the practical clinical application of CSCs in CRC is still limited, and further studies and efforts are needed for clinical applications.

Table 2 Methods for targeting colorectal cancer stem cells in the treatment of colorectal cancer

Ref.	Genes/transcription factors/protein	Inducing way	Role
Fumagalli *et al* [72]	Lgr5	-	Promoting the growth of CRC
Cheung *et al*[74]	CD16	-	Promoting the growth of CRC
Razi *et al*[75]	DCLK1	MiR-137 and Mir-15a-dependent manner	Promoting the growth of CRC
Park *et al*[76]	JAK2/STAT3/CCND2 axis	-	Causing drug resistance of CCSCs
Liu *et al*[77]	Sec62	Activating the Wnt/β-catenin pathway	Causing drug resistance of CCSCs
Izumi *et al*[78]	F-Box/WD repeat-containing protein 7	-	Causing drug resistance of CCSCs
Wei *et al*[79]	PD-L1	Activating HMGA1-dependent Signaling pathway	Maintaining CSCs self-renewal
Cho *et al*[80]	5-FU	Activing p53-mediated WNT/β-catenin pathway	Promoting the stemness of CRC
Chen *et al*[81]	Phenethyl isothiocyanate	Suppressing Wnt/β-catenin pathway	Inhibiting CCSCs
Chen *et al*[82]	(-)-Epigallocatechin-3-Gallate	Suppressing Wnt/β-catenin pathway	Inhibiting CCSCs
Dahal *et al*[83]	AGR2	Regulating Wnt/β-catenin pathway	Regulating the stemness maintenance of CCSCs
Jang *et al*[84]	Tankyrase inhibitors	Downregulating c-KIT tyrosine kinase	Inhibiting the growth of CD44-positive CCSCs
Liu *et al*[85]	PTK6 interacts with JAK2	Activating JAK2/STAT3 signaling	Reversing chemoresistance in CRC
Quarni *et al*[86]	Mitramycin A	-	Inhibiting CCSCs
Lim *et al*[88]	PrPC	Interacting with c-MET	Inhibiting CCSCs

CCSC: Colorectal cancer stem cells; CRC: Colorectal cancer; CSCs: Cancer stem cells.

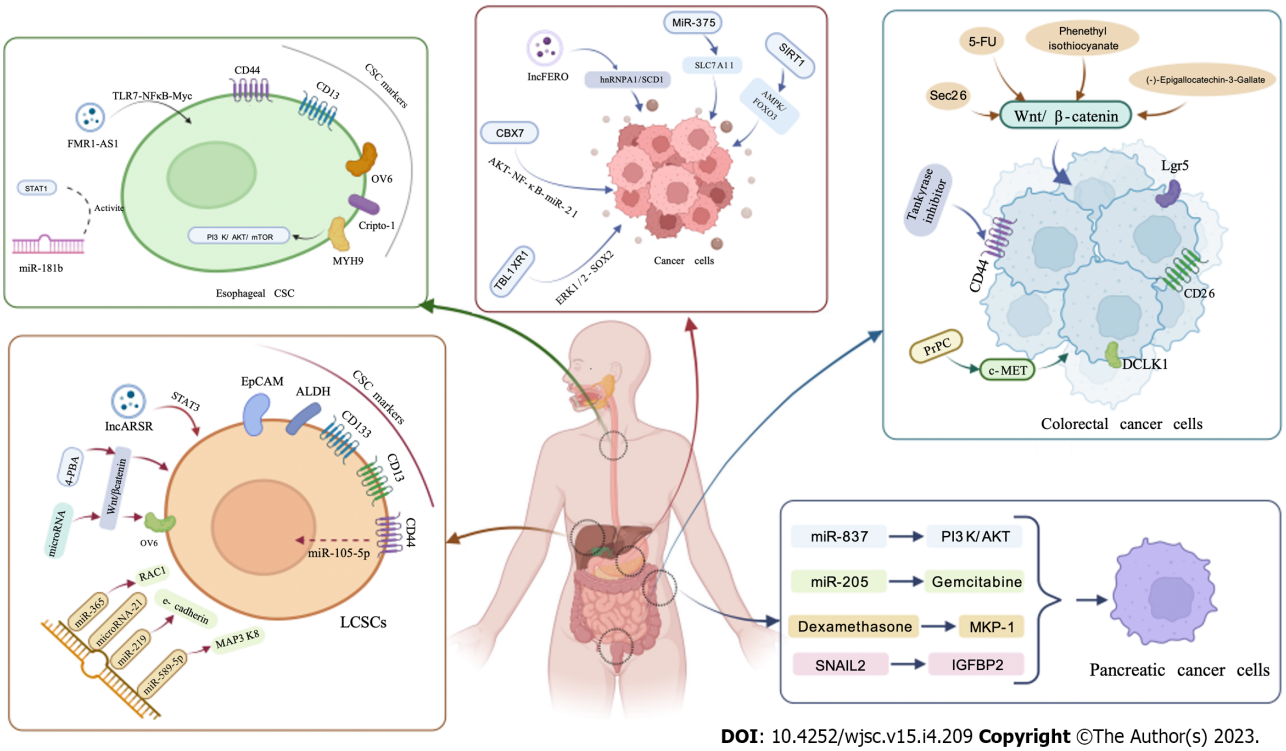

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Figure 1 The roles of cancer stem cells in gastrointestinal cancers. CSC: Cancer stem cells; LCSC: Liver cancer stem cells.

ROLES OF CSCS IN PANCREATIC CANCER

Pancreatic cancer is one of the most deadly human malignancies, the survival rate is 8% and the prognosis is the worst of all GI tumors[89-91]. It is now the fourth most common cause of cancer associated deaths worldwide. The tumorigenesis capacity of pancreatic cancer cells is different, and the proliferation and growth of pancreatic cancer are highly dependent on the presence of a limited subgroup of pancreatic cancer cells, called pancreatic CSCs (PCSCs)[92]. The concept of CSCs is recognized and some of the identified molecules and signaling pathways are associated with cancer diagnosis and treatment.

PCSCs contribute to the development and invasion of pancreatic cancer. It has been found that PCSC CD9 can promote the plasma membrane localization of glutamine transporter ASCT2, improving glutamine uptake in pancreatic cancer cells and promoting tumor growth[93]. Leng *et al*[94] found that SIRT1 coordinating with CRL4B can regulate PCSCs to promote tumorigenesis. In PCSCs, PAF1 interacts with DDX3 and PHF5A to regulate the expression of NANOG and other genes that regulate stemness. Therefore, knockdown of PAF1 reduced the development and progression ability of *in situ* pancreatic cancer in mice and its CSCs[95]. Bao *et al*[96] found that pancreatic CSLCs can promote tumor formation and rapid tumor growth by activating FoxQ1. Masuo *et al*[97] demonstrated that SNAIL2 can promote the tumorigenicity and chemotherapy resistance of PCSCs by regulating IGFBP2.

PCSCs have an important role in pancreatic cancer therapy. Studies have shown that PCSCs have longer telomeres and higher telomerase activity than tumor cells, which is associated with the expression of pluripotent genes (Nanog, Sox2, Oct3/4). Therefore, telomerase inhibition can lead to apoptosis of PCSCs, which is a suitable therapeutic approach against CSCs, especially in pancreatic cancer[98]. Yang *et al*[99] found that miR-873 could inhibit self-renewal and proliferation of PCSCs by blocking the PLEK2-dependent PI3K/AKT pathway. In addition, miR-205 can resensitize gemcitabine-resistant pancreatic cancer cells to gemcitabine and act as a tumor suppressor miRNA[100]. JNK is required for PCSCs self-renewal and tumor initiation, as well as its survivin expression. Dexamethasone was found to induce the expression of MKP-1 through glucocorticoid receptor activation, thereby inactivating JNK and inhibiting tumor growth[101]. Urtasun *et al*[102] showed that simultaneous blockade of IGF-IR and EGFR/Her-2 using NVP-AEW541 and lapatinib could inhibit drug resistance in pancreatic cancer.

The role of CSCs in pancreatic cancer metastasis, recurrence and treatment has become very important. A large number of studies have allowed tumor stem cell markers to achieve metastasis, progression and resistance of pancreatic cancer cells, which provide potential targets and therapeutic directions for the treatment of pancreatic cancer. Nevertheless, there are still few practical clinical applications of PCSCs, and further studies are needed for clinical applications.

Recently, many studies have shown that CSCs are closely related to tumor recurrence, metastasis and drug resistance, especially in EC, GC, LC, CRC and pancreatic cancer (Figure 1). CSCs can be identified by a series of surface markers, including OV6, EpCAM, CD13, CD133 and CD44. Surface markers of CSCs are useful for cancer diagnosis and prognosis prediction. In addition, CSCs regulate tumor progression and therapeutic resistance through multiple mechanisms, including Notch, Wnt/β-catenin and other signaling pathways. There are many other elements that make CSC impressions, such as the tumor microenvironment and non-coding RNAs, including miRNAs and long non-coding RNAs.

CONCLUSION

Currently, we have developed a combination of chemotherapeutic agents and small molecule inhibitors to reduce CSCs and effectively treat GI cancer. As CSCs have many similar features to stem cells, the molecular signal pathway or mechanism that distinguishes the two cell subgroups is still unknown. Therefore, stem cell therapy is limited, and it is necessary to further study the biological difference between normal stem cells and LCSCs. Research on the therapy of GI cancer with regard to CSCs is still at the *in vitro* and animal experimental stage, and the precise molecular mechanism of CSCs in GI cancer requires further study. Therefore, more research is needed to promote the application of CSCs in clinical practice.

FOOTNOTES

Author contributions: Wu J and Dong P designed study and revised the manuscript. Xuan SH, Hua ML and Xiang Z analyzed data and performed manuscript drafting; He XL, Huang L and Jiang C searched the literature and collected data; Dong P and Wu J reviewed the results and made critical comments on the manuscript; all authors reviewed and approved the final version; Xuan SH and Hua ML contributed equally to this work; Wu J and Dong P contributed equally to this work.

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MINIREVIEWS

Harnessing and honing mesenchymal stem/stromal cells for the amelioration of graft-versus-host disease

Tang-Her Jaing, Tsung-Yen Chang, Chia-Chi Chiu

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Abstract

Allogeneic hematopoietic stem cell transplantation is a deterministic curative procedure for various hematologic disorders and congenital immunodeficiency. Despite its increased use, the mortality rate for patients undergoing this procedure remains high, mainly due to the perceived risk of exacerbating graft-versus-host disease (GVHD). However, even with immunosuppressive agents, some patients still develop GVHD. Advanced mesenchymal stem/stromal cell (MSC) strategies have been proposed to achieve better therapeutic outcomes, given their immunosuppressive potential. However, the efficacy and trial designs have varied among the studies, and some research findings appear contradictory due to the challenges in characterizing the *in vivo* effects of MSCs. This review aims to provide real insights into this clinical entity, emphasizing diagnostic, and therapeutic considerations and generating pathophysiology hypotheses to identify research avenues. The indications and timing for the clinical application of MSCs are still subject to debate.

Key Words: Mesenchymal stem/stromal cells; Graft-versus-host disease; Immunomodulatory; Adaptive immunity; Exosomes

Core Tip: This article provides insights into the use of validated mesenchymal stem/stromal cells (MSCs) as a potential treatment strategy for graft-versus-host disease (GVHD) in hematopoietic stem cell transplantation (HSCT). Current prevention and treatment options involve immunosuppression, which can hinder immune recovery and limit the graft-versus-tumor effect. By using MSCs, clinicians can effectively treat GVHD, identify high-risk patients, and stratify patients based on disease severity. Therefore, MSCs can aid in promoting engraftment, ameliorating acute GVHD, and preventing chronic GVHD, making them an attractive option for HSCT.

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INTRODUCTION

Mesenchymal stem/stromal cells (MSCs) are multipotent cells with self□renewal abilities[1] that can be derived from different tissue sources. They attach to tissue culture dishes and express CD73, CD90, and CD105 but lack the expression of CD45, CD34, CD14, or CD11b, CD79α or CD19, and HLA-DR surface molecules. In vitro, MSCs can differentiate into osteoblasts, adipocytes, or chondroblasts[2,3]. MSCs can be effectively harvested without significant ethical concerns and have low immunogenicity. They have emerged as a promising cell source due to their regenerative and immunomodulatory potentials, limited ethical concerns, and low risk of tumor formation[4-6].

Malignancy relapse is a significant challenge in allogeneic hematopoietic stem cell transplantation (HSCT). Chronic graft-versus-host disease (GVHD) is associated with lower relapse rates, but the diagnosis, staging, and risk stratification of GVHD are challenging[7]. In this scoping review, we highlight recent evidence on different types of MSCs studied for GVHD, including bone marrow (BM), umbilical cord blood, placenta, adipose tissue, and others. MSCs have been found to inhibit immune cell proliferation and cytotoxic action, making them a potential treatment option for GVHD[8].

This review aims to provide a critical overview of the mechanisms by which MSC can treat GVHD, including immunomodulation, migration, homing, and clinical applications of MSC therapy. We searched peer-reviewed literature in PubMed and Embase to gather the latest information on this topic.

THERAPEUTIC STRATEGY OF GVHD

Immune pathways in GVHD

One of the most significant challenges in improving the prognosis for patients undergoing allogeneic HSCT is GVHD. This condition can be characterized as a rapid escalation in immune activation caused by massive target tissue apoptosis. The prevention of GVHD is primarily based on the use of calcineurin inhibitors and methotrexate, while the treatment of ongoing GVHD involves the use of corticosteroids. GVHD manifests as acute GVHD (aGVHD) in 53%-62.5% of the patients and chronic GVHD (cGVHD) in 20%-50.4% of patients[9,10], and the development of this complication may contribute to 6.3% of deaths following HSCT[9]. Although the administration of calcium inhibitors such as calcium sulphoaluminate can prevent the development of GVHD in some cases, about 19% of aGVHD II-IV cases are often resistant to all conventional therapy, resulting in a high mortality rate for these patients. Several potential second-line options have been proposed, including the use of MSCs. MSCs have attracted significant interest because they can actively undergo apoptosis by recipient cytotoxic cells [11]. Figure 1 illustrates the immune pathways involved in GVHD and the sites where therapy is used to block GVHD development.

Danger signals in aGVHD development

In a typical case of aGVHD, which occurs following a triptych course, symptoms begin with the prodromal phase caused by the underlying disease and conditioning regimens that secrete proinflammatory cytokines, mainly tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6)[12]. Host conditioning facilitates donor cell grafting. Donor allograft T-cells are the primary effector cells for GVHD. However, tissue damage leads to the release of alarmins and the expression of pathogen-recognition receptors, triggering the next phase. This phase activates the innate immune system and, in turn, the adaptive immune system. Alarmins and exogenous pathogen-associated molecular patterns (PAMPs) elicit similar responses to relevant signals, and they belong to the group of damage-associated molecular patterns (DAMPs)[13]. DAMPs and PAMPs are potent stimulators for

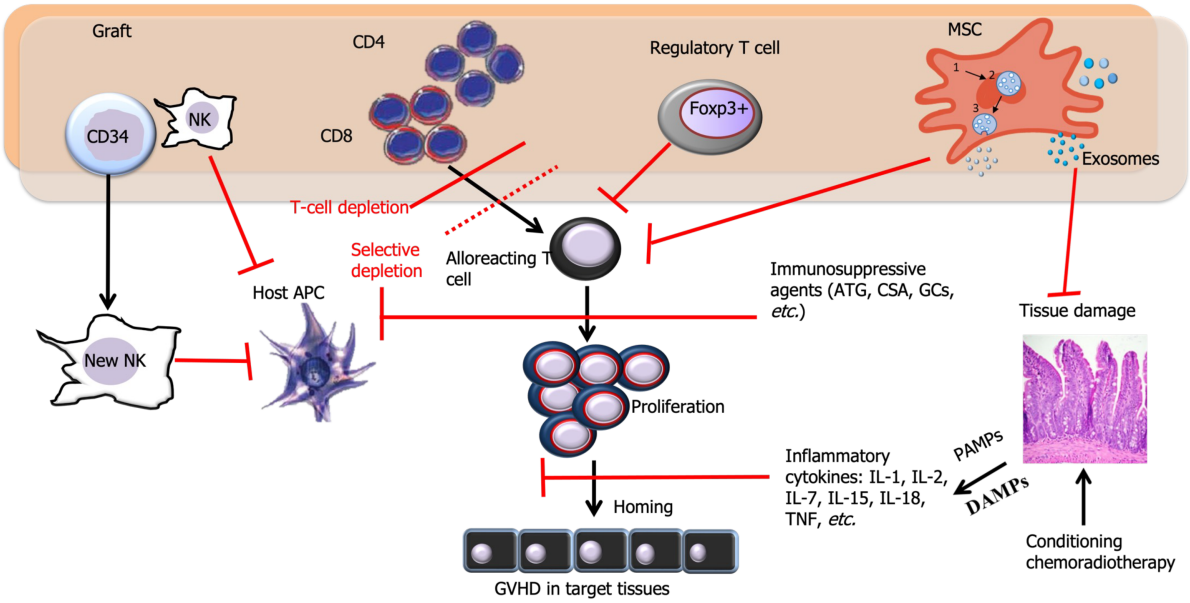

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Figure 1 Immune pathways in graft-versus-host disease and sites where therapy is used to block graft-versus-host disease development (red bars). APC: Antigen-presenting cell; ATG: Antithymocyte globulin; CSA: Cyclosporine A; DAMPs; Damage-associated molecular patterns; Foxp3+: Forkhead box P3; GCs: Glucocorticosteroids; GVHD: Graft-versus-host disease; MSC: Mesenchymal stem cell; TNF: Tumor necrosis factor; NK: Natural killer cells; PAMPs: Pathogen-associated molecular patterns.

host and donor-derived antigen-presenting cells (APCs), which activate and enhance the responses of alloreactive donor T cells[14].

The immunosuppressive effects of MSCs are classified into soluble factor-mediated effects and cell-cell contact-mediated effects. MSCs suppress the proliferation and survival of activated T lymphocytes and reduce the release of inflammatory factors such as IL-2, TNF-α, IL-1β, and IFN-γ. By the same means, MSCs also reduce the number of Th1/Th2 and Th17 cells. Through cell-to-cell contacts, MSCs can stimulate the expression of transcription complexes related to Runt 1 (RUNX1), RUNX3, and CBFβ in Treg-specific regulatory regions to improve the stability of Foxp3[15]. MSCs have also been shown to be highly effective in inhibiting the cytotoxic effect, proliferation, and secretion of different cytokines of NK cells by directly contacting these cells and transforming their phenotype.

The effects of MSCs on B cells involve inhibiting their cell cycle progression by inducing G0/G1 cell cycle arrest and suppressing their proliferation. Additionally, the differentiation of B cells into IgM-, IgG-, and IgA-secreting cells is impaired by MSCs, thereby limiting their antibody production. Furthermore, MSCs can affect the chemotactic function of B cells[16].

GENETIC BASIS OF GVHD

Humanized mouse models

Most relevant models for studying human adaptive immune responses use immunocompromised mice whose immune system is reconstituted with human immune cells and immune system components. Lee *et al*[17] used a model of NSG mice reconstituted with human CD34 cells to evaluate the immunological safety of therapeutically compromising human MSCs. As major histocompatibility complex (MHC) molecules are the primary mediators of the allogeneic immune response, MHC expression levels are critical in the potential immunogenicity of cells. To investigate MSCs as a cellular therapy in GVHD, Tobin *et al*[18] treated NSG-PBMC humanized mice with human MSCs as a GVHD model. MSC treatment resulted in a reduction in liver and intestinal pathology and a significant increase in the survival of the GVHD NSG mouse.

In contrast to aGVHD, some MHC-mismatched animal models may mimic the features of cGVHD. However, due to the pathological resemblance between cGVHD and autoimmune diseases, there is a clear connection between the two entities, and the difference in cGVHD is primarily caused by the donor lymphoid graft[19]. These findings provide compelling evidence for the essential role of human leukocyte antigen (HLA) disparity in both aGVHD and cGVHD. The expression pattern of minor histocompatibility antigens (miHAs) determines the target organ involvement in aGVHD. The miHAs exhibit hierarchical immunodominance, which may contribute to the variability in GVHD variability [20].

Translation and clinical advances in GVHD

aGVHD: HLA mismatching is one of the most significant risk factors for aGVHD and cGVHD risk. HLA proteins are specifically encoded by MHC. In vitro studies have demonstrated that most T cells associated with GVHD are naïve T cells, whereas memory T cells mediate immunity against pathogens and the graft-versus-leukemia (GVL) effect[21]. Regardless of the graft source or conditioning intensity, the incidence of aGVHD is closely related to the number of HLA disparities. Although the impact of HLA disparity has been analyzed in the outcomes following allogeneic HSCT, relatively few studies have tried to correlate it with the incidence and severity of cGVHD. Some studies reported an association between HLA-A, -B, and -C disparity and aGVHD[22].

Although MHC antigens guarantee HLA matching, the donor and recipient may differ in various proteins presented in the form of HLA-peptide complexes to T cells that act as miHAs. The genomes include more than 10^7 polymorphic sequences outside HLA, and the role of miHAs is supported by genome-wide analysis of single-nucleotide polymorphisms[7]. The disparity in a single immunodominant miHA is insufficient to cause aGVHD, although T cells primed against a single miHA may induce tissue damage in a human skin explant model[23]. It is unknown whether the number of miHAs triggering a GVL response in a given transplant is significant or whether a small number of antigens play a dominant role[24].

cGVHD: In contrast, cGVHD has been considered an autoimmune disease based on its clinical features [25]. Some experimental studies have shown that T cells from animals with cGVHD are specific for a public determinant of MHC class II molecules and are therefore considered autoreactive. These autoreactive cells of cGVHD are often associated with an injured thymus and adverse selection.

Recent clinical data has highlighted a significant link between immune responses against ubiquitous miHAs and cGVHD. Since cGVHD usually occurs after allogeneic HSCT, aGVHD is its related risk factor. Unlike syngeneic GVHD, which results from deficient thymic selection[26], cGVHD typically arises after allogeneic HSCT and is characterized by chronic T-cell activation due to continuous exposure to miHAs. This chronic stimulation can cause target organ damage that resembles autoimmune features, where the target is miHAs for cGVHD and non-polymorphic autoantigens for autoimmune diseases. A study on female-to-male HSCT demonstrated a good correlation between the presence of antibodies to the Y-chromosome-encoded gene and cGVHD[27]. A study in female-to-male HSCT demonstrated a good correlation between the presence of antibodies to Y-chromosome-encoded genes and cGVHD, suggesting that miHAs may indeed be the targets. However, it is not yet clear whether the miHAs targeted in cGVHD are the same as those targeted in aGVHD. A murine study had shown that the type and selection of immunodominant miHAs can determine the target and character of GVHD damage[28].

Epitope spreading and the failure of appropriate regulatory mechanisms in aGVHD may result in donor T cells recognizing both non-polymorphic and miHA epitopes, perpetuating cGVHD. In contrast, T cells directed against miHAs with hematopoietic restriction may also mediate a GVL response in the absence of GVHD[29]. However, the relevant immunogenic targets for cGVHD remain speculative and confidential.

Potent immunomodulatory role of MSCs-derived exosomes in preventing GVHD

The safety and effectiveness questions regarding using MSCs remain unresolved, and conflicting effects have been noted due to the heterogeneity observed among MSCs. MSCs-derived exosomes (MSCs-Exo), a subgroup of extracellular vesicles released by MSCs, have shown therapeutic benefits for inflammatory diseases and cancers due to their ability to transport proteins and nucleic acids from donor cells to recipient cells of the same or different tissues, making it a suitable candidate for cell-free therapy. MSCs-Exo have been found to reduce inflammation and fibrosis in the skin, lungs, and liver, and inhibit Th17 cells while inducing Treg cells, making it a potential alternative method for the treatment of cGVHD. The activation of CD4+ T cells and their infiltration into the inflamed mouse lung were reduced in MSCs-Exo-treated mice[30]. MSCs-Exo, extracted from healthy donors' BM, suppress the expression of pro-inflammatory factors TNF-α and IL-1β but increase the level of anti-inflammatory factor TGF-β during *in vitro* culture[31].

Typically, MSCs-Exo are characterized by endosomes that bud inward and package into multivesicular bodies (MVBs). These MVBs fuse with the plasma membrane and deliver the exosomes into the intracellular space. However, exosomes can enrich several molecules as cargo, such as proteins/cytokines, DNA, RNA, and other nucleic acids. Exosomes, as secretory components of MSCs, transport cytokines, and growth factors of immunoregulation, such as transforming growth factor beta-1 (TGF-β 1), IL-6, IL-10, hepatocyte growth factor, signaling lipids, mRNAs, and regulatory miRNAs, which exert biological effects on recipient cells, such as cell-to-cell communication, tissue regeneration, metabolism, immune modulation, and homing of immune cells[32,33]. Diverse immune cells establish complex interactions with each other. MSCs-Exo might represent a novel cell-free therapy with unique competitive advantages over parent MSCs, such as no apparent risk of tumor formation or lower immunogenicity.

IMMUNE CELL LANDSCAPE OF GVHD

Antigen-presenting cells

Antigen-presenting cells (APCs) play a critical role in inducing aGVHD, with dendritic cells (DCs) being one of the most formidable cells in this regard[26]. Innate immunity activation during acute inflammation leads to DCs maturation and subsequent T cell priming, which is central to the potential antitumor benefits of aGVHD. Experimental data suggest that modulating perceptible DC subsets can influence aGVHD[34]. For instance, the absence of RelB signaling in host DCs or enhancing host CD8+ lymphoid DC subsets following HSCT significantly reduces aGVHD[35]. Other APCs, such as monocytes/macrophages, also play a crucial role in this phase. Some data suggest that the host B cells may reduce aGVHD in specific contexts. Although the precise mechanisms remain unclear when acting as APCs, MSCs from the donor, or host also reduce aGVHD.

Natural killer, γδ T, and natural killer T cells

Natural killer (NK) cells can directly kill tumor cells without specific immunization and also have a modulatory effect on aGVHD. In an allo-HSCT donor-to-F1 model, NK cells recognize the absence of donor class I on host APCs and eliminate them, resulting in a reduction of aGVHD reduction. Upon activation, NK cells may induce apoptosis of target cells through contact-dependent cytotoxicity primarily *via* perforin and granzyme[36]. Pro-apoptotic granzymes enter through perforin pores in the plasma membrane of target cells. Besides the cytotoxic activity, NK cell activation increases the secretion of various cytokines and chemokines, such as IFN-γ. However, the role of NK cells in GVHD remains controversial.

The infusion of donor γδ T cells may increase aGVHD, while the absence of host γδ T cells may reduce APC activation and aGVHD in an MHC-mismatched model. Conversely, in the absence of host γδ T cells, GVHD severity was not modified in an MHC-matched, miHA-disparate model of cGVHD. aGVHD could be more significant in patients with more considerable donor γδ T cells. The significance of γδ T cells in aGVHD and cGVHD is not fully understood and may reflect differences in immunobiology between the two or be solely a consequence of variation in the experimental models.

NKT cells, which are CD1d-reactive, are believed to play an immunoregulatory role in suppressing dysfunctional immune reactions, including GVHD[37]. The cumulative frequency of regulatory T cells (Tregs) is negatively correlated with GVHD development[38], and exogenous NKT cell infusion can reduce the degree of GVHD[39]. However, Treg populations have unstable Foxp3 expression, particularly those expanded in vitro. Because the expression Foxp3 is needed for the suppressive function, further research is necessary to determine if Foxp3 expression can be simplified, especially under pro-inflammatory conditions characteristic of the GVHD milieu[40].

T cells

The complex interactions between MSCs and T cells have been extensively studied, particularly *in vitro* culture techniques. MSCs may facilitate activated T cells in the phase G0/G1 cell cycle, yet apoptosis is not applicable[41-43]. MSCs may suppress or downregulate the proliferation of both naïve and memory T cells through cell-cell contact or mitogenic stimuli. This suppression is generally not MHC-restricted. MSCs can further decrease IFN-γ producing T cells and contribute to the T-cell skewing toward Th2 cells producing IL-4. cGVHD is a Th2 cell dominant disease process[12].

Regulatory T cells

MSCs activate *immune* responses that induce the expression of Tregs, which are a cluster of cells with a $CD4^+CD25^+$ $Foxp3^+$ phenotype that regulate the body's immune response. Tregs highly and constitutively express CTLA-4, which binds to CD80, and CD86 on DCs, leading to impaired DC maturation and blocking CD80/CD86 to CD28 on conventional T cells, thereby preventing costimulation, and T-cell activation. Lower Tregs and deficient Foxp3 expression have been associated with cGVHD in peripheral blood and mucosal biopsies. However, levels of Foxp3 mRNA in the CD25+ T cell compartment do not predict the development of cGVHD, demonstrating that the presence, or absence of Tregs must be considered in the context of their impact on aGVHD and cGVHD. An intriguing possibility is that the negative impact of calcineurin inhibitors on Tregs could exacerbate cGVHD as a consequence of the suppression of the alloreactive donor cytopathic and Tregs.

B cells

Host B cells attenuate aGVHD in an IL-10-dependent manner. Recent data provide a rationale for the pathogenic role of donor B cells in cGVHD[12], including a robust correlation between cGVHD and (1) The effects of antibodies against Y-chromosome-encoded miHA; (2) higher numbers of B cells with altered TLR9 responses; (2) levels of a B-cell-activating factor, which enhances survival and differentiation of activated B cells; and (4) in animal models, levels of autoantibodies. Besides, emerging data from the depletion of B cells with rituximab further supports the theory of the pathogenic action of B cells in cGVHD[44]. However, whether B cells are the effectors or inducers of cGVHD remains unknown.

SEARCH STRATEGY, STUDY SCREENING, AND SELECTION

We systematically searched the electronic bibliographic databases MEDLINE, EMBASE, and Google Scholar for studies published before November 2022 using the keywords: "graft-versus-host disease" OR "acute GVHD" OR "chronic GVHD" AND "mesenchymal stem cells" AND "mesenchymal stromal cells" AND "treatment response" AND "outcome." Publications were included if they met the following inclusion criteria: (1) Original research; (2) published in 2002 or later; and (3) specifically reporting on the use of MSCs in GVHD patients. Publications were excluded based on the following criteria: (1) Non-English literature; (2) small populations (n < 20 patients) or case studies; and (3) mixed population with non-GVHD patients. A meta-analysis was not performed for the limited number of published studies meeting the inclusion criteria. Pre-post design studies and case series were not included for lack of sustainability of the results. Additionally, reference lists of retrieved articles were cross-referenced for additional eligible articles.

RESULTS

This review provides an overview of clinical studies, animal models, and limited human patient trials regarding MSCs. MSCs have been widely studied and increasingly used in GVHD treatment since the first report of promising results by Le Blanc *et al*[45] However, the studies have reported varying outcomes, which could be contributed to differences in cell concentration and MSC infusion dose. While MSC infusion has shown quite promising results following GVHD prophylaxis failure, some clinicians still prefer using methylprednisolone and calcineurin inhibitors before cell therapy with MSCs.

In addition to suppressing inflammation, MSCs have other beneficial effects, including increased angiogenesis, reduced apoptosis, and modified extracellular matrix dynamics. These cells mediate immune system components like macrophages and neutrophils, improving tissue microenvironments. After the injury, MSCs can either promote or suppress the immune system to guide the whole-tissue regeneration process[1]. Clinical responses to MSC infusion assessed as early as one week after treatment may predict patients' overall survival, indicating the potential of MSCs in treating GVHD[45].

Although the paracrine effects of MSCs are known to mediate the modulation of the immune response, the mechanisms underlying this modulation are not yet fully understood. However, it has been found that under conditions of chronic hypoxia or co-stimulation with IFN-γ, MSCs express proteins that have the immunosuppressive capacity, such as IDO, HLA-G, PGE2, and FasL, which can modulate the immune response[46]. While other cytokines play a crucial role in immunosuppression, blocking highly expressed proteins can result in the setback of the human immunosuppressed state, leading to the growth, and proliferation of immune cells. Moreover, MSCs do not trigger the activation of immune cells as they lack CD40, CD80, CD86, and HLA-DR-stimulating molecules. Given that GVHD occurs following the infusion of immune cells donated by the same donors, suppressing the immune activity can improve the patient's prognosis. MSCs' expression of paracrine effects can regulate these donor immune cells through various mechanisms (Table 1)[47-62].

When MSCs are exposed to an insult, such as injury, or bacterial infection, MHC-II molecules facilitate the presentation of bacterial antigens, which induces further activation of T cells expressing IFN-γ. MHC-II is downregulated at high levels of IFN-γ, while B7-H1 is upregulated[45]. These presentation pathways are illustrated in Figure 2.

MSCs have been used to treat various conditions, including diabetes mellitus (DM), cardiovascular diseases, GVHD, and autoimmune diseases. Despite persistent questions, the immunomodulatory effects of MSCs make them a top choice for cell therapy. MSCs are early multipotent progenitors and non-hematopoietic cell populations that can be expanded ex-vivo to achieve large numbers necessary for *in vivo* use. Recently, adipose tissues, umbilical cord, placenta, and dental pulp have been recognized as multipotent sources of MSCs. MSCs can differentiate into a variety of cell types capable of osteogenic, chondrogenic, adipogenic, myogenic, and neurogenic differentiation. However, not all individual cells cultivated in tissue culture flasks result in the same degree of multipotency. Self-renewing progenitors can be identified in human BM, and it is currently unknown whether MSCs from other tissues exhibit this property. BM-MSCs are a critical source of multipotent stem cells and serve as a standard for comparing MSCs from different sources (Table 2)[49,63-85].

The term "mesenchymal stem cells" has been proposed as a more appropriate term than MSCs. These cells possess not only multipotency but also significant immunomodulatory and engraftment-promoting properties. They create a specialized microenvironment for HSCs by promoting the secretion of various inflammatory cytokines, chemokines, growth factors, extracellular matrix, and extracellular vesicles that are crucial for HSC differentiation, proliferation, and maintenance[86-88]. After *in vivo* biological application, MSCs secrete a range of cytokines and regulatory molecules with anti-inflammatory, wound healing, and regenerative effects, promoting the repair of endogenous tissues or tissue replacement. Beres *et al*[40] demonstrated that even in otherwise immunocompetent humans, allogeneic MSCs may graft, and differentiate through significant histocompatible barriers.

Table 1 Immunosuppressive effect exerted by mesenchymal stem/stromal cells from different sources on immune cells

MSC types	Mechanism of immunosuppressive effect	Ref.
BM-MSCs	Recipient-derived MSCs from patients with GVHD are analogous to MSCs from healthy volunteers	Copland *et al*[47]
	After MSC infusion, the ratio of Th1 cells to Th2 cells was reversed, with an increase in Th1 and a decrease in Th2 achieving a new balance	Zhou *et al*[48]
	BM-MSCs reduce the incidence and severity of GVHD by improving thymic function and induction of Tregs but not increase the risks of infections and tumor relapse	Zhao *et al*[49]; Selmani *et al*[50]
	HLA-G5 secreted by MSCs is critical to the suppressive functions of MSCs	Selmani *et al*[51]
MenSCs	MenSCs exhibit a higher capacity to migrate into the intestine and liver and not to their anti-inflammatory capacities	Luz-Crawford *et al*[52]
FL-MSCs	FL-MSCs demonstrates much longer-lasting immunomodulatory properties by inhibiting directly the proliferation and activation of CD4+ and CD8+ T cells	Yu *et al*[53]
UC-MSCs	UC-MSCs showed minimal expression of HLA-DR after activation and posed minimal risk of initiating an allogeneic immune	Kim *et al*[54]
	UC-MSCs alleviate SLE through upregulating Treg cells, which was partly dependent on HLA-G	Chen *et al*[55]
	UC-MSCs ameliorate GVHD and spare GVL effect *via* immunoregulations	Wu *et al*[56]
WJ-MSCs	WJ-MSCs exert immunosuppressive effects by cell-cell contact with activated T cells and in part through the soluble factor indoleamine 2,3-dioxygenase	He *et al*[57]
MC-, WJ- and BM-MSCs	The mixed populations of MSCs displayed all of the positive attributes of WJ-MSC and BM-MSC	Mennan *et al*[58]
AT-MSCs	The use of AT-MSC rather than BM-MSC could further preserve NK cell activity and favor GVL	Blanco *et al*[59]
hG-MSCs	hG-MSC treatment inhibited local inflammation of injured skin by suppressing inflammatory cells, reducing pro-inflammatory cytokine tumor necrosis factor-α, and increasing anti-inflammatory cytokine interleukin-10, which was promoted by hypoxia	Jiang *et al*[60]
CP-, BM- and AT-MSCs	CP-MSCs may have additional advantage over the other MSCs in terms of immunomodulation	Lee *et al*[61]
DP-MSCs	Immunomodulation and expression of trophic factors by dental MSCs increase their resistance to allogeneic NK cell lysis and their potential in vivo lifespan	Martinez *et al*[62]

AT: Adipose tissue; BM: Bone marrow; CP: Chorionic plate; DP: Dental pump; FL: Fetal liver; hG: Human gingiva; GVHD: Graft-versus-host disease; GVL: Graft-versus-leukemia; MenSC: Menstrual blood-derived mesenchymal stem cell; MSC: Mesenchymal stem cell; UC: Umbilical cord; WJ: Wharton jelly.

Similar to hematopoietic stem cells, MSCs have multi-organ specificity, and plasticity. In 2006, the International Society for Cellular Therapy officially defined MSCs as plastic practitioners under standard growing conditions, expressing CD73, and CD90 surface molecules while lacking CD11b, CD14, CD19, CD34, CD45, CD79a, and HLA-DR[2]. In addition, MSCs can differentiate into various mesodermal lineages including osteoblast, adipocyte, and chondroblast, to different degrees.

MSCs are capable of modulating both innate and adaptive immunity through the release of various soluble factors, including indoleamine 2,3-dioxygenase[11], IL-10, prostaglandin 2, nitric oxide, transforming growth factor-β, HLA-G5, and anti-inflammatory molecule TNF-α-induced gene/protein 6 [89]. These molecules are believed to play a key role in the immunomodulating effects of MSCs, which have been shown to be beneficial in certain immunopathological diseases, such as aGVHD, and type 1 DM. However, the precise mechanisms underlying this therapeutic potential are not yet fully understood. The literature suggests that the immunomodulating potential of MSCs involves interactions with both humoral and cellular components of the innate and adaptive immune systems. The literature refers to several fundamental cellular interactions. An integrated perspective on the utility of MSCs for GVHD has been strengthened by the recent findings that MSCs are induced to undergo necrosis/apoptosis by the recipient's cytotoxic cells and that this process is assumed to elicit MSC-induced immunosuppression[90]. This finding made it possible to reconcile the dilemma between the effectiveness of MSC and its apparent lack of engraftment and highlighted the crucial role of the patient in the promotion and administration of immunosuppression of MSCs. Recent research has shed light on the role of the patient in promoting and administering immunosuppression of MSCs, with evidence suggesting that MSCs are induced to undergo necrosis/apoptosis by the recipient's cytotoxic cells, leading to MSC-induced immunosuppression[90]. Table 2 provides an overview of recent studies on this topic, with 97 articles selected for full-text evaluation based on agreed-upon title and abstract criteria.

Innate immunity is primarily centered around the complementary system, with C3, and C5 being cleaved into anaphylatoxins C3a and C5a by convertases at the sites of inflammation. The labile C3

Table 2 Effects of different mesenchymal stem/stromal cells on refractory acute graft-versus-host disease

Study type	Patient No.	Indication	MSC type	Response criteria	Main findings	Ref.
Phase 2	55	Steroid-resistant, severe, aGVHD	BM	Glucksberg	CR: 30/55, better OS/TRM for complete responder	Le Blanc *et al* [63], 2008
Phase 2	31	Gr. II-IV aGVHD	BM	Glucksberg	CR: 77%; PR: 16%	Kebriaei *et al*[64], 2009
Pilot study	20	Co-transplantation with NMA mismatched HSCT	BM	Glucksberg	Decreased 1 yr GVHD death (10% *vs* 31%, $P = 0.04$). Better NRM & OS	Baron *et al*[65], 2010
Retrospective	37	Resistant Gr. III-IV aGVHD	BM	Glucksberg	CR: 65%, better TRM and OS	Ball *et al*[66], 2013
Multicenter trial	50	Resistant Gr. IV aGVHD	BM	Not mentioned	OR: 33%, CR: 17%, initial response and young age have better survival	Resnick *et al*[67], 2013
Prospective, single-arm, open-label	75	Severe refractory aGVHD	BM	IBMTR SI	OR on day +28: 61.3%, better OS for responder on day +100 (78.1% *vs* 31.0%; $P < 0.001$)	Kurtzberg *et al* [68], 2014
Phase 1	40	Resistant Gr. II-IV aGVHD	BM	Glucksberg	CR: 27.5%, OR: 67.5% on day +28; more CR in pediatric group	Introna *et al*[69], 2014
Phase 2	25	Refractory aGVHD	BM	Glucksberg	71% responded, CR 11/24, better OS for CR	Sánchez-Guijo *et al*[70], 2014
Prospective, nonrandomized	28 *vs* 19 without MSC	Refractory aGVHD	BM	Glucksberg	Decreased incidence and severity of cGVHD. Better OR and CR.	Zhao *et al*[49], 2015
Phase 2	48	Steroid-resistant aGVHD	BM	Glucksberg	CR: 25% on day 28, 50% lasting > 1 mo, with better OS	Te Boome *et al* [71], 2015
Compassionate use	58	Steroid-resistant aGVHD	BM	IBMTR SI	OR: 47%, but no improvement in OS	von Dalowski *et al*[72], 2016
Phase 2/3	25	Refractory Gr. III-IV aGVHD	BM	Glucksberg	Better OS for OR at 4-wk (CR: 6/25, PR: 9/25)	Muroi *et al*[73], 2016
Pilot study	33	Refractory aGVHD	BM	IBMTR SI	CR: 18/33, PR: 7/33, better OS in CR, no TRM in CR	Erbey *et al*[74], 2016
Compassionate use	26	Severe resistant aGVHD	BM	Not mentioned	OR: 77% on day +28 (CR: 5/26, PR: 15/26)	Kuçi *et al*[75], 2016
Phase 2 prospective RCT	62 *vs* 62 without MSC	cGVHD prophylaxis in haplo	Cord	NIH score	cGVHD: 27% (MSC) *vs* 49% in 2 yr ($P = 0.021$)	Gao *et al*[76], 2016
Phase 1/2	26	Steroid-refractory aGVHD	BM	Glucksberg	OR: 62% on day 28. Higher response rate in children. High NRM in adults	Salmenniemi *et al* [77], 2017
Pilot study	22	Refractory GVHD (Gr. 2-4 a or cGVHD)	BM or adipose tissue	Glucksberg/NIH score	CR: 45.8%, PR: 33.3%, better OS in CR/PR	Cetin *et al*[78], 2017
Retrospective	46	Refractory Gr. III/IV aGVHD	BM	Not mentioned	50% responded with better OS ($P = 0.0004$)	Dotoli *et al*[79], 2017
Phase 1/2	33	Steroid-refractory aGVHD	BM	Glucksberg	CR: 34%, PR: 50% on day 28. Better OS on day 90 and 1 yr ($P = 0.006$, 0.002)	Fernández-Maqueda *et al* [80], 2017
Phase 1/2	69	Refractory aGVHD	BM	Glucksberg	OR: 83% on day 28	Bader *et al*[81], 2018
Observational study	34 *vs* 34 without MSC	aGVHD	BM or adipose tissue	IBMTR SI	Better OS compared with historical control, $P = 0.0678$. MSC has no association with risk of infectious complication	Stoma *et al*[82], 2018
Retrospective	11 (study group 2)	Severe refractory aGVHD	Placenta derived decidual stromal cell	Glucksberg	73% 1 yr OS in study group 2 (albumin), 47% in group 1 (AB plasma), $P = 0.016$	Ringden *et al* [83], 2018
Retrospective	22	Severe refractory aGVHD	Cord	IBMTR SI	CR: 45.5%, PR: 13.6%	Bozkurt *et al*[84], 2019

Phase 3 RCT	151 *vs* 72 placebo	Severe refractory aGVHD	BM	IBMTR SI	Difference of durable CR (lasting > 28 d) not achieved (35% *vs* 30%, P = 0.42); Pediatric pts had better OR (64% *vs* 23%, P = 0.05)	Kebriaei *et al*[85], 2020

aGVHD: Acute graft-versus-host disease; BM: Bone marrow; cGVHD: Chronic graft-versus-host disease; CR: Complete remission; IBMTR SI: International Bone Marrow Transplant Registry severity index; MSC: Mesenchymal stem/stromal cells; OS: Overall survival; PR: Partial remission; RCT: Randomized controlled trial.

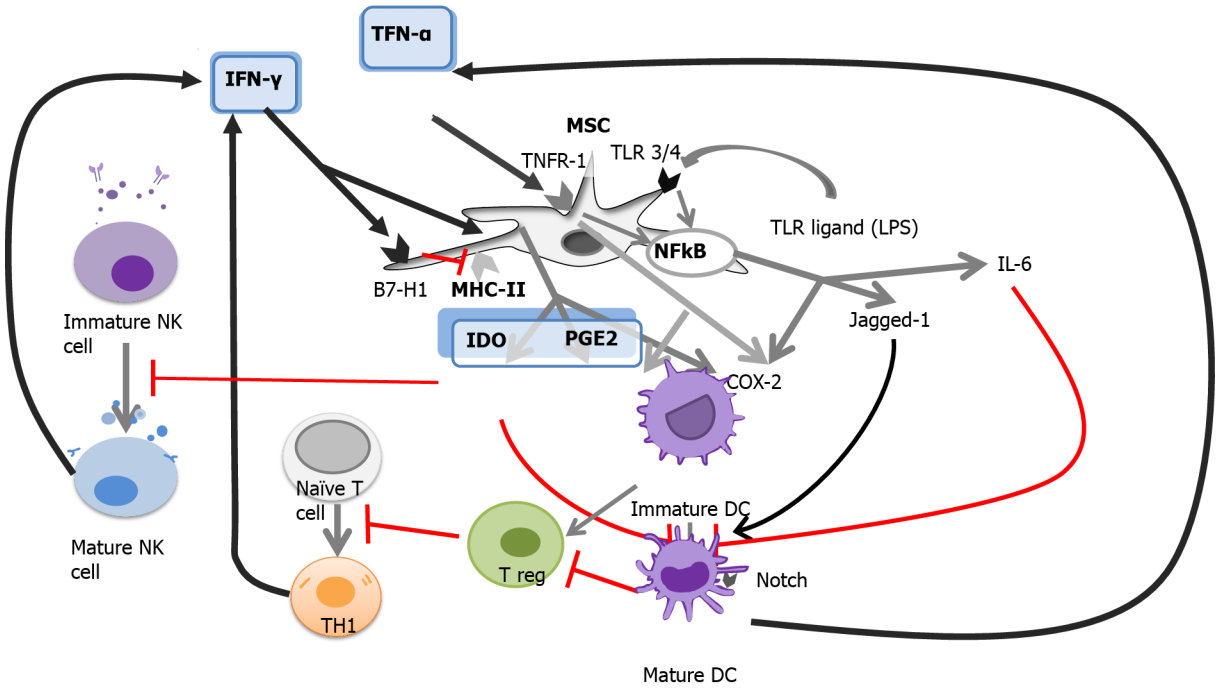

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Figure 2 The complex network of antigen presentation and immunomodulation. Mesenchymal stem/stromal cells (MSCs) exert immunomodulatory functions mainly *via* interactions with immune cells through cell-to-cell contacts and paracrine activity. The MSC secretome includes several cytokines, growth factors, and chemokines, and their immunomodulatory functions vary depending on the source of the MSCs, the target cells, and the microenvironment. COX-2: Cyclooxygenase-2; IDO: Indoleamine-pyrrole 2,3-dioxygenase; IFN: Interferon; IL: Interleukin; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; PGE2: Prostaglandin E2; TLR: Toll-like receptors; TNF-α: Tumor necrosis factor-α; DC: Dendritic cell; LPS: Lipopolysaccharide; NK: Natural killer cells.

convertases cleave C3 into C3a and C3b which can thereafter participate in forming distinct complexes and activate pathways for proliferation and protection against apoptosis through receptor binding. MSCs also secrete the factor H, which inhibits complement activation by limiting the activity of C3 and C5 convertases. In mice, MSCs promote pro-inflammatory repolarization and produce chemostatic cytokines, including IL-6, IL-8, GM-CSF, and macrophage inhibitory factors. IL-8, in particular, is a pro-inflammatory chemokine produced by multiple cell types that recruits leukocytes to sites of infection or tissue injury. Additionally, MSCs can inhibit mast cell degranulation and histamine release by binding allergens to allergen-specific IgE *via* FcRε on mast cells, providing a potential therapeutic benefit for allergic reactions[91].

The molecular interaction between NK cells and MSCs is complex and depends on the immune microenvironment and NK cell activation status. MSC can inhibit cytokine proliferation and production and interfere with NK cell cytotoxicity. They also inhibit monocyte maturation and differentiation into DCs, which are the primary type of APC and play a key role in T lymphocyte activation through antigen presentation. Monocytes and macrophages are important for tissue development, homeostasis, and injury repair. Activated MSCs produce chemokines that attract circulating monocytes to sites of inflammation and injury[92].

MSCs can regulate the adaptive immune system through multiple redundant pathways. They suppress the proliferation of T cells, IFNγ production, CD4 T cell differentiation, and CD8 T-cell cytotoxicity. Di Nicola *et al*[41] reported that MSCs can suppress T lymphocyte proliferation *in vitro* with autologous and allogeneic MSCs, including T lymphocytes cultured with DCs or lymphocytes in mixed lymphocyte reactions. MSCs can express and secrete programmed death-ligand 1 and 2, which suppress T-cell proliferation in the presence of MSCs, secrete IL-2, induce apoptosis, and promote the induction of an irreversible hyporeactive state[93]. In vivo studies suggested that MSCs may restore the balance

between T helper 1 and 2 cells in diseases associated with a shift to dominance of these T cell subpopulations[94]. *In vitro* models have shown that MSCs induce Tregs and maintain survival and suppressive phenotypes[95].

CONCLUSION

This article provides insights into the use of validated MSCs as a potential treatment strategy for GVHD in HSCT. Current prevention and treatment options involve immunosuppression, which can hinder immune recovery and limit the graft-versus-tumor effect. By using MSCs, clinicians can effectively treat GVHD, identify high-risk patients, and stratify patients based on disease severity. Therefore, MSCs can aid in promoting engraftment, ameliorating aGVHD, and preventing cGVHD, making them an attractive option for HSCT.

FOOTNOTES

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MINIREVIEWS

Role of brahma-related gene 1/brahma-associated factor subunits in neural stem/progenitor cells and related neural developmental disorders

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Abstract

Different fates of neural stem/progenitor cells (NSPCs) and their progeny are determined by the gene regulatory network, where a chromatin-remodeling complex affects synergy with other regulators. Here, we review recent research progress indicating that the BRG1/BRM-associated factor (BAF) complex plays an important role in NSPCs during neural development and neural developmental disorders. Several studies based on animal models have shown that mutations in the BAF complex may cause abnormal neural differentiation, which can also lead to various diseases in humans. We discussed BAF complex subunits and their main characteristics in NSPCs. With advances in studies of human pluripotent stem cells and the feasibility of driving their differentiation into NSPCs, we can now investigate the role of the BAF complex in regulating the balance between self-renewal and differentiation of NSPCs. Considering recent progress in these research areas, we suggest that three approaches should be used in investigations in the near future. Sequencing of whole human exome and genome-wide association studies suggest that mutations in the subunits of the BAF complex are related to neurodevelopmental disorders. More insight into the mechanism of BAF complex regulation in NSPCs during neural cell fate decisions and neurodevelopment may help in exploiting new methods for clinical applications.

Key Words: Neural stem/progenitor cell; BRG1/BRM-associated factor complex; Subunit; Proliferation; Differentiation; Neural developmental disorders

Core Tip: There are several reviews in the literature contributed to the role of BRG1/BRM-associated factor (BAF) complex in neural cell specification and neural development diseases. We review recent progress indicating that BAF complex plays an important role in neural stem/progenitor cells (NSPCs) during neural development and neural developmental disorders. More progresses in the role of BAF complex subunits in balancing self-renewal and differentiation of NSPCs and neurodevelopment could finally be involved in highlighting new methods for clinical application.

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INTRODUCTION

Neural stem/progenitor cells (NSPCs) are self-renewing neural cells capable of differentiating into neurons, astrocytes and/or oligodendrocytes[1-3]. During development, NSPCs are critical for the establishment of the central nervous system (CNS)[4-6]. In the adult central neural system, neurogenesis plays a key role in fundamental processes, for example, memory, learning, the maintenance of normal tissue homeostasis, and the autonomous repair of pathological brain tissues[7,8]. NSPCs can be isolated from three canonical neurogenic niches in the spinal cord and brain, namely, the central canal (CC) in the spinal cord, the subgranular zone (SGZ) of the dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricle (LV) in the brain[9-13]. In addition, NSPCs have been identified in the developing cerebral cortex[14], olfactory epithelium[15] and outside the CC[16]. The fact that NSPCs can be isolated and propagated *in vitro* opens up new opportunities for medical research, and we hope they can be used to compensate for cell loss that features in several serious neurological disorders. Moreover, NSPCs can be created *in vitro* through induced differentiation from induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs), expanding the pool of models for studying stem cells in health and illness[17,18]. For cell-based therapy approaches targeting the brain and spinal cord, NSPCs have emerged as focal points. However, the small amount of NSPCs present in this tissue has restricted the clinical uses of these cells. Although recent advancements in ESCs and iPSC research have meant that these cells can be novel sources of NSPCs, understanding NSC molecular regulation and NSPC applications, which are important for disease modeling and regenerative medicine, remain challenges[19-21].

The BRG1/BRM-associated factor (BAF) complex, which is a mammalian chromatin remodeler, regulates chromatin structure and transcription by providing subunit organization and nucleosome recognition in an ATP-dependent manner. Structurally, the BAF complex contains three modules, an ATPase, an actin-related protein (ARP) and a base module[22]. More than 30 major subunits of these modules can combine to form the complex[22-24].

By controlling chromatin structure, controlling the differentiation of NSPCs to produce different types of brain cells, and regulating transdifferentiation between cell types, BAF complexes perform crucial roles in the maintenance of a gene expression program. At various phases of brain formation, BAF complexes have specific roles and seem to be produced by the combinatorial assembly of their subunits. Moreover, intimate interactions between the BAF chromatin-remodeling complex and transcriptional machinery control the development of NSPCs[25]. With the combinatorial assembly of homologous component families, which enables their nonredundant functions, distinct BAF complex structures are made possible. ESCs, NSPCs, and postmitotic neurons all exhibit developmental stage-specific BAF assemblages during mammalian brain development. Neuronal BAF (nBAF) function is required for the development of mature postmitotic neuronal features along with long-term memory, and neural progenitor-special BAF (npBAF) structures are particularly important for modulating the rates and modalities of neural progenitor cell replication. Given the high prevalence of BAF subunit mutagenesis in neurological illnesses, it is obvious that BAF complexes perform a crucial role in controlling the rate of neuronal development, homeostasis, and plasticity. Understanding how BAF complex subunits influence BAF complex function and the roles played by different subunits will reveal disease pathogenesis and lead to novel treatments for related human disorders ultimately[26,27].

Here, we summarize recent progress in comprehending the role of the BAF complex in NSPCs. We focus on NSC-/NPC-specific BAF complex subunits and describe how they determine the balance between the self-renewal and differentiation of NSCs and are involved in several human neural developmental disorders. Supplementary Table 1 shows the abbreviations and their descriptions.

BAF COMPLEX SUBUNITS AND THEIR MAIN CHARACTERISTICS IN NSPCS

Among the major BAF complex subunits, there are 12 subunits expressed in NSPCs in the embryo cortex, olfactory bulb (OB) and spinal cord, adult brain and spinal cord, and human and mouse retina (detailed information and references are shown in Table 1).

BAF190A (BRG1/ SMARCA4)

Brg1 (Brahma-related gene 1) is necessary for NSPCs to remain in a state where they can react to gliogenic signals and to suppress neuronal commitment. Lack of Brg1 in NSPCs led to precocious neurogenesis, such that cells in the ventricular zone developed into postmitotic neurons before the start of gliogenesis in conditional brg1- mutant mice. As a result, these animals significantly lost the ability to differentiate astrocytes and oligodendrocytes. In addition, in vitro Brg1 deletion reduced growth factor-induced astrocyte development in gliogenic progenitors. Furthermore, it has been discovered that the levels of proteins associated with stem cell maintenance, such as Pax6, Sox1, and Musashi-1, are significantly lower in the ventricular zones of the mice with brg1-mutations[28]. The hippocampus experienced abnormal adult neurogenesis as a result of Brg1 deletion in nestin-expressing NSPCs. This abnormal adult neurogenesis initially decreased the number of adult NSPCs in the hippocampus, inhibited progenitor maintenance, and later decreased NSPC responsiveness to physiological stimulation. Brg1 deletion appears to hinder cell cycle progression mechanistically, which is partly because of increased p53 pathway activation and p21 expression. Defects in neurosphere formation brought on by Brg1 deletion were repaired by knocking down p53. These findings suggest that aNSPC and progenitor cell maintenance and responsiveness during neurogenesis are determined by epigenetic chromatin remodeling *via* a Brg1- and p53/p21-dependent mechanism[29].

Many transcription factors manipulating neuronogenesis have been ascertained in the adult and developing brain, and notably, the neurogenic transcriptional regulation factor Pax6, which can directly interact with the Brg1-carrying BAF complex in adult neural progenitor cells. Abolition of either Pax6 or Brg1 in the subependymal zone (SEZ) caused the offspring of adult NSPCs to switch to the SEZ ependymal cell lineage. In the interim, shifting neuroblasts changed to distinct glial cell lineages during or after reaching the OB. Several studies have revealed that there is a network with tripartite effector programing neuronal fate, which can be activated by Pax6-BAF and promote neurogenesis and the transformation from glia to neurons. According to the whole-genome analysis, the downstream regulatory factors of Pax6-BAF include Nfib, Sox11 and Pou3f4. While Brg1 is absent in SEZ and OB, the binding sites of Pax6 carried by Brg1 and most down-regulated genes will be down-regulated at the same time[30]. In the study of Jayaprakash *et al*[31], they find that Brg1 appears as a slender spherical structure so that it can provide a lager surface and form the further complex by binding to BAF57 and BAF60A. Furthermore, a neural Brg1 isoform interacts with the central neurodevelopmental transcriptional repressor REST/NRSF, suggesting that Brg1 can interact with transcription regulators and factors to conduct the BAF complex to specific sites[31].

BAF155 (SMARCC1) and BAF170 (SMARCC2)

Recent study found that conditional deletion of BAF155 can lead to the decrease of basal intermediate progenitors (bIPs), while the delamination of apical radial glial progenitors (RGs) can cause the increase of basal RGs (bRGs). During the progress, BAF155 has been proved necessary for the normal activity of PAX6 to regulate the gene expression. BAF155 can control the expression of the CDC42 effecting protein CEP4 in the Pax6-dependent way in order to regulate the stratification of progenitor. In addition, BAF155-dependent chromatin remodeling is also involved in regulating several human RG-specific genes, thereby acting on the generation of basal progenitors (BPs)[32].

The study of Narayanan *et al*[33] in 2015 revealed a molecular mechanism of controlling the global chromatin state and transcriptional program during development, which is mediated by the BAF complex. They found that BAF155 and BAF170 knockout can cause elimination of the entire BAF complex with all kinds of BAF subunits in cortical development, with the overall increase of the abundance of the repressive marks (H3K27me2/3), decrease of active chromatin mark H3K9Ac, and down-regulation of gene expression. Both BAF155 and BAF170 subunits are expressed in early cortical progenitors (E10.5-E14.5), and BAF170 is replaced by BAF155 in late cortical progenitors[34]. Specifically, between E12.5 and E14.5, apical progenitors express BAF170 and BAF155. They exhibit comparable expression patterns at the height of upper layer neurogenesis (E15.5), with BAF170 being missing from VZ progenitors but expressed in the cortical plate but expressed in the cortical plate[34]. While BAF170 overexpression resulted in the acquisition of a feature similar to that acquired by the Pax6-loss brain, with depletion of overlying layer neurons, BAF170 loss in Emx1t cortical progenitors exerted an effect opposite to that of Pax6 elimination and caused an aberrant growth of IPs and a bias toward the acquisition of an overlying layer identity[34]. By the recruitment of the transcriptional suppressor REST to Pax6 targets, the BAF170-containing npBAF complex indirectly suppresses neurogenesis in its early stages[34,35]. Interestingly, the transcription of BAF170 and Brm subunits, which replace BAF155 and Brg1 subunits in the esBAF, respectively, begins at the same time when ES cells commit to developing into neural progenitors[33,36]. According to a recent functional invest-

Table 1 BRG1/BRM-associated factor complex subunits in neural stem/progenitor cells

BAF complex subunits (aliases)	NSPC types	Function	Interaction signalings/factors	Related neural developmental diseases	Ref.
BAF45A (PHF10)	oNSC, E10.5-E11.5 cortical NPCs, E10.5-E16.5 spinal cord VZ	NSPC proliferation	BRG1	-	Bachmann *et al*[15], 2016; MacDonald *et al*[89], 2022; Lessard *et al*[41], 2007
BAF45D (DPF2)	SGZ, SVZ, spinal cord central canal GFAP + radial glial cells	PAX6 expression, contributes to NSPC induction	PAX6, BRG1	CSS	Liu *et al*[42], 2017; Wang *et al*[44], 2019; Chen *et al*[43], 2022; Vasileiou *et al*[69], 2018; Knapp *et al*[70], 2019; Milone *et al*[71], 2020
BAF47 (SMARCB1)	oNSC	-	BRG1	CSS, kleefstra syndrome, Nicolaides-Baraitser syndrome	Bachmann *et al*[15], 2016; Tsurusaki *et al*[72], 2012; Santen *et al*[74], 2013; Wieczorek *et al*[75], 2013; Kleefstra *et al*[88], 2012; Gossai *et al*[73], 2015
BAF53A (ACTL6A)	oNSC, NPCS in the neural tubes of E11.5 embryos	BAF53A is essential for NPC proliferation	miR-9 and miR-124	-	Bachmann *et al*[15], 2016; Lessard *et al*[41], 2007; Yoo *et al*[45], 2009
BAF55A (SS18)	NPCs in VZ	SS18 is required for NSC self-renewal	BRG1	-	Staahl *et al*[48], 2013
BAF57 (SMARCE1)	Adult human OBNSCs	NSC proliferation	Neuregulin-1	CSS	Wieczorek *et al*[75], 2013; Kosho *et al*[67], 2014; Marei *et al*[52], 2012; Zarate *et al*[76], 2016; Pirotte *et al*[63], 2010
BAF60A (SMARCD1)	Oligodendrocyte precursors	-	BRG1, BAF155 and BAF170	CSS, Nicolaides-Baraitser syndrome	(Yu *et al*[54], 2013; Hsiao *et al*[53], 2003; Machol *et al*[78], 2019; Nixon *et al*[77], 2019
BAF60C (SMARCD3)	NPCs in human retinas as well as mouse retina, cortex and spinal cord	Keeps the progenitors in a proliferative state -	Notch signaling	-	Lamba *et al*[55], 2008
BAF155 (SMARCC1)	oNSC, adult human OBNSCs	Proliferation and maintenance of ONSCs forebrain development	BRG1, PAX6	ASD	Lessard *et al*[41], 2007; Marei *et al*[52], 2012; Narayanan *et al*[33], 2015; Neale *et al*[84], 2012
BAF170 (SMARCC2)	oNSC, in postnatal DG, in RGL progenitors	oNSC proliferation, affects NSC proliferation, differentiation, forebrain development	BRG1	CSS, ASD, Nicolaides-Baraitser syndrome	Lessard *et al*[41], 2007; Machol *et al*[78], 2019; Narayanan *et al*[33], 2015; Neale *et al*[84], 2012; Tuoc *et al*[38], 2017
BAF190A (BRG1/SMARCA4)	Mouse VZ, SGZ, SEZ, NSCs/NPCs, NCC	NSC maintenance and neuronal differentiation	PAX6, BAF45D	CSS, ASD	Tsurusaki *et al*[72], 2012; De Rubeis *et al*[85], 2014; Matsumoto *et al*[28], 2006; Petrik *et al*[29], 2015; Ninkovic *et al*[30], 2013
BAF250A (ARID1A)	Developing cortex	Regulates NSPC proliferation and differentiation during cortical development	BRG1	CSS	Tsurusaki *et al*[72], 2012; Liu *et al*[40], 2021

BRG1: Brahma related gene 1; BRM: Brahma; BAF: BRG1/BRM-associated factor; NSPCs: Neural stem/progenitor cells; oNSCs: Olfactory neural stem cells; CSS: Coffin-siris syndrome; VZ: Ventricular zone; OBNSCs: Olfactory bulb-derived neural stem cells; ASD: Autism spectrum disorder; KSS: Kleefstra syndrome spectrum; DG: Dentate gyrus; RGL: Radial glial-like; SEZ: Subependymal zone; NCC: Neural crest cells.

igation, Pax6 is directly associated to the BAF complex in aNSCs that contained the Brg1, and its neurogenic activity was abolished in the lack of the Brg1[30]. BAF170 and BAF45 also carry domains with putative DNA-binding capacities[37]. In the neonatal DG, the BAF170 member of the complex is found in radial glial-like (RGL) cells and cell types implicated in following stages of adult neurogenesis together with mature astrocytes. The pool of RGL cells in the DG is reduced by conditional knockout of BAF170 during late cortical neurogenesis and also adult brain neurogenesis, which encourages terminal astrocyte differentiation. A moderate loss in spatial learning was produced by the induced abolition of BAF170 in the DG throughout adult brain neurogenesis, with the reversal test showing the greatest impact on spatial learning. These findings indicate a specialized role for adult neurogenesis in the DG

regulating adaptive behavior and show the engagement of BAF170-reliant chromatin modification in neurogenesis and cognition of hippocampus[38].

BAF250A (ARID1A)

The core ATPase, Brg1 or Brm, BAF250A or BAF250B, a homologous dimer of BAF155, or a heterologous dimer of BAF170 and BAF155 are just a few examples of the different subunits that npBAF can contain[27]. Mammal BAF is significantly different from its yeast analogue, losing some members while obtaining new core members, such as BAF45a/b/c/d, BAF57, BAF250A/B, SS18/CREST, beta-actin, BRD7, and BRD9. Notably, BAF250A/B are the most commonly mutated subunits of BAF complex in related human neurological diseases[36]. Loss of BAF250A or BAF250B in mouse models leads to early embryonic lethality[39], indicating that, despite their homology, both genes are essential for early development.

Recent genome sequencing and many clinical studies have revealed that mutations of BAF250A is closely related to microcephaly and mental retardation. Liu *et al*[40] generated a *baf250a* conditional knockout mouse line and found that cortical thickness was reduced in the developing cortex. Radial glial cell proliferation is inhibited by loss of BAF250A function, which also increases the rate of cell damage during late cortical neurogenesis and causes abnormal expression of genes involved in proliferation and differentiation. Therefore, for the purpose of better understanding the pathogenic mechanisms and creating new treatments for neurodevelopmental disorders brought on by its mutations, BAF250A may be one of the gene possibilities that is worthwhile being researched[40].

BAF45A and BAF45D (DPF2)

BAF45 family proteins, including BAF45A, BAF45B, BAF45C and BAF45D, are subunits of the BAF complex[41]. BAF45A, an npBAF, is required for the self-renewal and proliferative features of neural cells[41]. BAF45A is identified in olfactory neural stem cells (oNSCs), E10.5-E11.5 cortical NPCs, and E10.5-E16.5 spinal cord ventricular zone NSCs[15,41]. BAF45D is expressed in the SGZ of the LV, SGZ of the DG, and the CC of the adult spinal cord. Co-expression of BAF45D and glial fibrillary acidic protein (GFAP), a marker protein of radial glial cell-like cell was found in the SGZ, SVZ, and CC of the adult spinal cord. According to the results of quantitative examination, BAF45D is selectively expressed in the adult neurogenic regions of neurons in CNS. What's more, BAF45D is necessary for the induction of PAX6, a determinant for neuroectoderm that regulates adult neural stem/progenitor cell self-renewal and specific neural fate, during neuroectodermal differentiation of H9 cells[42].

Recently, BAF45D has been shown to regulate spinal cord neural stem/progenitor cell (SCNSC/SCNPC) fate mediated through the SMAD-PAX6 axis. SCNSCs exhibit increased DNA-BAF45D compared to that in human ESCs[43]. It has been reported that postmitotic neurons express BAF45B/C protein but lack the paralogous BAF45A/D, which confers neuronal properties[25-27,41]. The protein expression of BAF45D in adult rat NSPCs and neurons but not astrocytes may imply a role for BAF45D in the differentiation of NSPCs[44].

BAF53A (ACTL6A)

The BAF53 subunit is encoded by the BAF53A (ACTL6A) or BAF53B (ACTL6B) gene, with the latter exclusively expressed in differentiated neurons[41].

BAF53A is specific to the npBAF complex and can be replaced by BAF53B in the nBAF complex in neurons[26,45]. miR-9 and miR-124 control the transition of postmitotic neurons from the BAF complex's BAF53A to BAF53B subunit[46]. These miRNAs' expression is derepressed in neurons during cell development, which encourages the replacement of BAF53B in the nBAF complex by suppressing the expression of BAF53A. Increased progenitor proliferation can result from BAF53A's continued expression[46]. These miRNAs have an interacting location in the 3′UTR of BAF53A, and their expression is constrained in progenitors by REST and its corepressors[47]. As a result of decreased chromatin accessibility at particular neural transcription factor-binding sites brought on by BAF53A loss, cell cycle-related genes are repressed, preventing the advancement of the cell cycle and cell differentiation[48].

BAF57 (SMARCE1)

BAF57, subunit in the BAF complex core, is highly conserved in the BAF complexes of vertebrates[49]. The DNA-binding capabilities of its primary structural characteristic, a high-mobility group domain, imply that BAF57 may perform topological functions when the BAF complex moves in or out a nucleosome. BAF57 specifically interacts with a variety of proteins which aren't part of the BAF complex. BAF57 demonstrates particular functionalities as a result of these interactions. For example, in the embryo, it interacts with the transcriptional cosuppressor Co-REST to enable the neuronal genes inactivation in nonneuronal cells throughout development[50]. Interestingly, global gene expression profiling results indicated that BAF57A was specifically upregulated in human NSPCs of OB (OBNSPCs) but not in human embryonic NSPCs (hENSC)[51]. In cultured NSCs, BAF57 interacts with the overexpressed intracellular domain of neuregulin-1 and leads to a decrease in the NSC proliferation rate[52].

BAF60A (SMARCD1)

The BAF60A/B subunits define an ESC-specific assembly of BAF, named esBAF[27]. The BAF subunits BAF60A binds to BRG1, the primary factor that drives the BAF complex to particular genomic locations, and interacts with transcription regulators and factors[31]. Nuclear receptors and the BRG1 complex may have an additional important and direct relationship that is necessary for recruitment of promoter and subsequent chromatin modification[53]. Low levels of BAF60A and BAF45B/D expression are found in oligodendrocyte precursors, and these expression levels are upregulated upon differentiation [54]. However, their specific roles in NSPCs are still unclear.

BAF60C (SMARCD3)

During development, another crucial BAF complex component called BAF60C also has neuronal progenitor-specific actions. BAF60C is expressed in neural progenitors in the mouse and human retina, as well as the human cortex and spinal cord[55]. BAF60C expression decreases during neural differentiation, and through its interaction with the Notch pathway, its overexpression keeps progenitors in a proliferative condition. Finally, Muller glia that resume the cell cycle following neurotoxic damage express BAF60C once more[55].

BAF55A (SS18)

BAF55A, also called SS18, is a member of the npBAF complex. In NSCs, SS18 knockdown results in cell cycle exit and self-renewal failure[48]. Moreover, SS18 is adaptively activated to respond to ethanol exposure, protecting fetal NSCs against complete loss of miR-9-2[49].

BAF47

BAF47, a ubiquitous BAF complex subunit, is expressed in oNSCS[15]. However, the exact function of BAF47 remains largely unknown.

BAF COMPLEX FUNCTIONS IN BALANCING SELF-RENEWAL AND DIFFERENTIATION OF NSPCS

The number of neural stem cells expands through symmetrical division, and through asymmetrical divisions, they maintain the capability to self-renew and produce other differentiated cells[56]. Normal neural development requires the maintenance of the dynamic balance between the proliferative cells and differentiated cells of neural stem cells, which controls the number of stem cells and neurons and protects the body from diseases such as glioblastoma[57]. Furthermore, neural stem cells respond to several molecular signals during neurogenesis, especially those mediated by the Wnt and Notch signaling pathways[58]. For instance, Notch signaling is a significant mechanism regulating the balance between the quiescence and differentiation of neural stem cells[59]. By suppressing the Notch pathway, the homeostasis of neural stem cells can be altered, promoting neural differentiation[60].

Several BAF family members, which act as components or modulators of the Notch and Wnt/β-catenin signaling pathways, have been shown to exert either positive or negative effects on the differentiation of neural stem cells[55,61]. On the one hand, neural differentiation can be promoted by BAF155 and BAF170, which act on regulators of Wnt/β-catenin signaling-related genes, including H3K27me3 and H3K4me2[15,62]. On the other hand, BAF45a and BAF53a contribute to the proliferation and self-renewal of neural stem cells[41]. The repression of BAF53a during neural differentiation is realized through its 3'-UTR and the cooperative influence of miR-9 and miR-124[45]. Brm and BAF57 interact with the neuregulin-1 (Nrg-1) intracellular domain, reduce proliferation and promote differentiation of neural stem cells in vitro, possibly by reversing the direction of signaling in a pathway[63]. Furthermore, it has been shown that the overexpression of BAF60c maintains the proliferation of neural progenitors by interacting with the Notch signaling pathway[55]. The BAF complex may also make direct contact with the neurogenic transcription factors that are critical for neural stem cell specification or differentiation. Notably, the subunit composition of BAF complexes undergoes a profound switching during differentiation; for instance, BAF40c is replaced by BAF40a, the function of which is activated by Pax6, Tbr1, and Tbr2[64]. Moreover, BAF155 in the primate brain has been shown to be involved in the normal activity of Pax6, thereby regulating the specification of progenitors during cortex development [32].

Overall, BAF complexes function as both agonists and inhibitors of neural differentiation. Notably, certain similarities and differences have been revealed through the approaches used in the aforementioned studies. A study by Narayanan *et al*[32] was carried out by generating cortex-specific BAF155-knockout mice, while in another study, Nguyen *et al*[62] adopted a transgenic mouse model in which both BAF155 and BAF170 were knocked out, eliminating the entire BAF complex during late cortical neurogenesis. Their double-knockout model offered a novel and practical method for examining the function of complete BAF complexes during cerebral development[65]. Furthermore, a study by Bi-Lin *et al*[66] was conducted after the neural crest-specific knockout of BAF155 and BAF170 in $Wnt1^{Cre/+}$ and

Pax3$^{Cre/+}$ mice, and the results demonstrated that the BAF complex modulated the gene expression network and pathways, including the Notch signaling pathway, critical for neural crest development.

NSPC-RELATED BAF COMPLEX SUBUNITS IN NEURAL DEVELOPMENTAL DISEASES

Given the importance of BAF complexes in creating and sustaining a chromatin state that keeps proper transcriptional output during NSPC proliferation, differentiation and responses to exogenous or endogenous stimuli, it is clear that several neural developmental disorders have been linked to NSPC-related BAF complex subunits (Table 1 and Figure 1).

Coffin-Siris syndrome

Coffin-Siris syndrome (CSS) is a unusual congenital neural developmental disorder characterized clinically by intellectual disability (ID), progressive facial thickening, hirsutism, recurrent infections, difficult feeding, and restricted growth of the distal fifth phalanx and nails[67,68]. Genes encoding NSPC-related BAF complex subunits have been found to have mutations recently thanks to extensive human exome sequencing and genome-wide association research, including BAF45D[69-71], BAF47[72-74], BAF57[67,75,76], BAF60A[77], BAF170[78], BAF190A/BRG1[72], and BAF250A[72]. Different brain midline abnormalities, similar to those seen in people with CSS, were shown to be caused specifically by BAF47, BAF57, and BAF250B mutations in mice with a heterozygous neural system-specific SMARCB1 defect and a partial dysfunctional mutation in a BAF core member gene, indicating significant clinical suggestions for BAF complex-related ID/neurodevelopmental diseases[79].

Within a cohort of 15 unrelated individuals on the CSS pathway disorders registry, who were carriers of a SMARCA4 variant, they showed differences in the severity and number of learning disabilities and health problems. Two of them with novel nonsense variants appeared to acquire a non-organ/system affected phenotype with minor learning/behavioral differences[80]. A recent report described a Chinese woman presenting with on the CSS pathway disorders registry, who were carriers of a SMARCA4 variant, they showed differences in the severity and number of learning disabilities and health problems. Two of them with novel nonsense variants appeared to acquire a non-organ/system affected phenotype with minor learning/behavioral differences and endocrine dysfunction. Whole-exome sequencing was used and led to the identification of a heterozygous missense variant in the SMARCC2 gene of the proband[81].

Nicolaides-Baraitser syndrome

The clinical features of patients with nicolaides-Baraitser syndrome (NCBRS) caused by mutations in the SMARCA2 gene, remarkably resemble those of CSS patients[82]. The characteristics of hands and feet are where NCBRS and CSS most clearly diverge. While typical CSS patients have hypoplasia or agenesis of the fifth fingernail with or without engagement of the nail phalanges, typical NCBRS patients have pronounced distal phalanges and interphalangeal joints[75]. Five individuals with mutations in BAF60A exhibited developmental delay, small hands and feet, hypotonia, ID, and difficult feeding. According to TRIO exome sequencing, these mutations developed in four of the five patients[77]. A missense mutation (p. Arg366Cys) of BAF47 also has been reported to be associated with NCBRS[75]. Furthermore, variants in BAF170 have also been found in patients presenting with overlapping ID syndromes related to other BAF subunits, such as those identified in CSS and NCBRS[78].

Autism spectrum disorder

Autism is a neurodevelopmental disease which is caused by both environmental and genetic factors, presented as speech problems, limited and repetitive behaviors and social skills barrier[83]. Recent exome-sequencing studies on individuals with autism have found mutations in the BAF155, BAF170[84] and BAF190A[85] genes. But at least seventy percent of the cases, the fundamental genetic cause is still unknown.

Kleefstra syndrome spectrum

Kleefstra syndrome spectrum (KSS) is a recognizable syndrome characterized by ID and induced by a hybrid mutation in the euchromatin histone methyltransferase 1 (EHMT1) gene[86,87]. According to the research of Kleefstra *et al*[88], KSS could result from mutations in any complex protein including MLL3, MBD5, NR1I3, BAF47, or EHMT1. The EHMT1 mutation is not present in every patient with the primary symptoms of Kleefstra syndrome. In fact, de novo mutations in four genes that code for epigenetic regulators, including BAF47 (SMARCB1), were found in four of the nine individuals who did not have EHMT1 mutations[88]. And their research using a Drosophila model showed that MLL3, MBD5, NR1I3, BAF47, and EHMT1 directly interact with one another[88].

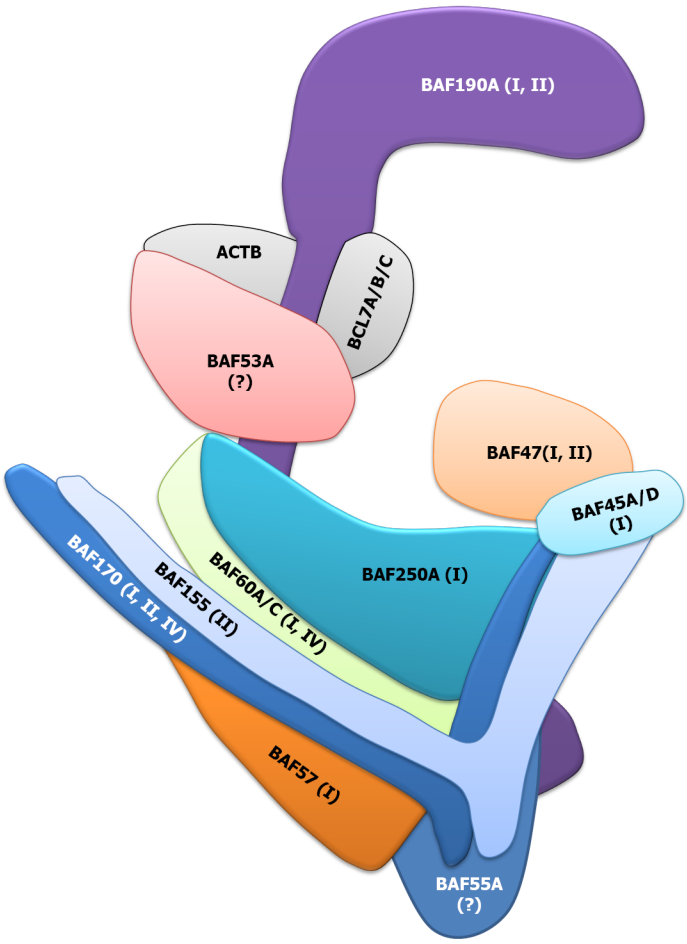

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Figure 1 BRG1/BRM-associated factor complex subunits in neural stem/progenitor cells and the related neural developmental diseases. The BRG1/BRM-associated factor (BAF) complex subunits in neural stem/progenitor cells are shown (in colors), including BAF45A/D, BAF47, BAF53, BAF55A, BAF57, BF60A/C, BAF155, BAF170, BAF190A, and BAF250A. The other two types of the BAF complex subunits, β-actin and BCL7/A/B/C, are shown in grey. Neural developmental disease types are listed as, I: Coffin-Siris syndrome; II: Autism spectrum disorder; III: Kleefstra syndrome; IV: Nicolaides-Baraitser syndrome; "?": Unknown. BAF: BRG1/BRM-associated factor.

CONCLUSION

Considering recent research progress, we suggest that three aspects of BAF complexes should be investigated in the near future. First, BAF complexes are crucial for development of neural system and differentiation of neural cells, including the specification of neural fate and functionality[4]. BAF complex subunits play key roles in the manipulation of gene expression, and the distinct ontogenetic stage-specific BAF complex functions in neural stem/progenitors and postmitotic neuronal cells that are derived from the combinatorial organization of these subunits[26]. Sequencing of human whole-exome and genome-wide association studies have recently demonstrated the link between mutants of BAF complex subunits and neurodevelopmental illnesses like CSS, NCBS, KSS and ASD[89-92]. Focus must be placed on the roles played by BAF complex organization during neural development as well as the ways in which mutations in well-known BAF complex subunits contribute to certain neurodevelopmental illnesses[93]. Secondly, both loss- and gain-of-function techniques to evaluating neural network development at the molecular, single-cell, and network activity levels should be used in order to comprehend how BAF complex gene mutations might cause the phenotypic convergence of CSS, NCBS, KSS, and ASD. For example, ASD and KSS genes focus primarily on the level of neural network communication and provide insight into the pathophysiology of phenotypic consistency disorders[79, 94]. Thirdly, recent investigations have shown that BAF complexes are frequently involved in human cerebral development abnormalities, providing fresh mechanisms and corresponding paths for therapeutic intervention, all of which should be looked into. In recent years, small compounds that target the BAF complex subunits have become viable therapeutic agents. Researchers in the field have been motivated to recognize and take use of the whole spectrum of therapeutic options as a result of the creation and evaluation of novel drugs that target a variety of BAF complex subunits[95].

FOOTNOTES

Author contributions: Ke NY and Zhao TY collected the data and wrote the manuscript; Wang WR and Qian YT collected the data; Liu C conceived the manuscript, collected the data, and wrote the manuscript.

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Bone marrow mesenchymal stem cell-derived exosomal microRNAs target PI3K/Akt signaling pathway to promote the activation of fibroblasts

Fang-Qi Li, Wen-Bo Chen, Zhi-Wen Luo, Yi-Sheng Chen, Ya-Ying Sun, Xiao-Ping Su, Jun-Ming Sun, Shi-Yi Chen

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Abstract

BACKGROUND

Fibroblast plays a major role in tendon-bone healing. Exosomes derived from bone marrow mesenchymal stem cells (BMSCs) can activate fibroblasts and promote tendon-bone healing *via* the contained microRNAs (miRNAs). However, the underlying mechanism is not comprehensively understood. Herein, this study aimed to identify overlapped BMSC-derived exosomal miRNAs in three GSE datasets, and to verify their effects as well as mechanisms on fibroblasts.

AIM

To identify overlapped BMSC-derived exosomal miRNAs in three GSE datasets and verify their effects as well as mechanisms on fibroblasts.

METHODS

BMSC-derived exosomal miRNAs data (GSE71241, GSE153752, and GSE85341) were downloaded from the Gene Expression Omnibus (GEO) database. The candidate miRNAs were obtained by the intersection of three data sets. TargetScan was used to predict potential target genes for the candidate miRNAs. Functional and pathway analyses were conducted using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, respectively, by processing data with the Metascape. Highly interconnected genes in the

protein-protein interaction (PPI) network were analyzed using Cytoscape software. Bromodeoxyuridine, wound healing assay, collagen contraction assay and the expression of COL I and α-smooth muscle actin positive were applied to investigate the cell proliferation, migration and collagen synthesis. Quantitative real-time reverse transcription polymerase chain reaction was applied to determine the cell fibroblastic, tenogenic, and chondrogenic potential.

RESULTS

Bioinformatics analyses found two BMSC-derived exosomal miRNAs, has-miR-144-3p and has-miR-23b-3p, were overlapped in three GSE datasets. PPI network analysis and functional enrichment analyses in the GO and KEGG databases indicated that both miRNAs regulated the PI3K/Akt signaling pathway by targeting phosphatase and tensin homolog (PTEN). *In vitro* experiments confirmed that miR-144-3p and miR-23b-3p stimulated proliferation, migration and collagen synthesis of NIH3T3 fibroblasts. Interfering with PTEN affected the phosphorylation of Akt and thus activated fibroblasts. Inhibition of PTEN also promoted the fibroblastic, tenogenic, and chondrogenic potential of NIH3T3 fibroblasts.

CONCLUSION

BMSC-derived exosomes promote fibroblast activation possibly through the PTEN and PI3K/Akt signaling pathways, which may serve as potential targets to further promote tendon-bone healing.

Key Words: Exosome; MicroRNA; Fibroblast; Mesenchymal stem cell; Tendon-bone healing

Core Tip: Exosomes derived from bone marrow mesenchymal stem cells (BMSCs) can activate fibroblasts and promote tendon-bone healing *via* the contained microRNAs (miRNAs). Supported by bioinformatics tools, this study identified two BMSC-derived exosomal miRNAs, has-miR-144-3p and has-miR-23b-3p, were overlapped in three GSE datasets. Bioinformatic analysis revealed that both miRNAs regulated the PI3K/Akt signaling pathway by targeting phosphatase and tensin homolog (PTEN). Experiments *in vitro* confirmed that interfering with PTEN can affect the phosphorylation of Akt and thus the activation of fibroblasts. These results suggested a potential mechanism by which BMSC-derived exosomes promote tendon-bone healing.

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INTRODUCTION

Tendon-bone insertion (TBI) injury, such as rotator cuff tears and anterior cruciate ligament injuries, is one of the common injuries in daily life and sports. Thus, how to promote tendon-bone healing becomes an important issue in research and clinical practice. The normal TBI has a transitional structure consisting of four gradated layers including bone, mineralized fibrocartilage layer, non-mineralized fibrocartilage layer and tendon[1,2]. This complex structure can disperse force from bone to tendon, preventing stress concentration[3]. However, current therapeutic strategies cannot restore this structure, hence raising the risk of re-injury.

Fibroblasts play a key role in tendon-bone healing. In the early stages of TBI injury, α-smooth muscle actin positive (α-SMA+) fibroblasts help form Sharpey-like fibers to withstand strength at TBI and participate in early ligament remodeling by producing collagen and restoring in situ tension[4,5]. Mesenchymal stem cells (MSCs) are a kind of stem cells with strong proliferation capacity and multilineage differentiation potential, which can differentiate into myoblasts, osteoblasts, adipocytes and chondroblast[6]. MSCs can be extracted from bone, adipose tissue, blood, and amniotic membrane [7]. They have multiple functions including immunomodulatory, anti-inflammatory, anti-apoptotic, and angiogenesis promotion, making them ideal candidate cells for tissue engineering research[8]. Recent evidence indicates that conditioned medium, primarily containing exosomes of MSCs, can stimulate the activation of fibroblasts, thereby promoting tendon-bone healing[9-12]. However, the underlying mechanism is not comprehensively understood.

Bioinformatics has played an important role in exploring disease mechanisms by allowing parallel processing of large volumes of high-throughput sequencing data. To identify key bone marrow MSC (BMSC)-derived exosome microRNAs (miRNAs), three Gene Expression Omnibus (GEO) datasets were interrogated using bioinformatics tools in this study. TargetScan, a software designed to predict miRNA binding sites, is often used to analyze target genes of miRNAs[13]. Metascape, a powerful gene functional annotation analysis tool, enables pathway enrichment and biological process (BP) annotation [14]. STRING database allows for assessing the functional associations among target genes[15]. Cytoscape software enables visualization of miRNA-target gene pairs.

Therefore, using the above database tools, this study aimed to identify overlapped BMSC-derived exosomal miRNAs in three GSE datasets and verify their effects as well as mechanisms on fibroblasts, so as to facilitate further studies to verify the role of exosomal miRNAs and their potential in promoting tendon-bone healing.

MATERIALS AND METHODS

The workflow of this study was described (Figure 1).

Data collection

Three BMSC-derived exosomal miRNA expression microarray datasets (GSE71241, GSE153752, and GSE85341) were retrieved from the GEO repository on 20 April 2022. (https://www.ncbi.nlm.nih.gov/geo). GSE71241[16], GSE153752[17], and GSE85341[18] included nine, four and one BMSC-derived exosome samples respectively. The above datasets were produced independently using the GPL18743, GPL16791, and GPL22300 platforms, respectively.

Venn diagram analysis and prediction of target genes

After obtaining the top 100 BMSC-derived exosomal miRNAs in each dataset, Venn diagram analysis was used to identify overlapped miRNAs in the three datasets through online tool Venny (version 2.1) (https://bioinfogp.cnb.csic.es/tools/venny). The overlapped miRNAs were considered as candidate miRNAs. Potential target genes for the candidate miRNAs were predicted by TargetScan, an experimentally validated database of miRNA-target interactions[13].

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses

Functional and pathway analyses of the predicted target genes were conducted using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, respectively, by processing data with the Metascape[14].

Construction of target gene-protein-protein interaction and miRNA-gene networks

Target gene data was uploaded to the STRING database[15] to assess the functional associations among the target genes of candidate miRNAs. Highly interconnected (hub) genes in the protein-protein interaction (PPI) network were analyzed using Cytoscape software (version 3.9.1). After candidate exosomal miRNAs and their hub target genes were identified, Cytoscape was used to visualize the resulting miRNA-gene network.

Cell culture and treatment

The NIH3T3 fibroblast (Shanghai Institutes for Biological Sciences) was used as a model *in vitro* to further determine the effect of SF1670 [a specific phosphatase and tensin homolog (PTEN) inhibitor] on fibroblasts[19]. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. When the cells reached 80% confluence, they were digested with 0.25% trypsin and sub-cultured 1:3. To determine the effect of SF1670, NIH3T3 cells were starved for 24 h with DMEM containing 0.5% FBS and then treated with or without SF1670 2 μM for the indicated time. NIH3T3 fibroblast was seeded and cultured into six-well plate.

Western blot assay

Protein was extracted and analyzed using an established method[20]. Anti-phosphorylated Akt (p-Akt) (CST, United States, 1:1000) and anti-Akt (CST, United States, 1:1000) were used as primary antibodies. Each group contains 3 protein samples for calculation.

Quantitative real-time reverse transcription polymerase chain reaction

RNA was extracted and analyzed using the previous method[21]. Total RNA was obtained by the Trizol reagent (Invitrogen, Carlsbad, CA) and quantified by Nanodrop. RNA was then reversely transcribed by the PrimeScript RT reagent kit (Takara Bio). Specific primers used in the experiment are from PrimerBank (Table 1). The operation was performed on the ABI7900 Real-Time PCR System (Applied

Table 1 Primers used in the experiment

Genes	Primers (5' - 3')
PTEN	F: TCAGCCGTTACCTGTGTGTG
	R: TCCTTGTCATTATCTGCACGC
α-SMA	F: GACAATGGCTCTGGGCTCTGTAA
	R: CTGTGCTTCGTCACCCACGTA
Vimentin	F: GACGCCATCAACACCGAGTT
	R: CTTTGTCGTTGGTTAGCTGGT
Tenomodulin	F: CCATGCTGGATGAGAGAGGTT
	R: CCGTCCTCCTTGGTAGCAGT
Col I	F: CCCGGGTTTCAGAGACAACTTC
	R: TCCACATGCTTTATTCCAGCAATC
Sox 9	F: GAGCCGGATCTGAAGAGGGA
	R: GCTTGACGTGTGGCTTGTTC
Col II	F: GGGAATGTCCTCTGCGATGAC
	R: GAAGGGGATCTCGGGGTTG
GAPDH	F: ACTCCACTCACGGCAAATTC
	R: TCTCCATGGTGGTGAAGACA

PTEN: Phosphatase and tensin homolog; α-SMA: α-smooth muscle actin positive.

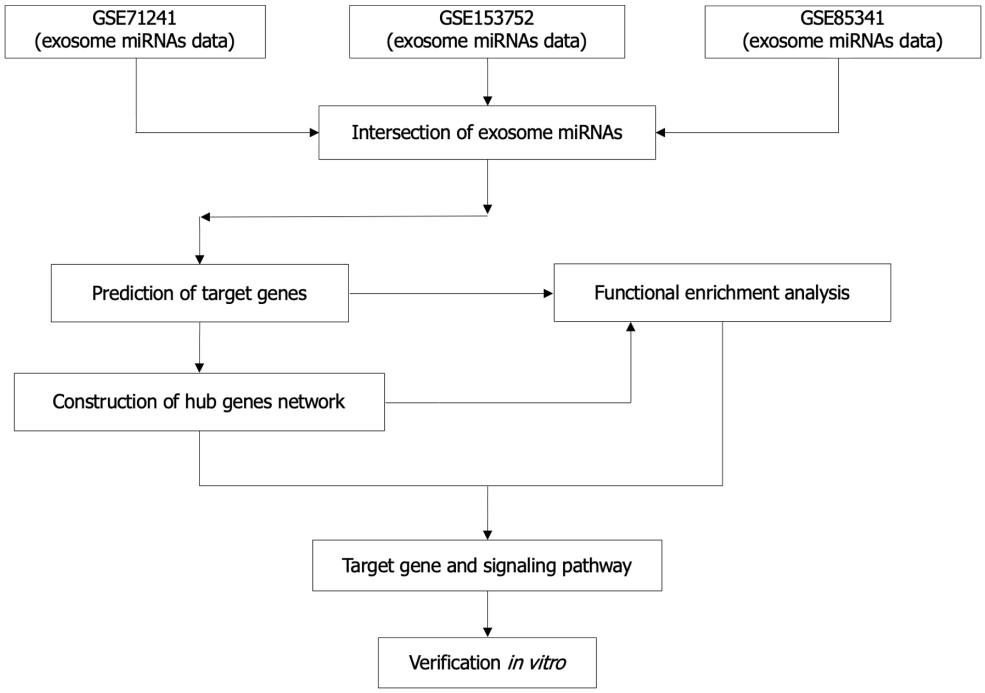

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Figure 1 The flowchart of this study. miRNAs: MicroRNAs.

Biosystems). The expression of mRNAs relative to the expression of GAPDH was calculated and normalized to the control group.

Luciferase reporter assay

Validation of miR-144 and miR-23b binding to 3′-UTR was performed using Dual-Glo Luciferase assay

system (Promega) as recommended by the manufacturer. The relative luciferase activities were determined by calculating the ratio of firefly luciferase activities over Renilla luciferase activities. All experiments were repeated three times in triplicate. Two constructs of pmirGLO luciferase reporter plasmid were generated: MUT-PTEN (with mutation of part of miRNA binding site sequence) and WT-PTEN [containing the wild-type (WT) miRNA binding site sequence]. 500 ng of the pmirGLO luciferase reporter plasmid and appropriate miRNA plasmid were co-transfected with lipofectamine 3000 (Invitrogen) into NIH3T3 cells. 48 h after transfection, the luciferase expression was determined using the Dual-Glo Luciferase Reporter Assay Kit (Promega) according to the manufacturer's protocol. The pRL-TK vector (Promega) containing Renilla luciferase was also co-transfected for normalization in all relevant experiments.

Bromodeoxyuridine assays

The Bromodeoxyuridine (BrdU) incorporation assay was performed using Cell Proliferation ELISA kits (1647229; Roche Applied Science, Mannheim, Germany). Briefly, NIH3T3 cells were plated at 5000 cells/well in 96-well culture plates in complete media. After attaining 70% confluence, cells were treated with media containing 10% FBS. BrdU solution (10 μM) was added after intervention. The cells were dried and fixed, and cellular DNA was denatured with FixDenat solution (Roche Applied Science) for 30 min at room temperature. A peroxidase-conjugated mouse anti-BrdU monoclonal antibody (Roche Applied Science) was added to the culture plates and cells were incubated for 90 min at room temperature. Tetramethyl-benzidine substrate was added and the plates were incubated for 15 min at room temperature. The absorbance of samples was measured using a microplate reader at 450-620 nm.

Wound healing assay

Cells at logarithmic growth stage were taken and placed in a six-well plate with a cell density of 5×10^5/well. Three multiple wells were set for each group. When the cells were adherent to the wall in a single layer, a pipetting tip was used to vertically scratch the six-well plate to avoid tilting. The suspension cells were cleaned and removed with PBS and cultured in an incubator with 5% CO_2 at 37 °C. Photographs were taken at 0 and 24 h under the microscope. The wound healing area was measured using ImageJ[22].

Collagen gel contraction assay

Cell contraction was determined using a kit from Cell Biolabs (San Diego, CA). Briefly, cells were seeded in collagen solution according to the manufacturer's instructions and release from the plates with a sterile spatula. Gel images were taken after release and the surface area of each gel was measured using ImageJ[22].

Immunofluorescence

NIH3T3 cells were cultured in a 24-well plate. The cells were fixed with 4% paraformaldehyde and incubated with 0.5% Triton X-100 (Sigma) and then blocked with goat serum (Biyuntian Company, China) for 1 h. The cells were then incubated overnight at 4 °C with the primary antibody. The secondary antibody was applied, and the cells were incubated for 1 h in the dark. Finally, the nuclei were counterstained with DAPI for 15 min. The stained cells were photographed using a fluorescence microscope.

Statistical analysis

All experiments were performed at least three times. Data were analyzed with GraphPad Prism 7.0 (GraphPad Software, La Jolla, United States) and were presented by mean ± SD. Significance was typically analyzed by student's *t*-test, one-way ANOVA followed by post hoc LSD test, and two-way ANOVA followed by multiple *t*-tests. $P < 0.05$ was regarded as significant.

RESULTS

Identification of candidate exosomal miRNAs and target genes

Three independent BMSC-derived exosomal miRNA expression microarray datasets (GSE71241, GSE153752, and GSE85341)[16-18] were downloaded from the GEO database. Subsequently, top 100 BMSC-derived exosomal miRNAs in each dataset were obtained. The Venn diagram analysis demonstrated two miRNAs in the intersection of the three datasets (Figure 2). The target gene interactions of two candidate miRNAs, hsa-miR-144-3p and hsa-miR-23b-3p, were assessed by the TargetScan[13]. In total, 1048 and 105 potential target genes were identified for miR-144-3p and miR-23b-3p, respectively.

Functional enrichment analysis of miRNAs' target genes

GO functional annotation analysis revealed that the most enriched terms for the target genes of miR-

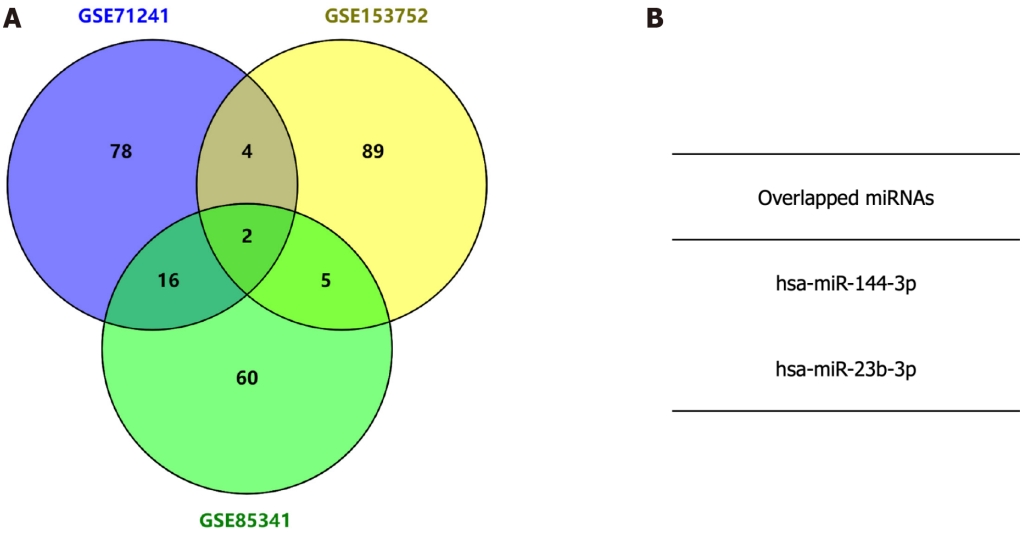

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Figure 2 Identification of candidate microRNAs by intersecting three exosome microRNA expression microarray datasets. A: Venn diagram analysis showing the top 100 exosome microRNAs (miRNAs) in the three Gene Expression Omnibus (GEO) datasets; B: Identification of two miRNAs overlapping between the three GEO datasets. miRNAs: MicroRNAs.

144-3p were 'head development' and 'regulation of kinase activity' in the BP, 'postsynapse' in the cellular component (CC), and 'transcription factor binding' and 'kinase activity' in the molecular function (MF) (Figures 3A-D). On the other hand, the target genes of miR-23b-3p were mostly enriched in 'phospholipid metabolic process' in the BP, 'nuclear speck' and 'centrosome' in the CC, and 'chromatin binding' in the MF (Figures 4A-D). KEGG pathway analysis revealed that most of target genes of miR-144-3p enriched in 'pathways in cancer' and 'PI3K-AKT signaling pathway' (Figure 3E); most of target genes of miR-23b-3p enriched in 'pathways in cancer' (Figure 4E).

Construction of target gene-PPI and miRNA-hub gene networks

A PPI network was constructed through the STRING database[15]. Interactions with an overall score of more than 0.4 were considered significant. Further processing was carried out using Cytoscape software. Then, the cytoHubba plugin was used to determine the top 10 hub genes based on the Degree algorithm. The top 10 predicted hub genes of miR-144-3p and miR-23b-3p were presented (Figures 5A and B). GO and KEGG analysis revealed that these hub genes were mostly enriched in 'regulation of MAPK cascade', 'regulation of phosphatidylinositol 3-kinase signaling' and 'negative regulation of cell differentiation' in the BP (Figure 5C, Table 2), 'fibrillar center' and 'focal adhesion' in the CC (Figure 5D, Table 3), and 'protein kinase activity' and 'transcription factor binding' in the MF (Figure 5E, Table 4). Most of these genes were mainly enriched in PI3K-Akt signaling pathway (Figure 5F, Table 5). Interactional analysis showed that PTEN and sonic hedgehog (SHH) are potentially co-regulated by miR-144-3p and miR-23b-3p (Figure 5G).

PTEN is verified as the targets of miR-144-3p and miR-23b-3p

The phosphatase and tensin homolog deleted on chromosome ten (PTEN), was predicted as the hub gene of miR-144-3p and miR-23b-3p (Figures 6A and B). To further validate the putative binding sites, WT and mutated 3'-UTR of PTEN were cloned into the pmirGLO vector. The transcripts of the target gene were then evaluated by dual luciferase reporter assays. Compared with the control group, luciferase reporter assays showed that miR-144-3p mimics and miR-23b-3p mimics significantly decreased the luciferase activity of WT-PTEN but did not show a significant effect on the luciferase activity of MUT-PTEN (Figures 6C and D). In addition, miR-144-3p mimic significantly down-regulated the mRNA levels of PTEN, while miR-144-3p inhibitor up-regulated the mRNA levels of PTEN (Figure 6E). Similarly, miR-23b-3p mimic down-regulated the mRNA level of PTEN, while miR-23b-3p inhibitor up-regulated the mRNA level of PTEN (Figure 6F). These results indicated that miR-144-3p and miR-23b-3p repressed the expression of PTEN by specifically binding with and subsequently inducing the degradation of mRNA.

miR-144-3p and miR-23b-3p regulate the proliferation, migration, and collagen synthesis of NIH3T3 fibroblasts

To evaluate the effect of the identified miRNAs on fibroblast biology and activation, analysis of proliferation and migration was then performed 24 h after transfection of miR-144-3p and miR-23b-3p respectively. Compared with the control group, proliferation and migration capacity of NIH3T3 cells in

Table 2 Top 10 clusters with Gene Ontology Biological Processes analysis of hub-gene targets

Term	Description	LogP	Log(q-value)	Count
GO:0048732	Gland development	-12.279	-7.927	9
GO:0048608	Reproductive structure development	-10.269	-6.495	8
GO:0061458	Reproductive system development	-10.245	-6.495	8
GO:0030900	Forebrain development	-8.922	-5.472	7
GO:0043408	Regulation of MAPK cascade	-8.603	-5.240	8
GO:0045596	Negative regulation of cell differentiation	-8.549	-5.238	8
GO:0022612	Gland morphogenesis	-8.482	-5.208	5
GO:0014066	Regulation of phosphatidylinositol 3-kinase signaling	-8.316	-5.077	5
GO:0060322	Head development	-8.138	-4.931	8
GO:0060284	Regulation of cell development	-8.045	-4.868	7

Table 3 Top 10 clusters with Gene Ontology Cellular Components analysis of hub-gene targets

Term	Description	LogP	Log(q-value)	Count
GO:0045121	Membrane raft	-4.441	-2.311	4
GO:0098857	Membrane microdomain	-4.436	-2.310	4
GO:0001650	Fibrillar center	-4.076	-2.035	3
GO:0009898	Cytoplasmic side of plasma membrane	-3.849	-1.860	3
GO:0098562	Cytoplasmic side of membrane	-3.638	-1.688	3
GO:0005770	Late endosome	-3.205	-1.308	3
GO:0005788	Endoplasmic reticulum lumen	-3.103	-1.227	3
GO:0031983	Vesicle lumen	-3.041	-1.174	3
GO:0005925	Focal adhesion	-2.726	-0.896	3
GO:0030055	Cell-substrate junction	-2.699	-0.873	3

Table 4 Top 10 clusters with Gene Ontology Molecular Functions analysis of hub-gene targets

Term	Description	LogP	Log(q-value)	Count
GO:0002020	Protease binding	-5.941	-3.326	4
GO:0004712	Protein serine/threonine /tyrosine kinase activity	-5.299	-2.910	5
GO:0004672	Protein kinase activity	-4.802	-2.546	5
GO:0003682	Chromatin binding	-4.726	-2.507	5
GO:0008134	Transcription factor binding	-4.694	-2.487	5
GO:0031490	Chromatin DNA binding	-4.457	-2.319	3
GO:0016773	Phosphotransferase activity, alcohol group as acceptor	-4.435	-2.310	5
GO:0019904	Protein domain specific binding	-4.395	-2.280	5
GO:0061629	RNA polymerase II-specific DNA-binding transcription factor binding	-4.331	-2.228	4
GO:0016301	Kinase activity	-4.267	-2.193	5

transfection groups were significantly increased (Figures 7A and B). Furthermore, overexpression of miR-144-3p or miR-23b-3p upregulated the expression of Col I and a-SMA in NIH3T3 cells (Figure 7C). These *in vitro* transfection data indicated that increment in intracellular miR-144-3p or miR-23b-3p stimulated NIH3T3 fibroblast proliferation and migration, and promoted fibroblast-myofibroblast differentiation and collagen synthesis.

Table 5 Top 10 clusters with Kyoto Encyclopedia of Genes and Genomes Pathway analysis of hub-gene targets

Term	Description	Log*P*	Log(*q*-value)	Count
hsa04510	Focal adhesion	-10.807	-6.755	7
hsa05200	Pathways in cancer	-9.488	-5.834	8
hsa04151	PI3K-Akt signaling pathway	-9.088	-5.538	7
hsa01521	EGFR tyrosine kinase inhibitor resistance	-9.046	-5.538	5
hsa05215	Prostate cancer	-8.593	-5.240	5
hsa05224	Breast cancer	-7.682	-4.608	5
hsa05165	Human papillomavirus infection	-7.560	-4.511	6
hsa05213	Endometrial cancer	-7.435	-4.444	4
hsa05225	Hepatocellular carcinoma	-7.392	-4.437	5
hsa05230	Central carbon metabolism in cancer	-7.102	-4.181	4

EGFR: Epidermal growth factor receptor.

Inhibition of PTEN promote cell proliferation, migration, collagen synthesis and change the gene expression of fibroblastic-, tenogenic-, and chondrogenic-related factors in NIH3T3 cells

To explore whether BMSC-derived exosome promoted tendon bone healing by directly targeting PTEN in fibroblasts, SF1670, a specific PTEN inhibitor that binds to the active site of PTEN and increases cellular PtdIns(3,4,5)P3 levels and phosphorylation of Akt *in vitro*[19], was used to examine the effect of PTEN inhibition on NIH3T3 cells. The results showed that SF1670 significantly increased the phosphorylation level of Akt compared with DMSO group (Figure 8A). Moreover, BrdU and wound healing assays showed that SF1670 significantly increased proliferation (Figure 8B) and migration (Figure 8C) of NIH3T3 cells. Collagen contraction assays indicated that SF1670 significantly enhanced the contractility of NIH3T3 cells (Figure 8D). Finally, the properties necessary for tendon-bone healing was examined by quantitative real-time reverse transcription polymerase chain reaction. The results showed that the mRNA level of fibroblast-related genes (α-SMA and vimentin), tenogenic-related genes (TNMD and Col I), and chondrogenic-related genes (SOX-9 and Col II) were significantly up-regulated by SF1670 (Figure 9).

DISCUSSION

Tendon-bone healing has always been an important challenge in orthopedics and sports medicine research and clinical practice. Fibroblasts play a key role in early ligament remodeling and a large number of studies have verified the effective role of MSC-derived exosomes in tendon-bone healing[9-12]. However, because the status of MSCs may be influenced by their generations or donors, the expression of exosomal miRNAs may be more or less different. This presents difficulties in identifying the roles associated with MSC-derived exosomal miRNAs. On ground of this, three GEO datasets were interrogated through bioinformatics tools to determine key BMSC-derived exosomal miRNAs. Two candidate miRNAs (miRNA-144 and miRNA-23b) were obtained by intersecting the top 100 exosomal miRNAs in each dataset (Figure 2). Their target genes and hub genes were obtained subsequently. GO annotation and KEGG pathway enrichment analysis suggested that these genes were involved in multiple cellular processes (*e.g.*, regulation of kinase activity, transcription factor binding, and phospholipid metabolic process) and signaling cascades (*e.g.*, focal adhesion, and PI3K-Akt pathways). A miRNA-target gene interaction network indicated that PTEN was co-regulated by miRNA-144 and miRNA-23b (Figure 5G).

PTEN functions primarily *via* dephosphorylation of PIP3 to PIP2, resulting in negative regulation of the PI3K/Akt activity[23]. The PTEN/PI3K/Akt nexus participates in various physiological and pathological conditions, and plays an important role in regulating cell growth, apoptosis, metabolism and other processes[24]. Being the direct target of PTEN, PI3K/Akt is also involved in activation of fibroblasts[25,26]. In this study, the effect of PTEN on fibroblasts was also demonstrated. The results showed that inhibition of PTEN increased the level of Akt phosphorylation and promoted the proliferation and migration of fibroblasts (Figure 8), which suggest that PTEN plays an important role in the activation of fibroblasts.

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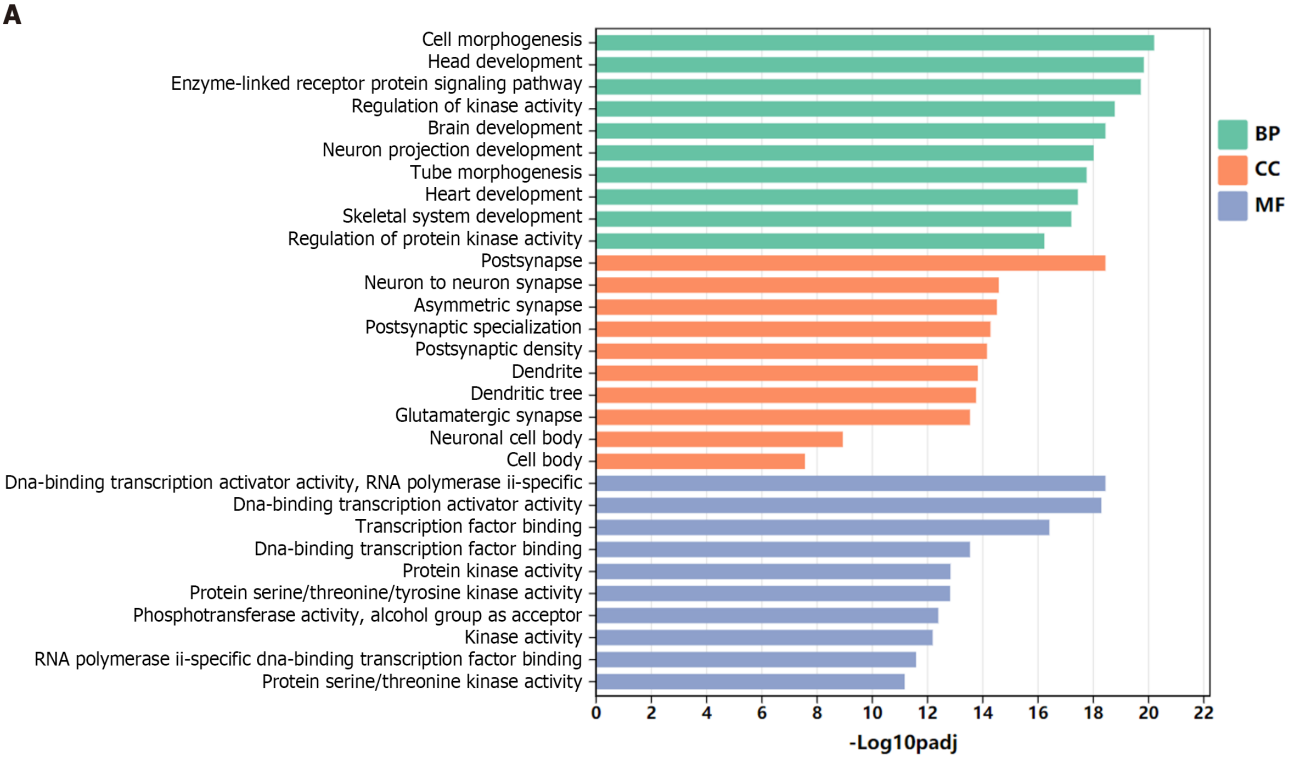
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Regulation of protein kinase activity
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Neuron to neuron synapse
Asymmetric synapse
Postsynaptic specialization
Postsynaptic density
Dendrite
Dendritic tree
Glutamatergic synapse
Neuronal cell body
Cell body
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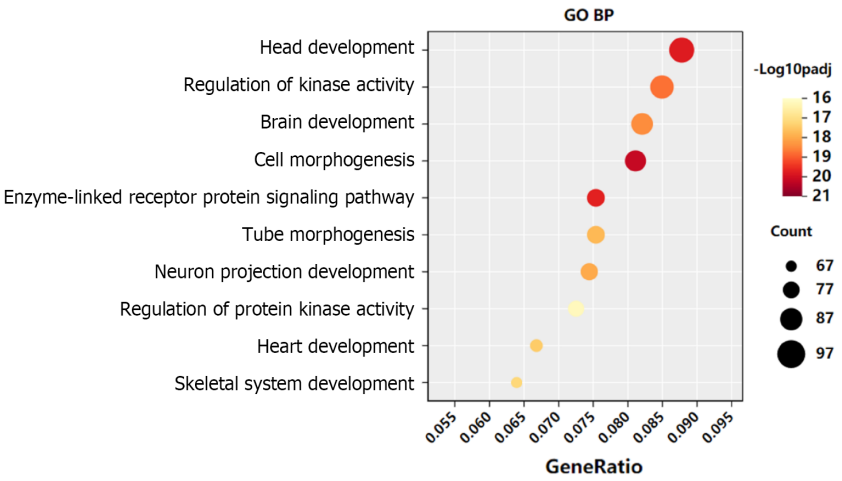
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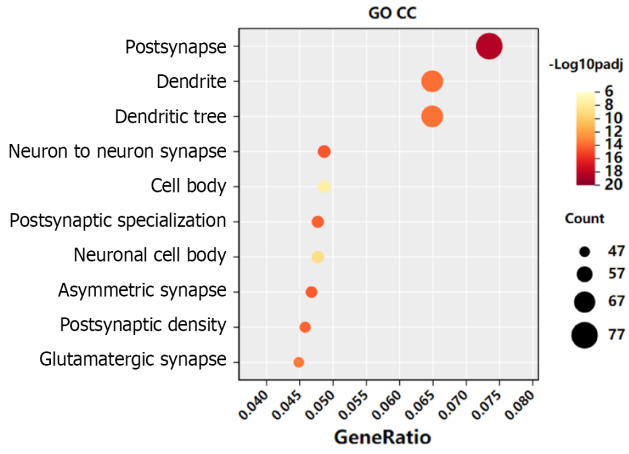
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Cell body
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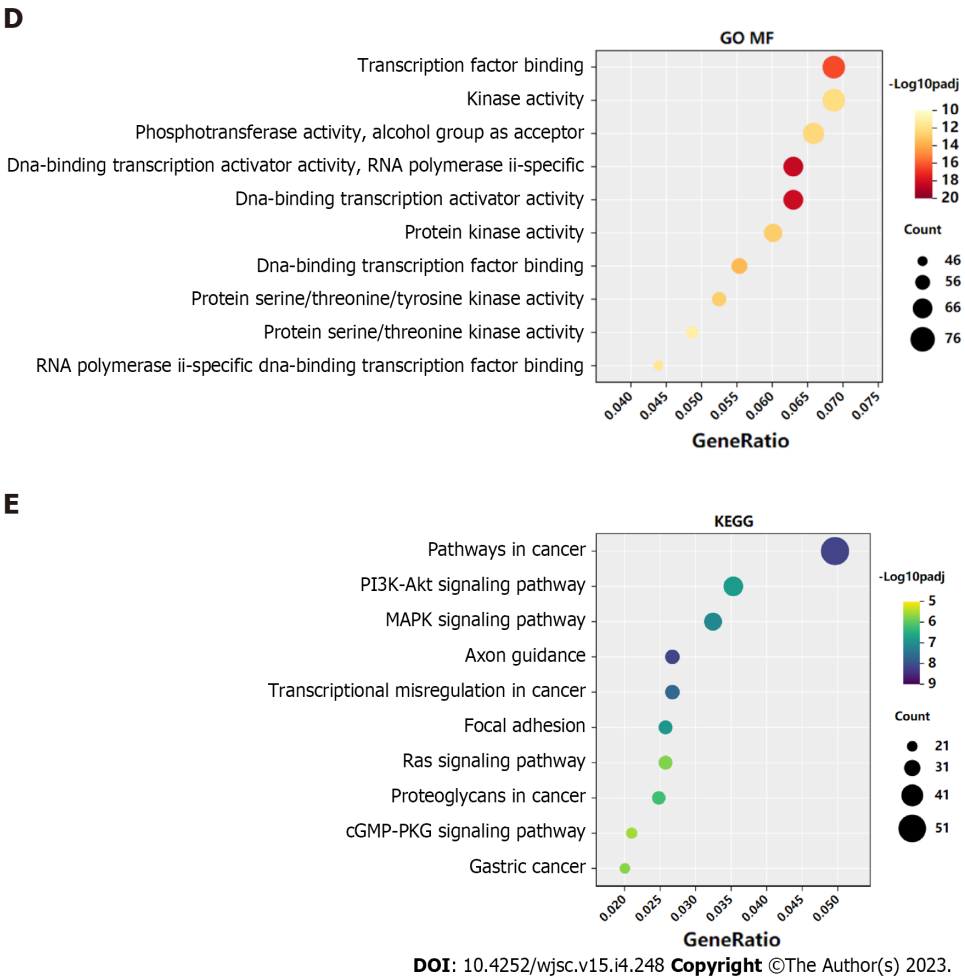

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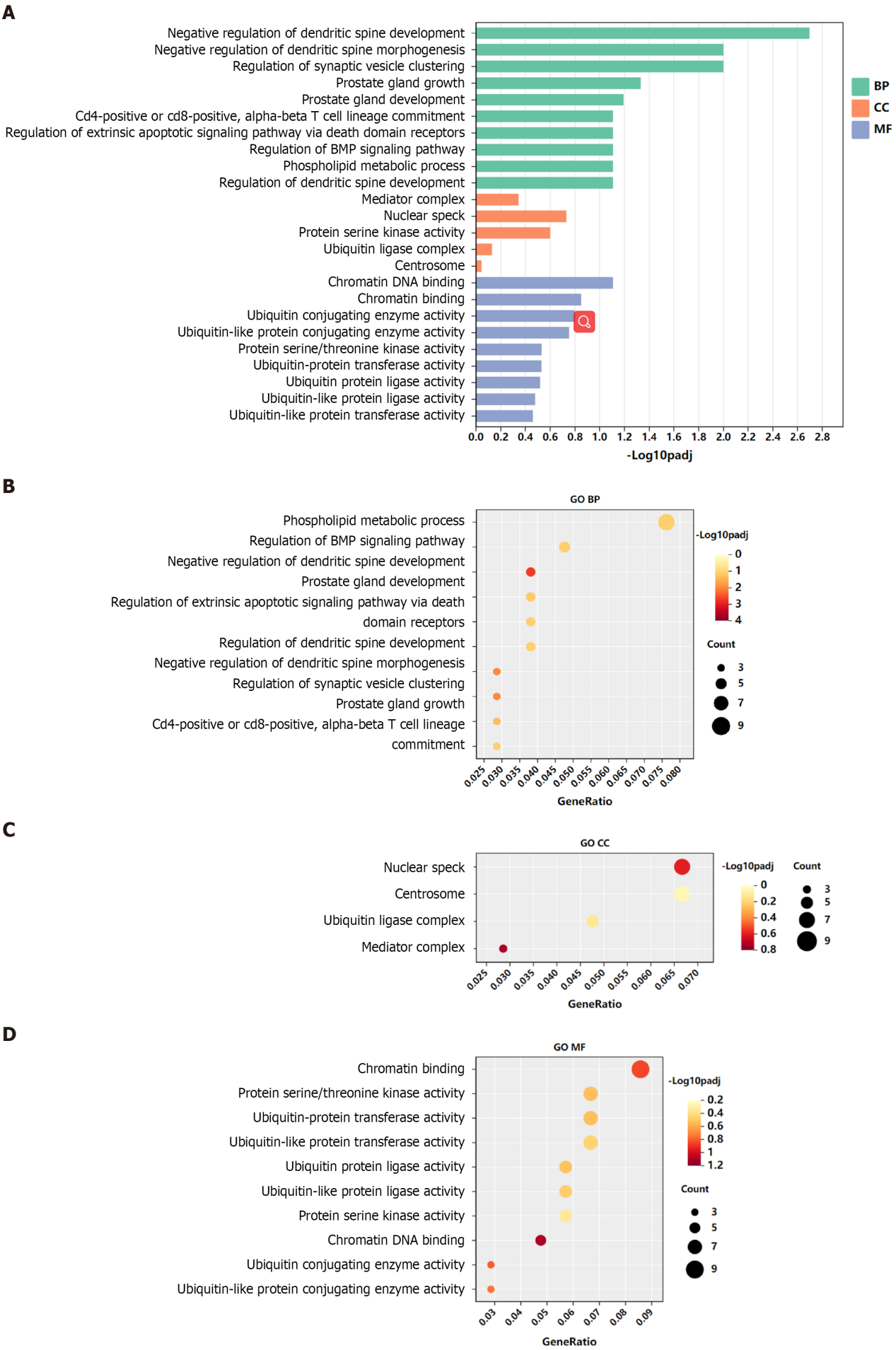
Figure 3 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis of genes targeted by hsa-miR-144-3p. A-D: Top 10 Gene Ontology biological process, cellular component, and molecular function terms enriched in target genes of hsa-miR-144-3p; E: Top 10 Kyoto Encyclopedia of Genes and Genomes pathways enriched in target genes of hsa-miR-144-3p. BP: Biological process; CC: Cellular component; MF: Molecular function; GO: Gene Ontology; KEEG: Kyoto Encyclopedia of Genes and Genomes.

Normal TBI has a transitional structure consisting of four graduated layers including bone tissue, mineralized fibrocartilage layer, non-mineralized fibrocartilage layer and tendon tissue[27], which means that fibrogenesis, tenogenesis and chondrogenesis are key characteristics of tendon-bone healing. As the markers of fibrogenesis, α-SMA and vimentin are expressed in activated fibroblasts[28]. Similarly, tenomodulin (TNMD) and collagen I, collagen II and Sox-9 are well-acknowledged indicators for representing the activation of tenogenic and chondrogenic process[29,30]. By inhibiting PTEN, the results showed increased expression of fibroblastic, tenogenic and chondrogenic markers in fibroblasts (Figure 9), implying that PTEN and PI3K/Akt pathway may be targets for promoting tendon-bone healing.

In addition, this study found that SHH was also regulated by both miRNA-144 and miRNA-23b (Figure 5G). HH signaling pathway is a highly conserved pathway involved in embryonic development, tissue homeostasis and stem cell maintenance[31]. HH signaling is also involved in regulating proliferation of MSCs in adult tissues and plays critical roles in promoting tendon-bone healing[32-34]. SHH, one of three HH family members in mammals, acts in the early stages of development to regulate patterning and growth[35], and plays a crucial role in bone healing[36]. These evidences suggested that MSC-derived exosomal miRNAs may promote tendon-bone healing in more than one way.

The outcomes of the current study were guaranteed by several factors. In this study, three BMSC-derived exosomal miRNA expression microarray datasets were searched from the GEO repository to avoid batch differences. Moreover, due to the costs, technical challenges, and lack of biomarkers suitable for specific exosomes, it is difficult to isolate large number of pure and specific exosomes from mixtures of different vesicle types in a large volume of solution[37]. Therefore, this study identified the key pathway of exosomal miRNA through comprehensive analysis, providing a theoretical basis for developing methods to stably regulate the activation of fibroblasts and thus promote tendon-bone healing.


A
Negative regulation of dendritic spine development
Negative regulation of dendritic spine morphogenesis
Regulation of synaptic vesicle clustering
Prostate gland growth
Prostate gland development
Cd4-positive or cd8-positive, alpha-beta T cell lineage commitment
Regulation of extrinsic apoptotic signaling pathway via death domain receptors
Regulation of BMP signaling pathway
Phospholipid metabolic process
Regulation of dendritic spine development
Mediator complex
Nuclear speck
Protein serine kinase activity
Ubiquitin ligase complex
Centrosome
Chromatin DNA binding
Chromatin binding
Ubiquitin conjugating enzyme activity
Ubiquitin-like protein conjugating enzyme activity
Protein serine/threonine kinase activity
Ubiquitin-protein transferase activity
Ubiquitin protein ligase activity
Ubiquitin-like protein ligase activity
Ubiquitin-like protein transferase activity
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8
-Log10padj
BP
CC
MF
B
GO BP
Phospholipid metabolic process
Regulation of BMP signaling pathway
Negative regulation of dendritic spine development
Prostate gland development
Regulation of extrinsic apoptotic signaling pathway via death
domain receptors
Regulation of dendritic spine development
Negative regulation of dendritic spine morphogenesis
Regulation of synaptic vesicle clustering
Prostate gland growth
Cd4-positive or cd8-positive, alpha-beta T cell lineage
commitment
0.025 0.030 0.035 0.040 0.045 0.050 0.055 0.060 0.065 0.070 0.075 0.080
GeneRatio
-Log10padj
0 1 2 3 4
Count
3 5 7 9
C
GO CC
Nuclear speck
Centrosome
Ubiquitin ligase complex
Mediator complex
0.025 0.030 0.035 0.040 0.045 0.050 0.055 0.060 0.065 0.070
GeneRatio
-Log10padj
0 0.2 0.4 0.6 0.8
Count
3 5 7 9
D
GO MF
Chromatin binding
Protein serine/threonine kinase activity
Ubiquitin-protein transferase activity
Ubiquitin-like protein transferase activity
Ubiquitin protein ligase activity
Ubiquitin-like protein ligase activity
Protein serine kinase activity
Chromatin DNA binding
Ubiquitin conjugating enzyme activity
Ubiquitin-like protein conjugating enzyme activity
0.03 0.04 0.05 0.06 0.07 0.08 0.09
GeneRatio
-Log10padj
0.2 0.4 0.6 0.8 1 1.2
Count
3 5 7 9

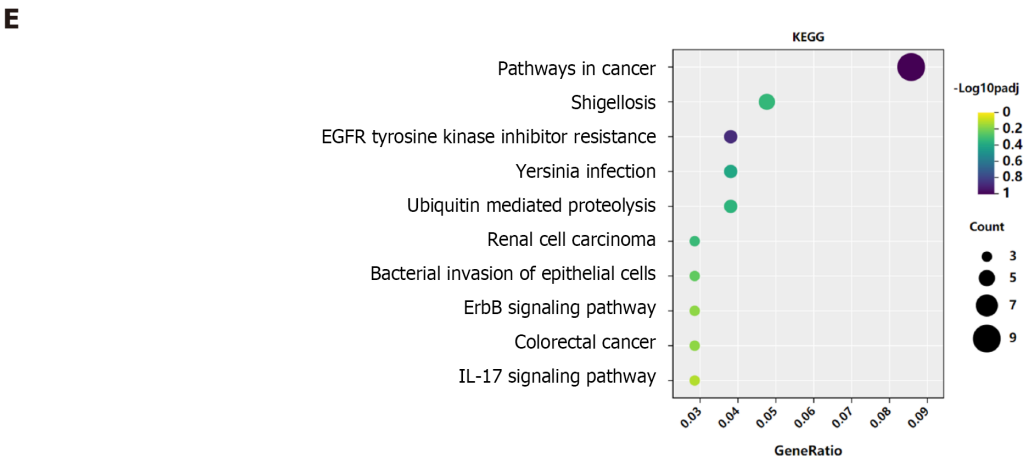


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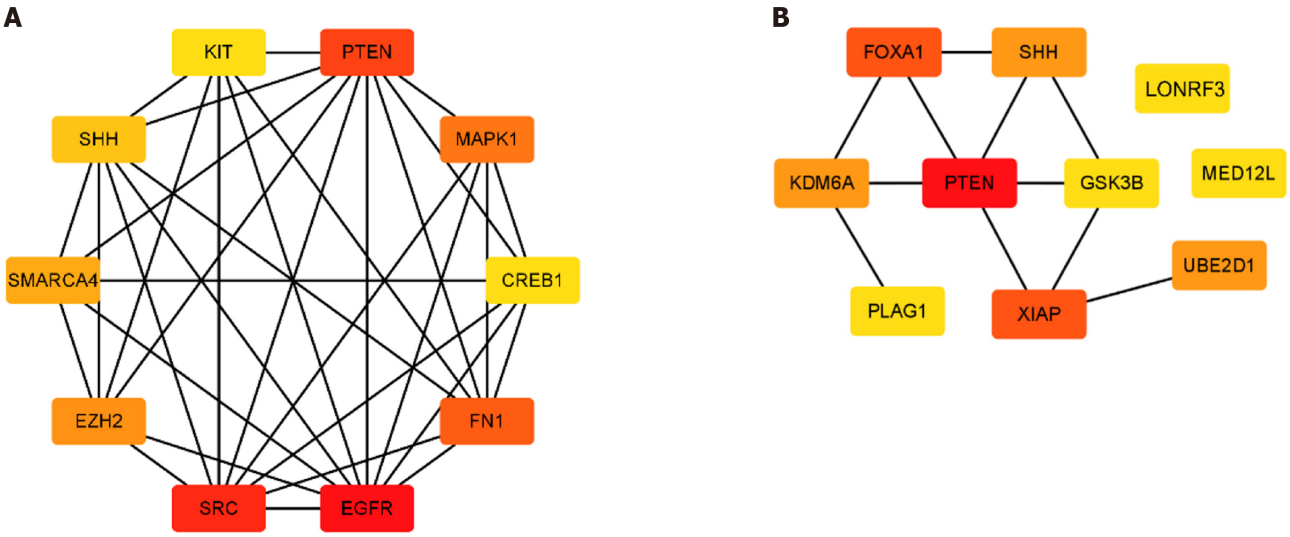
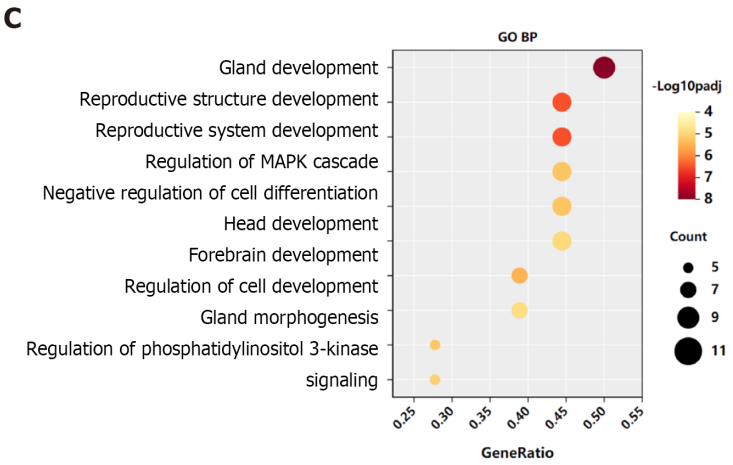
Figure 4 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis of genes targeted by hsa-miR-23b-3p. A-D: Top 10 Gene Ontology biological process, cellular component, and molecular function terms enriched in target genes of hsa-miR-23b-3p; E: Top 10 Kyoto Encyclopedia of Genes and Genomes pathways enriched in target genes of hsa-miR-23b-3p. BP: Biological process; CC: Cellular component; MF: Molecular function; GO: Gene Ontology; KEEG: Kyoto Encyclopedia of Genes and Genomes.





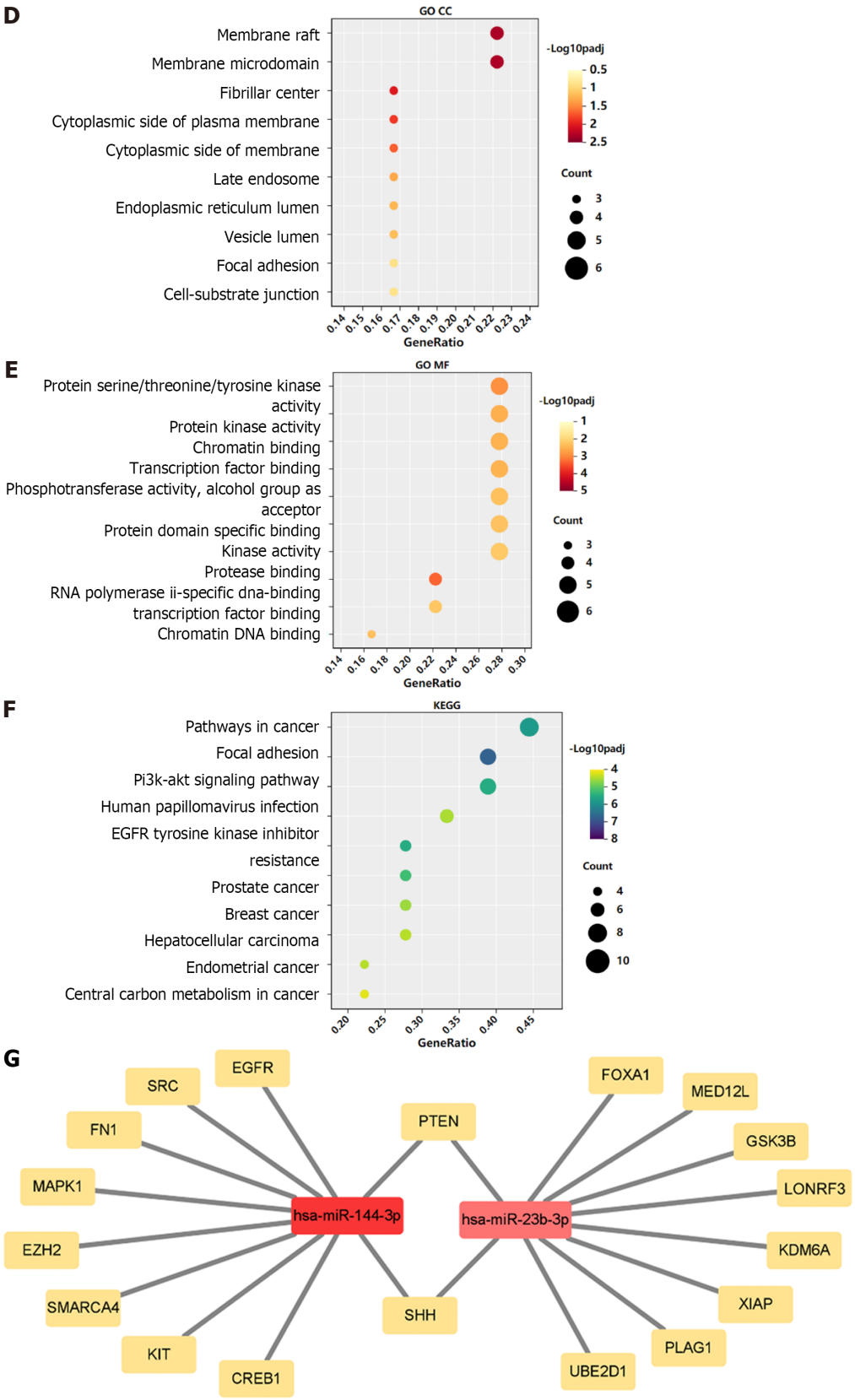


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Figure 5 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis of hub-gene targets. A: Top 10 hub-gene targets for hsa-miR-144-3p; B: Top 10 hub-gene targets for hsa-miR-23b-3p; C-E: Top 10 Gene Ontology biological process, cellular component, and molecular function terms enriched in the top 20 hub-gene targets; F: Top 10 Kyoto Encyclopedia of Genes and Genomes pathways enriched in the 20 hub-gene targets; G: Interaction network of the two microRNAs and their hub-gene targets.

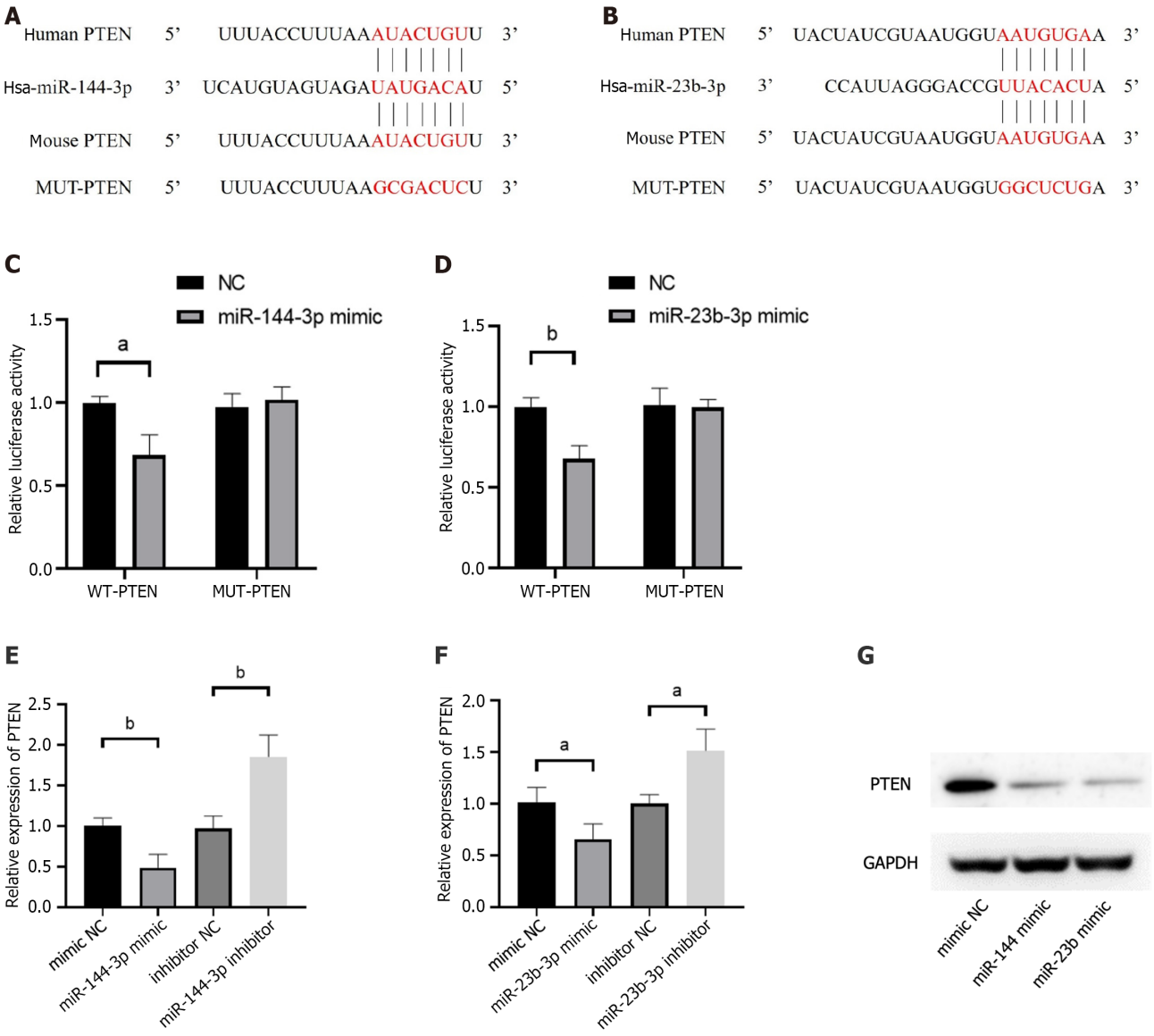

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Figure 6 Phosphatase and tensin homolog is verified as the targets of miR-144-3p and miR-23b-3p. A: Human and mouse sequence of phosphatase and tensin homolog (PTEN) for miR-144-3p binding site; B: Human and mouse sequence of PTEN for miR-23b-3p binding site; C: The relative luciferase activity was tested after co-transfection of WT/MUT pmirGLO-PTEN-3'UTR and miR-144-3p mimics or their control groups in NIH3T3 cells; D: The relative luciferase activity was tested after co-transfection of WT/MUT pmirGLO-PTEN-3'UTR and miR-23b-3p mimics or their control groups in NIH3T3 cells; E: The mRNA levels of PTEN in NIH3T3 cells transfected with miR-144-3p mimics or miR-144-3p inhibitors; F: The mRNA levels of PTEN in NIH3T3 cells transfected with miR-23b-3p mimics or miR-23b-3p inhibitors; G: Western blot showing the PTEN expression levels after transfected with miR-144-3p mimics and miR-23b-3p mimics. n = 3 per groups. Data are shown as mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$.

However, the outcomes should also be interpreted with caution. The biological functions of exosomal miRNAs are diverse, but this study only took the overlapped target gene, *i.e.*, PTEN, for analysis. Thus, further exploration is needed to pinpoint the specific functions of each miRNA. In addition, this study only used murine NIH3T3 cells *in vitro*. Experiments *in vivo* is needed to verify the reconstruction of the natural gradient structure of TBI.

CONCLUSION

BMSC-derived exosomes promote fibroblast activation possibly through the PTEN and PI3K/Akt signaling pathways, which may serve as potential targets to further promote tendon-bone healing.

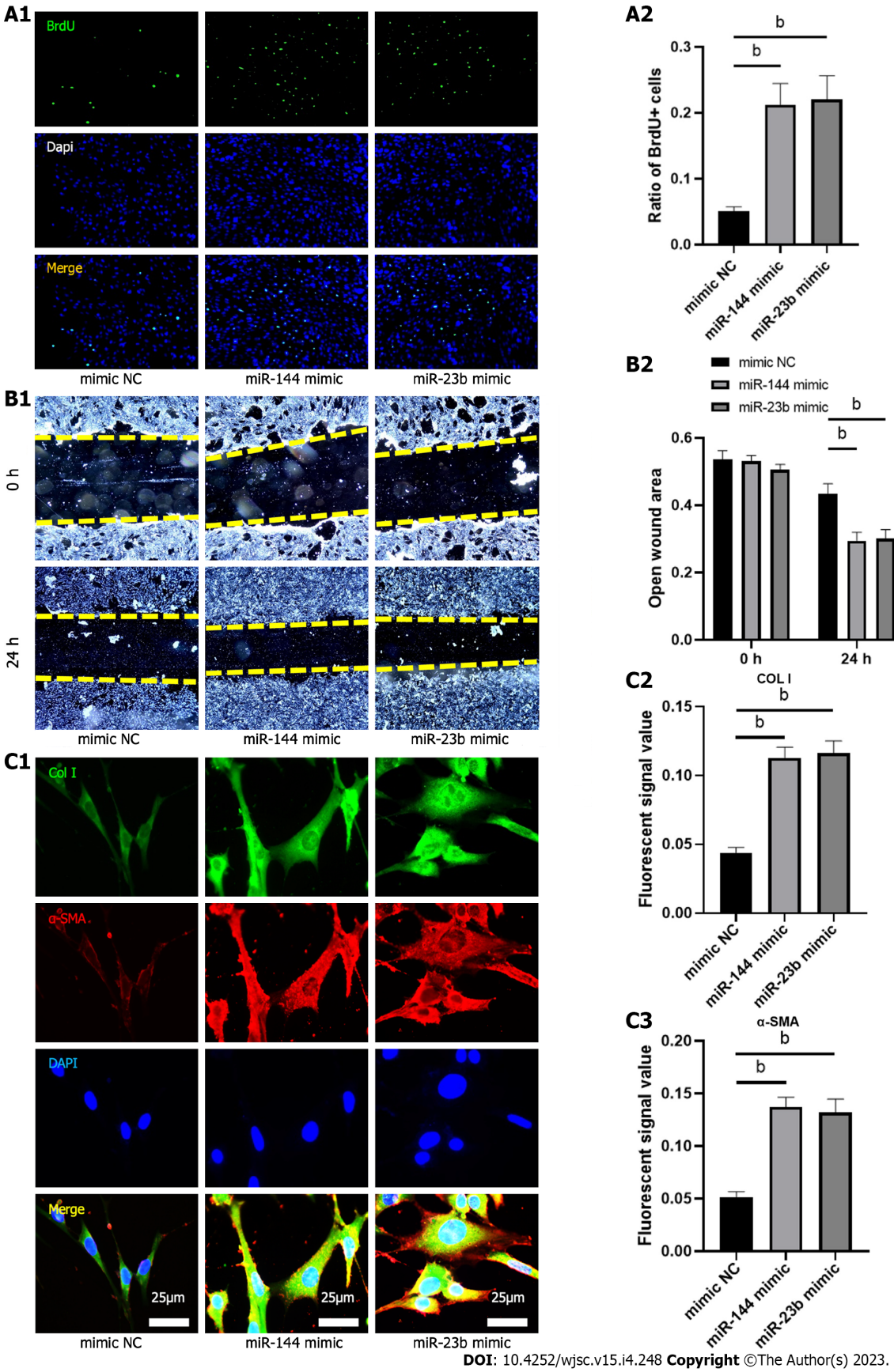

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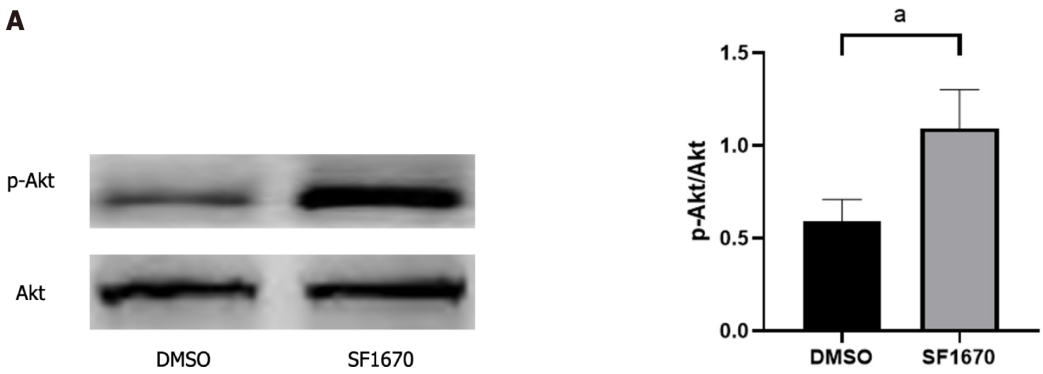
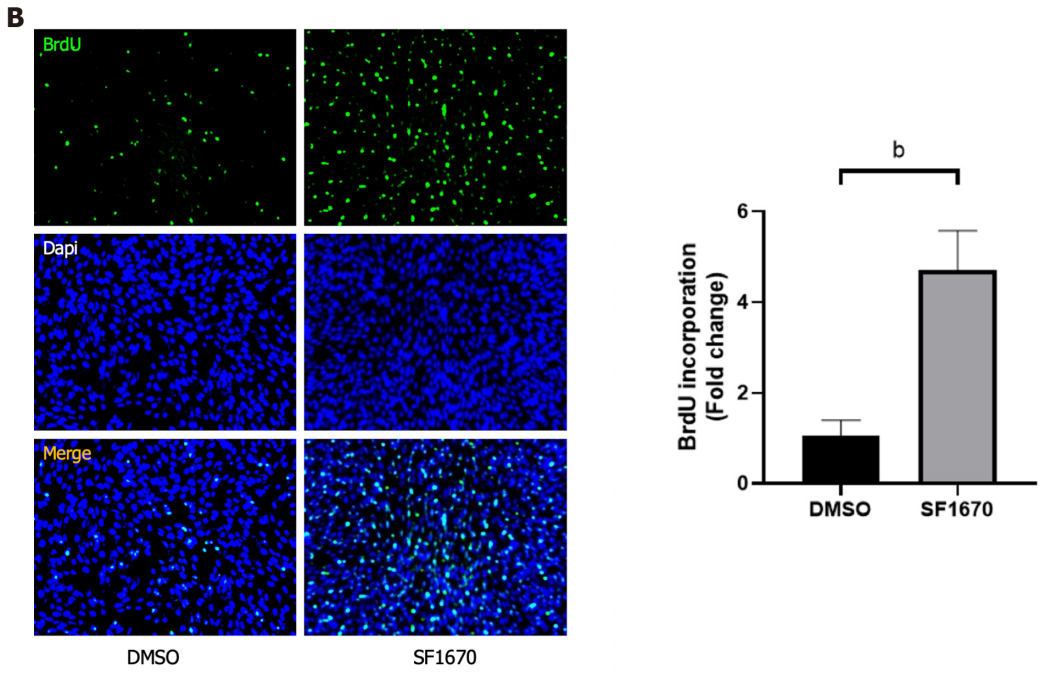
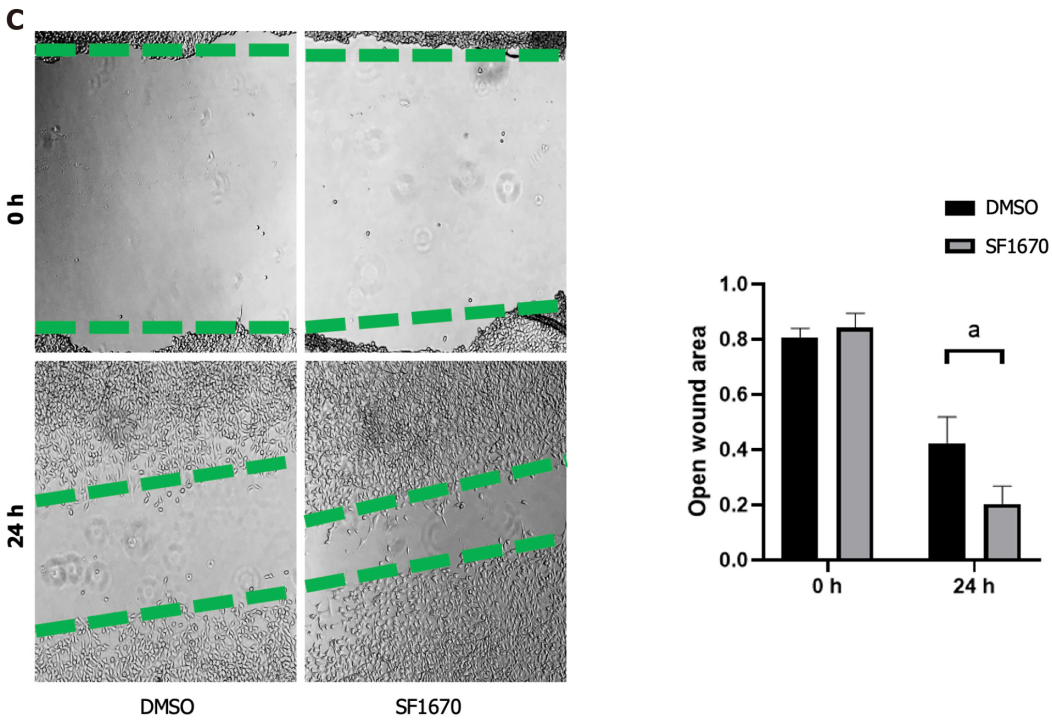
Figure 7 MiR-144-3p and miR-23b-3p regulate the proliferation, migration and collagen synthesis of NIH3T3 fibroblasts. A: Bromodeoxyuridine assay was performed to measure the proliferation ability; B: Wound healing assay was performed to measure the migratory capability; C: Immunofluorescence was performed to determine the effects of miR-144-3p and miR-23b-3p on COL I and α-smooth muscle actin expression. n = 3 per groups. Data are shown as mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$. α-SMA: α-smooth muscle actin.


A
p-Akt
Akt
DMSO
SF1670
a
p-Akt/Akt
1.5
1.0
0.5
0.0
DMSO
SF1670


B
BrdU
Dapi
Merge
DMSO
SF1670
b
BrdU incorporation (Fold change)
6
4
2
0
DMSO
SF1670


C
0 h
24 h
DMSO
SF1670
DMSO
SF1670
Open wound area
1.0
0.8
0.6
0.4
0.2
0.0
a
0 h
24 h

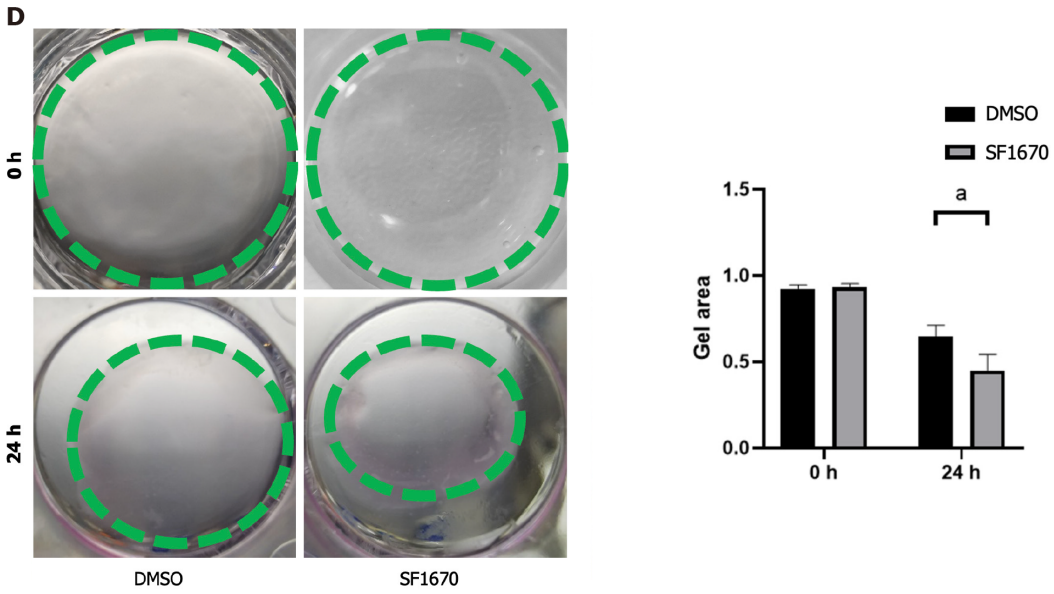


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Figure 8 Inhibition of phosphatase and tensin homolog promoted cell proliferation, migration, and collagen synthesis. A: The protein levels of p-Akt and Akt in NIH3T3 cells were determined by western blot; B: Bromodeoxyuridine assay was performed to measure the proliferation ability; C: Wound healing assay was performed to measure the migratory capability; D: Collagen contraction assay was performed to evaluate the cell contractility. *n* = 3 per groups. Data are shown as mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$.

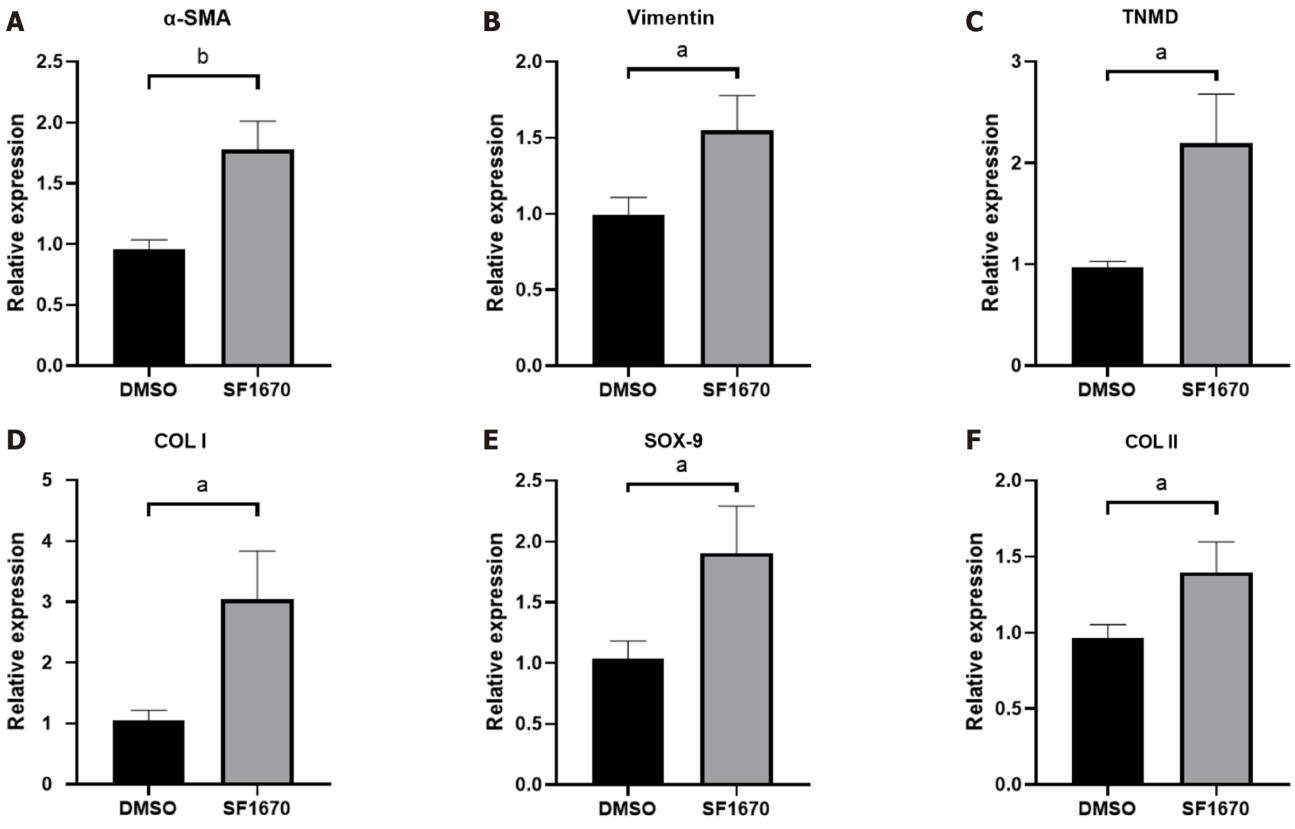

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Figure 9 Inhibition of phosphatase and tensin homolog change the gene expression of fibroblastic-, tenogenic-, and chondrogenic-related factors in NIH3T3 cells. The mRNA level of α-smooth muscle actin, vimentin, TNMD, Col I, Sox-9, and Col II in NIH3T3 cells treated with or without SF1670 was determined by quantitative real-time reverse transcription polymerase chain reaction. Data are shown as mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$. α-SMA: α-smooth muscle actin.

ARTICLE HIGHLIGHTS

Research background

The normal tendon-bone insertion has a transitional structure consisting of four gradated layers including bone, mineralized fibrocartilage layer, non-mineralized fibrocartilage layer and tendon. This complex structure can disperse force from bone to tendon, preventing stress concentration. However, current therapeutic strategies cannot restore this structure, hence raising the risk of re-injury.

Research motivation

Recent evidence indicates that conditioned medium, primarily contains exosomes of mesenchymal stem cells (MSCs), can stimulate the activation of fibroblasts, thereby promoting tendon-bone healing. However, the underlying mechanism is not comprehensively understood. Moreover, the expression of exosome microRNA (miRNA) may vary depending on the status of MSCs, which presents difficulties in identifying the roles associated with MSC-derived exosomal miRNAs.

Research objectives

To identify overlapped bone marrow MSC (BMSC)-derived exosomal miRNAs in three GSE datasets and verify their effects as well as mechanisms on fibroblasts.

Research methods

BMSC-derived exosomal miRNAs data were downloaded from the Gene Expression Omnibus database. The candidate miRNAs were obtained by the intersection of different datasets. TargetScan was used to predict potential target genes. Functional and pathway analyses were conducted using the Gene Ontology and Kyoto Encyclopedia of Genes and Genomes databases. Hub genes in the protein-protein interaction (PPI) network were analyzed using Cytoscape software. The transcripts of the target gene were assessed by dual luciferase reporter assays. BrdU, wound healing assay, collagen contraction assay and the expression of COL I and α-smooth muscle actin positive were applied to investigate the cell proliferation, migration and collagen synthesis.

Research results

Bioinformatics analyses showed two BMSC-derived exosomal miRNAs, has-miR-144-3p and has-miR-23b-3p, were overlapped in three GSE datasets. PPI network analysis and functional enrichment analyses indicated that both miRNAs regulated the PI3K/Akt signaling pathway by targeting phosphatase and tensin homolog (PTEN). *In vitro* experiments confirmed that both miRNAs stimulated proliferation, migration and collagen synthesis of NIH3T3 fibroblasts. Interfering with PTEN affected the phosphorylation of Akt and thus activated fibroblasts.

Research conclusions

BMSC-derived exosomes promote fibroblast activation possibly through the PTEN and PI3K/Akt signaling pathways, which may serve as potential targets to further promote tendon-bone healing.

Research perspectives

Further exploration is needed to pinpoint the specific functions of exosomal miRNAs. *In vivo* studies may better reveal the effect of exosomal miRNAs on tendon-bone healing.

FOOTNOTES

Author contributions: Li FQ, Chen WB, and Chen YS designed and performed most of the experiments, analyzed and interpreted the data, and wrote the manuscript; Luo ZW and Sun YY assisted during the acquisition, analysis, and interpretation of data and revised the manuscript; and all authors have reviewed and approved the final manuscript. Li FQ, Chen WB, and Luo ZW contributed equally to this study. Su XP (xiaopingsu2013@aliyun.com), Sun JM (sjm990205@163.com), and Chen SY (cshiyi@163.com) correspond to this study.

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

Data sharing statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Basic Study

Repetitive administration of cultured human CD34+ cells improve adenine-induced kidney injury in mice

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Abstract

BACKGROUND

There is no established treatment to impede the progression or restore kidney function in human chronic kidney disease (CKD).

AIM
To examine the efficacy of cultured human CD34+ cells with enhanced proliferating potential in kidney injury in mice.

METHODS
Human umbilical cord blood (UCB)-derived CD34+ cells were incubated for one week in vasculogenic conditioning medium. Vasculogenic culture significantly increased the number of CD34+ cells and their ability to form endothelial progenitor cell colony-forming units. Adenine-induced tubulointerstitial injury of the kidney was induced in immunodeficient non-obese diabetic/severe combined immunodeficiency mice, and cultured human UCB-CD34+ cells were administered at a dose of 1×10^6/mouse on days 7, 14, and 21 after the start of adenine diet.

RESULTS
Repetitive administration of cultured UCB-CD34+ cells significantly improved the time-course of kidney dysfunction in the cell therapy group compared with that in the control group. Both interstitial fibrosis and tubular damage were significantly reduced in the cell therapy group compared with those in the control group ($P < 0.01$). Microvasculature integrity was significantly preserved ($P < 0.01$) and macrophage infiltration into kidney tissue was dramatically decreased in the cell therapy group compared with those in the control group ($P < 0.001$).

CONCLUSION
Early intervention using human cultured CD34+ cells significantly improved the progression of tubulointerstitial kidney injury. Repetitive administration of cultured human UCB-CD34+ cells significantly improved tubulointerstitial damage in adenine-induced kidney injury in mice *via* vasculoprotective and anti-inflammatory effects.

Key Words: Chronic kidney disease; CD34+ cell; Adenine; Tubulointerstitial injury; Quality and quantity control culture; Umbilical cord blood

Core Tip: There is no established treatment for retarding the progression or improving advanced chronic kidney disease (CKD). Here, we provided effectiveness of cord blood derived cultured human CD34+ cell on progressive CKD model in mice. Kidney function and pathological damage were improved by repetitive cell therapy along with the improvement of microvasculature and inhibition of inflammatory cell infiltration in the kidney. Enhanced cell potential (proliferation and increase of endothelial progenitor cell colony-forming unit) by culture might be a key factor for improving progressive kidney injury.

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INTRODUCTION

Chronic kidney disease (CKD) is a progressive disease that leads to end-stage renal disease (ESRD) with increasing incidence and mortality[1]. Incomplete recovery from acute kidney injury (AKI) can also lead to CKD (AKI-CKD transition) through tubular atrophy and failed tubular recovery[2]. CKD affected nearly 750 million people globally in 2016 and constitutes a serious public health burden worldwide[3]. Several treatment strategies, including pharmaceutical therapies, such as renin-angiotensin-aldosterone blockers, and diet control, including salt and protein restriction, have been applied to impede CKD progression. However, satisfactory outcomes have not been achieved. Considering the progressive nature of CKD, which is associated with high mortality rates, especially in late-stage CKD patients, including those undergoing dialysis, there is a pressing need to develop novel therapeutic strategies to treat CKD.

The efficacy of cell-based regenerative therapy in animal models of CKD has been tested, and preclinical studies have provided favorable results[4]. Cell-based therapy has been shown to improve functional and histological parameters of kidney injury and delay the development of CKD in animal

models[4]. However, clinical trials of cell-based therapy for CKD in humans are still limited, with only three clinical trials published[5-7]. These trials used single administration of autologous bone marrow-derived mesenchymal stem cells[5,6] or granulocyte colony-stimulating factor-mobilized autologous peripheral blood-derived CD34+ cells[7]. Although the safety and tolerability of cell therapy were confirmed, the efficacy in improving or stabilizing renal dysfunction was not established by these early phase trials.

Adenine-induced renal injury in mouse is used as a model of chronic kidney injury caused by 2,8-dihydroxyadenine (DHA), a metabolic product of adenine[8,9]. The primary site of adenine-induced renal injury is the tubulointerstitial region. Accumulation of DHA crystals in the renal tubules and interstitial tissue causes progressive degeneration of tubular epithelial cells and induces interstitial fibrosis. Moreover, adenine-containing diet induces chronic and progressive renal injury in mice in a dose-dependent manner. This animal model is suitable for evaluating the efficacy of cell-based therapy for progressive tubulointerstitial injuries. However, to evaluate the efficacy of xenotransplantation of human cells, the animal's immune response has to be avoided. Therefore, we stablished an adenine-induced renal injury model in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, which lack mature T, B, and natural killer cells.

Human CD34+ endothelial progenitor cells (EPC) have high angiogenic and anti-inflammatory potential, and thus used for this study[10,11]. However, because EPCs from patients with several diseases have impaired function and are insufficient, allogeneic sources are considered as good alternatives. We therefore used umbilical cord blood (UCB)-derived CD34+ cells. However, in our preliminary study, uncultured human UCB-derived CD34+ cell administration could not effectively retard the progression of chronic kidney injury in this model. Hence, we cultured UCB-CD34+ cells to enhance their vasculogenic potential using the quality and quantity culture (QQc) method, which was established for EPCs[12], and used in our previous report[13]. Once or twice administration of human UCB-QQc-CD34+ cells could not improve the time-course of kidney injury. However, administration of human UCB-QQc-CD34+ cells once a week for 3 consecutive weeks demonstrated a significant improvement of kidney function and reduction of renal histological damages. This is the first report of the application of human UCB-QQc-CD34+ cells as an effective regenerative therapy for CKD.

MATERIALS AND METHODS

Cell preparation

Human UCB-CD34+ cells were purchased from the RIKEN Bioresource Center (Tsukuba, Japan), plated at a density of 1 × 10^4 cells/well in a 24-well tissue culture plate (BD Falcon, Bedford), and cultured in QQc media for 7 d as previously described[12,13]. QQc uses a serum-free StemSpan SFEM media (Stem Cell Technologies, Vancouver, BC, Canada) with an optimized combination of growth factors as well as cytokines (20 ng/mL thyroid peroxidase, 20 ng/mL interleukine-6, 100 ng/mL SCF, 100 ng/mL Flt-3 Ligand, and 50 ng/mL vascular endothelial growth factor). All reagents were purchased from PeproTech Inc. (Rocky Hill, NJ, United States). This culture method significantly enhanced the vasculogenesis, proliferation, and colony-forming units of CD34+ cells[10]. UCB-CD34+ cells from five separate samples were cultured, and cell populations of pre- and post-QQc human UCB-CD34+ EPCs were evaluated using FACS analysis (FACSVerse™, BD, United States). FlowJo™ (version 10.6, BD, United States) was used for data analysis.

EPC colony-forming assay

EPC colony-forming units (EPC-CFUs) of pre-QQc and post-QQc human UCB-CD34+ cells were assessed using a colony-forming assay as previously described[12,13]. The EPC colony-forming assay was designed to evaluate the vasculogenic potential of EPCs and categorize total EPC-CFUs (TEPC-CFUs) into two different types of EPC-CFUs: Primitive (small cell-sized) and definitive (large cell-sized). Primitive EPC-CFUs (PEPC-CFUs) have greater potential for proliferation, whereas definitive EPC-CFUs (DEPC-CFUs) are predominantly vasculogenic populations with a greater differentiation potential. Briefly, 500 human UCB-CD34+ cells per dish were seeded into a 35-mm hydrophilic tissue culture dish. Ten to fourteen days later, total EPC colony-forming unit (TEPC-CFUs), PEPC-CFUs, and DEPC-CFUs were counted under light microscopy by two investigators who were blinded to the experimental conditions. Five samples of UCB-CD34+ cells were used for EPC colony-forming assay. All experiments were performed in triplicate.

Mice

Six-week-old male NOD/SCID mice were purchased from Charles River Laboratories Inc. (Tsukuba, Japan) and used for all experiments. All animal use was in accordance with institutional and ARRIVE guidelines (https://arriveguidelines.org). The experimental protocol was approved by the Administration Committee of Experimental Animals at Kamakura Techno-science, Inc. (No. 20-034, 20-079, Kanagawa, Japan).

Adenine-induced chronic tubulointerstitial kidney injury

The CKD animal model (adenine-induced chronic tubulointerstitial kidney injury) using NOD/SCID mouse was developed in collaboration with Kamakura Techno-science, Inc. First, several doses of adenine were used to optimize and induce progressive tubulointerstitial kidney injury. Kidney damage showed a dose-dependent pattern, and the optimized dose and period of adenine diet ingestion were finally determined as 0.14% and 3 wk, respectively.

The experimental design is shown in Figure 1. Thirty-five male NOD/SCID mice were used in this experiment. All mice had free access to tap water, and were fed a 0.14% (w/w) adenine-containing diet from day 0 to day 21. The adenine-containing diet was stopped on day 21 and changed to standard laboratory chow diet (CRF-1 powder diet, Oriental Yeast Co., Ltd., Tokyo, Japan) from days 22 to 28 in all mice. Body weight and food intake were measured weekly. Blood sampling from the tail vein was performed on days 0, 7, 14, 21, and 28. After evaluating the serum creatinine levels on day 7, the mice were divided into two groups, *i.e.*, control group (n = 17) and the cell therapy group (n = 18). After blood sampling on day 28, mice were anesthetized using isoflurane, and kidney tissues were collected for histological analysis. All animal experiments were performed at Kamakura Techno-science, Inc. (Kanagawa, Japan).

Cell transplantation

Mice in the control group were injected with 100 μL of vehicle (Iscove's modified Dulbecco's medium: IMDM), and those in the cell therapy group were injected with 1 × 10^6 UCB-QQc-CD34+ cells in 100 μL IMDM *via* the tail vein. UCB-QQc-CD34+ cells were administered three times on days 7, 14, and 21. Cell purity and viability were evaluated just before cell therapy.

Kidney function and renal histology

Serial blood samples were drawn from the tail vein on days 0, 7, 14, 21, and 28, and serum creatinine levels were immediately measured using DRI-CHEM CRE-P III (FUJIFILM Co., Ltd., Tokyo, Japan). Kidney tissues were harvested and fixed in 4% paraformaldehyde, transferred to 70% ethanol, and embedded in paraffin. From these paraffin blocks, 2 μm-thick sections were prepared and stained with periodic acid-Schiff (PAS) and Masson's trichrome (MT) stain for analysis of tubular damage and interstitial fibrosis, respectively. To examine the extent of tubulointerstitial injury, 10 non-overlapping fields from the entire cortical and outer medulla areas in PAS-stained specimens and MT-stained specimens were captured under 100 × magnification. Tubulointerstitial injury in PAS-stained sections were categorized as tubular dilatation with epithelial and tubular atrophy. The degree of injury was scored on a scale of 0 to 4 according to the percentage of the damaged area; 0: 0%, normal; 1, 0%-25%; 2, 25%-50%;3, 50%-75%; and 4, 75% or more. Interstitial fibrosis in MT-stained sections was quantified as fibrotic area per total area (%) and measured using an automatic image analyzer (cellSens, Olympus, Japan). Tubulointerstitial injury score and interstitial fibrosis area were evaluated in each mouse (n = 17 in the control group and n = 18 in the cell therapy group), and mean values in each mouse were used to calculate the group mean value for further comparison between the control group and cell therapy group.

CD31 and F4/80 immunohistochemistry

Since CD31 antigen is constitutively expressed on the endothelial cell surface, the loss of CD31-positive staining most likely reflects capillary loss. We performed CD31 immunostaining to evaluate peritubular capillary (PTC) loss using 3 μm-thick paraffin sections. PTCs were identified by immunostaining with a rabbit monoclonal anti-CD31 (PECAM-1) antibody (1:100; #77699, Cell Signaling Technology, Danvers, MA, United States). Immunostaining was performed using a BOND-III autostainer (Leica Biosystems, Wetzlar, Germany) according to the manufacturer's protocol. Briefly, after deparaffinization of paraffin sections, heat-induced epitope retrieval was performed using BOND Epitope Retrieval Solution 2 (prediluted, pH 9.0; Leica Biosystems, Wetzlar, Germany) for 20 min at 100 °C. Sections were incubated sequentially with endogenous peroxidase block for 5 min, primary antibody for 30 min, secondary detection polymer for 10 min, diaminobenzidine for 10 min, and hematoxylin for 5 min.

PTC density was evaluated according to a previously published method with some modifications [11]. Twenty randomly selected fields encompassing the renal cortex and outer medulla were randomly captured by digital imaging (× 400). Each image was divided into 270 squares using a grid, and the number of squares with CD31-positive staining was counted. The PTC density was represented as a percentage of CD31 positive squares per total number of squares.

Macrophages were identified using a rabbit polyclonal anti-F4/80 antibody (1:100; ab100790, Abcam, Cambridge, United Kingdom). Ten non-overlapping fields in the cortical and outer medulla areas were randomly selected and captured by digital imaging (× 100). The degree of macrophage infiltration was calculated using the 270-square grid method, as described above. The rate of macrophage infiltration was represented as a percentage of F4/80- positive squares relative to the entire cortical and outer medulla areas. Macrophage infiltration rate and PTC density were assessed in the control group and cell therapy group.

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Figure 1 Experimental design. The adenine-containing diet was fed from day 0 to day 21, and switched to a standard laboratory diet from day 22 to day 28. Vehicle (IMDM) or UCB-CD34+ cells in IMDM were administered on days 7, 14, and 21 *via* the tail vein in the control group and cell therapy group, respectively. IMDM: Iscove's modified Dulbecco's medium; UCB: Umbilical cord blood.

Statistical analysis

All data are expressed as the mean ± SE, unless otherwise specified. Comparison between two groups were performed using the Mann-Whitney *U* test. Differences in time-course of serum creatinine levels during the experimental period between the control group and the cell therapy group were analyzed using repeated measures analysis of variance (ANOVA). Statistical analysis was performed using SPSS version 11.0 software (SPSS Inc., Chicago, IL, United States) and a P value < 0.05 was considered statistically significant.

RESULTS

Effect of the QQc on CD34+ cell expansion and EPC-CFUs

The QQc significantly increased the number of CD34+ cells. CD34+ positivity per the 1 × 10^4 initially seeded cells was significantly increased in post-QQc-CD34+ cells compared with that in pre-QQc-CD34+ cells (353821 ± 122429 *vs* 7341 ± 947, P < 0.001) (Figure 2A). Cell viability of QQc-CD34+ cells was higher than that of pre-QQc CD34+ cells (93.6% ± 1.3% *vs* 87.0% ± 0.8%, P < 0.01). After seven days of culturing using the QQc method, the number of CD34+ cells showed almost a 60-fold increase on day 7 compared with that on day 0 (59.0 ± 10.4 *vs* 1-fold, P < 0.001) (Figure 2B). Post-QQc CD34+ cells formed more TEPC-CFUs than did pre-QQc CD34+ cells (1138.0 ± 124.0 *vs* 27.0 ± 4.5, P < 0.001) (Figure 2C). Moreover, both PEPC-CFUs and DEPC-CFUs showed a significant increase after QQc compared with pre-QQc (PEPC-CFUs: 17.4 ± 3.0 *vs* 666.0 ± 127.5, P < 0.001; DEPC-CFUs: 9.4 ± 2.5 *vs* 472.0 ± 112.0, P < 0.01).

QQc-CD34+ cells improved kidney function

The body weight was not significantly different between the cell therapy and control groups on days 0, 7, 14, 21, and 28. Diet consumption was also comparable between the two groups throughout the experimental period (data not shown).

To evaluate the role of UCB-QQc-CD34+ cell therapy in renal injury, serum creatinine levels were determined in the cell therapy and control groups. The time-course in serum creatinine levels is shown in Figure 3A. Serum creatinine levels were not statistically different between the cell therapy and control groups (0.56 mg/ dL ± 0.02 mg/dL *vs* 0.61 mg/dL ± 0.03 mg/dL) on day 0. During adenine-containing diet feeding, serum creatinine levels showed a gradual and constant increase in the control group (1.51 mg/dL ± 0.14 mg/dL on day 21) demonstrating a state of renal dysfunction. After switching to the standard diet on day 22, serum creatinine levels in the control group decreased to 1.41 mg/dL ± 0.14 mg/dL on day 28. In the cell therapy group, serum creatinine levels increased until day 14 and then showed a gradual increasing trend on day 21 (1.29 mg/dL ± 0.09 mg/dL). After switching to the standard diet, serum creatinine levels decreased to 1.09 mg/dL ± 0.09 mg/dL on day 28 in the cell therapy group. Time-course of serum creatinine levels between the control and the cell therapy groups showed borderline significance (P = 0.06). However, the relative ratio of changes in serum creatinine levels from day 0 to day 28 showed a statistically significant difference between the control and the cell therapy groups (255.1% ± 25.9% *vs* 185.9% ± 18.5%, P = 0.035) (Figure 3B). Thus, repetitive administration of UCB-QQc-CD34+ cells significantly improved the time-course of adenine-induced chronic kidney injury.

QQc-CD34+ cells reduced interstitial fibrosis and tubular damage

The adenine-containing diet induced prominent tubular damage, including tubular dilatation and

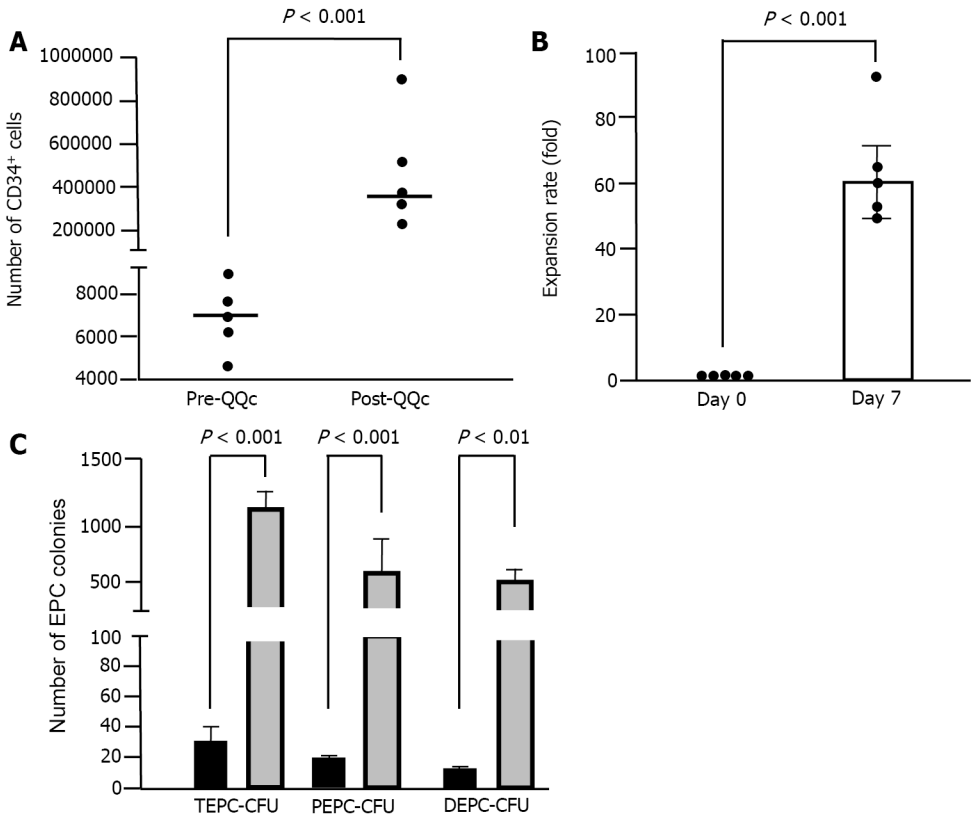

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Figure 2 CD34+ cell and endothelial progenitor cell colony-forming units expansion by quality and quantity culture method. A: Number of total CD34+ cells. The plot graph demonstrates CD34+ positivity per 1 × 10^4 fresh UCB-CD34+ sample and post-culture CD34+ cell number per 1 × 10^4 initially seeded cells; B: Expansion rate of CD34+ cells. The expansion rate of CD34+ cells from fresh CD34+ cells (day 0) to cultured CD34+ cells (day 7); C: Endothelial progenitor cells (EPC) colony-forming unit. The number of TEPC-CFUs, primitive EPC-CFUs, and definitive EPC-CFUs of post- quality and quantity culture (QQc) CD34+ cells. Gray column: Significantly increased compared with that of pre-QQc CD34+ cells; black column: Statistical significances in Figure 2A-C were determined using Mann-Whitney *U* test. UCB: Umbilical cord blood; EPC: Endothelial progenitor cell; CFU: Colony-forming unit; QQc: Quality and quantity culture; TEPC: Total endothelial progenitor cell; PEPC: Primitive endothelial progenitor cell; DEPC: Definite endothelial progenitor cell.

tubular atrophy accompanied by interstitial fibrosis (Figure 4A-D). In line with the reduced time-course of renal injury in cell therapy mice, the interstitial fibrosis area was significantly reduced in the cell therapy group compared with that in the control group (8.1% ± 5.4% *vs* 16.3% ± 7.0%, $P < 0.01$) (Figure 4E). The tubular damage score was also significantly decreased in the cell therapy group compared with that in the control group (1.48 ± 1.27 *vs* 2.83 ± 1.00, $P < 0.01$) (Figure 4F).

PTC density and macrophage infiltration

Representative images of PTC density as evaluated by CD31 immunohistochemical staining are shown in Figure 5. CD31+ staining was significantly decreased, especially around the dilated and degenerated tubules, in this model (Figure 5A). The cell therapy group showed significantly higher PTC density than did the control group (57.3% ± 6.4% *vs* 31.0% ± 6.1%, $P < 0.01$) (Figure 5B and C).

Macrophage infiltration is shown in Figure 6. Infiltration of F4/80-positive macrophages was mainly observed in the area with interstitial fibrotic changes and dilated and degenerated tubules in the control group (Figure 6A). However, macrophage infiltration was dramatically reduced in the cell therapy group compared with that in the control group (1.3% ± 0.3% *vs* 14.4% ± 1.1%, $P < 0.001$) (Figure 6B and C).

DISCUSSION

We demonstrated that repetitive administration of human UCB-QQc-CD34+ cells significantly improved the time-course of adenine-induced tubulointerstitial injury in mice. The relative changes in serum creatinine levels from baseline to day 28 were significantly improved in the cell therapy group compared with those in the control group. Interstitial fibrosis area and tubulointerstitial injury score were also significantly improved by cell therapy. The PTC network, visualized by CD31+ staining, was found to be severely damaged in adenine-induced kidney injury. However, PTC rarefaction was

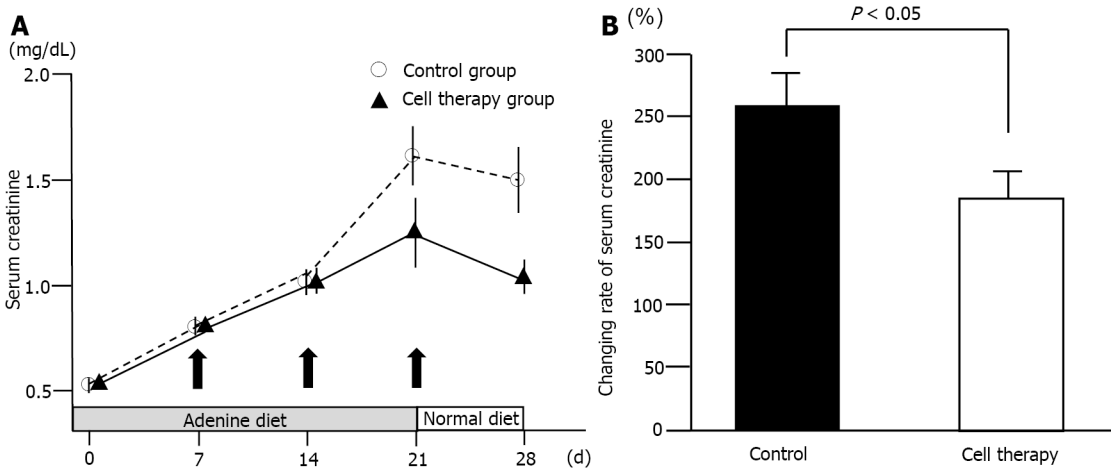

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Figure 3 Changes in serum creatinine levels. A: Time-course of serum creatinine levels in the control group (n = 17) and cell therapy group (n = 18). The P value between the control group and the cell therapy group on day 28 was 0.06; B: Change rate of serum creatinine levels from baseline to day 28 in the control and cell therapy groups. Statistical significance in Figure 3A was determined using repeated measures analysis of variance. Statistical significance in Figure 3B was determined using Mann-Whitney U test.

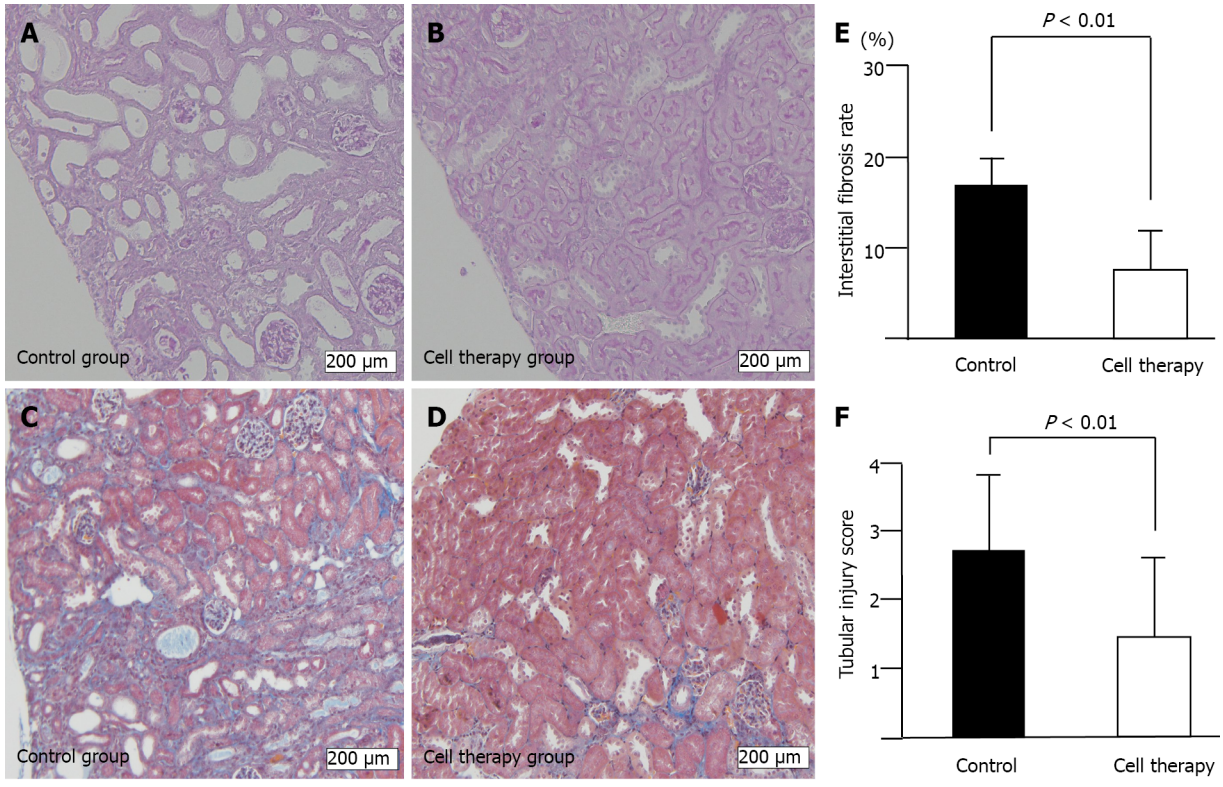

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Figure 4 Histological analysis of renal injury. A and B: Tubular injury including tubular dilatation, tubular epithelial atrophy, and tubular atrophy, evaluated using periodic acid-Schiff-stained renal tissue sections of control group (A) and cell therapy group (B); C and D: Interstitial fibrosis using Masson's trichrome stained sections of control group (C) and cell therapy group (D). Scale bar: 200 μm; E: Quantitative analysis of interstitial fibrosis rate, and F: semi-quantitative analysis of tubular injury score. Statistical significance of Figure 4E and F were determined using Mann-Whitney U test.

significantly improved by cell therapy. Furthermore, macrophage infiltration was dramatically reduced in the cell therapy group. These results might imply the significant potential of CD34+ cell-based therapy for clinical application in CKD.

CKD progression is caused by multiple factors, including diabetes, hypertension, and glomerulonephritis. Regardless of the diverse causes of CKD, tubulointerstitial damage is the final common pathway that results in its transition to ESRD[14]. Therefore, one major target of cell-based regenerative therapy for CKD is the arrest of chronic tubular and interstitial damage. Loss of integrity of the

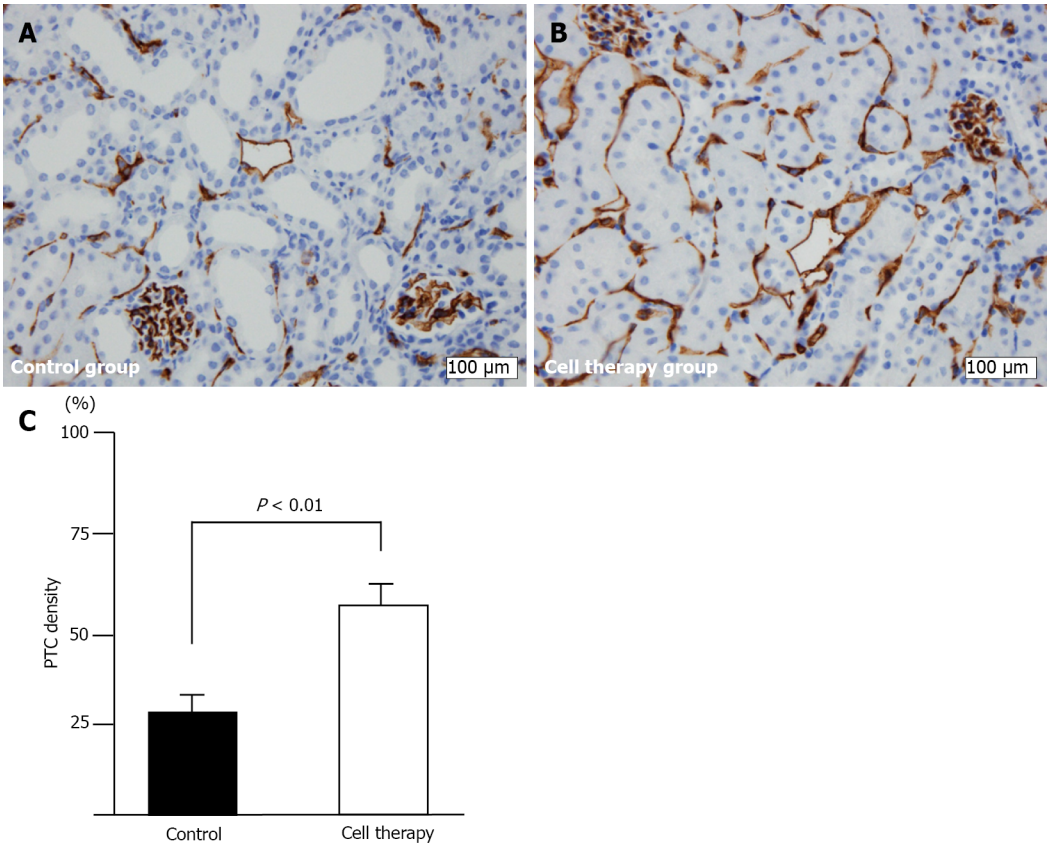

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Figure 5 Peritubular capillary density evaluation using CD31+ staining. A and B: CD31+ immunostaining of peritubular capillary (PTC) in control group (A) and cell therapy group (B); original magnification (× 400). Scale bar: 100 μm; C: Quantitative analysis of PTC density. Statistical significance in Figure 5C was determined using Mann-Whitney *U* test.

intrarenal microvasculature is also a major contributor to the progression of renal diseases[15-17]. Tubular damage, interstitial fibrosis, and microvasculature impairment are associates with each other and form a vicious cycle to promote CKD[18-24]. A close association between PTC rarefaction and tubulointerstitial fibrosis has been proven in the human kidney[20]. Hypoxia due to PTC rarefaction induces the production of local inflammatory cytokines[25], which in turn, worsen hypoxia in inflamed tissues. Thus, hypoxia also creates a vicious cycle of inflammation[26].

CD34+ cells have been used in regenerative therapy for several organ damages, including critical limb ischemia[27,28], myocardial ischemia[29], stroke[30], and acute kidney injury[31]. CD34+ cells possess two important properties that are considered necessary for regenerative therapy: Enhanced angiogenic and anti-inflammatory potential[10,11]. Externally administered CD34+ EPCs improved kidney injury such as endothelial cell damage in several animal models[13,15,19,32-35]. In cases of acute and severe renal endothelial cell injury, intrinsic repair may not function sufficiently[36]. Furthermore, endothelial disturbances related to ischemia induce permanent damage to PTCs[37]. Therefore, a strategy for vascular protection or angiogenesis might be important for the long-term preservation of kidney function. Recently, horizontal transfer of regenerative information *via* extracellular vesicles (EVs) between regeneration associated stem/progenitor cells and injured cells in the damaged organ was shown to be a major regenerative mechanism. Exosomes from human CD34+ cells, by themselves, have demonstrated angiogenic activity in *in vivo* and *in vitro* experiments[38,39]. Hence, CD34+ based cell therapy could be an excellent strategy to induce vasculogenesis and repair kidney function.

Regarding cell administration, frequency of transplantation as well as the number and regenerative potential of transplanted cells is thought to be very important. Multiple rather than single administration might show more advantages to improve organ damage. Lv *et al*[40] demonstrated that multiple administration of UCB-mesenchymal stem cells showed a superior therapeutic effect compared with that of single administration in an animal model of chemotherapy-induced premature ovarian failure. We also demonstrated that repetitive administration (3 times, once in a week) of cultured UCB-CD34+ cells significantly improved the time-course of adenine-induced chronic kidney injury. The causative signals for CKD, including oxidative stress, tissue hypoxia, and inflammatory cell infiltration with the release of inflammatory cytokines continuously injure the kidney. Therefore, multiple administration of regeneration-associated CD34+ cells at fixed time intervals might be a better treatment strategy for CKD. For the number of administered cells, 1 × 10^6 cells/20 g mouse is equivalent to about 50 × 10^6/kg

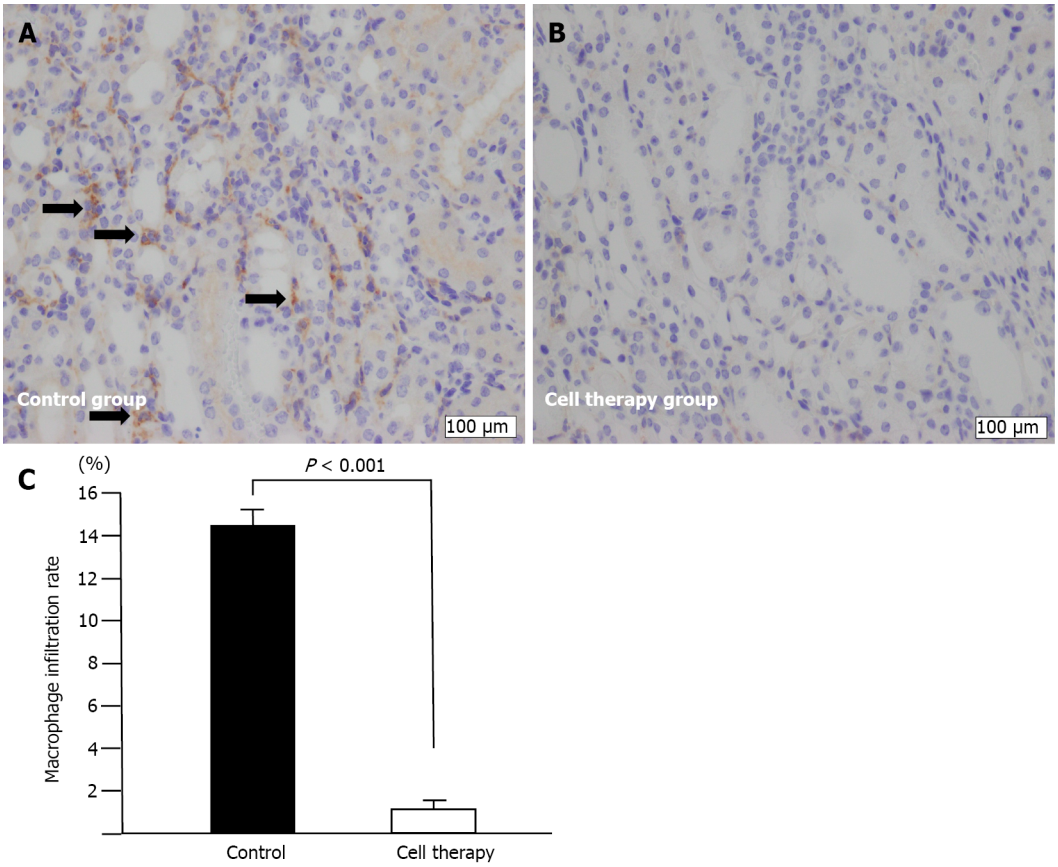

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Figure 6 Macrophage infiltration analysis. A and B: F4/80+ macrophage immunostaining (arrows) in control group (A) and cell therapy group (B); original magnification (× 400). Scale bar: 100 μm; C: Quantitative analysis of the rate of macrophage infiltration area. Statistical significance in Figure 6C was determined using Mann-Whitney *U* test.

in humans, an amount that is probably not achievable clinically. It is known that in cases of cell administration *via* the tail vein in mice, many cells are trapped in the lung, spleen, and bone marrow (extra-renal trap)[13]. Extra-renal trap might necessitate more cells to be administered in mice to deliver sufficient number of cells into the kidney. Therefore, future clinical trials should consider the route of cell administration. To get better effects in future clinical trials, cells should be designed for direct arterial administration.

Herein, we used human UCB-derived CD34+ cells. UCB-CD34+ cells can be used as an off-the-shelf treatment. Because EPCs from patients with several diseases have impaired function and are insufficient, allogeneic source is considered as a good alternative. Immune reaction against allogeneic cells might limit their use. However, a recent study reported the immunosuppressive effect of cultured human EPCs. The proliferation and activation of mouse T cells were significantly suppressed by co-culture with allogeneic human UCB-EPCs[41]. Therefore, cultured UCB-EPCs with enhanced potential might have a chance of emerging as a possible tool for allogeneic cell therapy. One major limitation to using UCB-CD34+ cells is the limited number obtained from one UCB donor. Regarding this, QQc of UCB-CD34+ cells significantly increased the number of CD34+ cells (almost 60 folds) in one week, and EPC-CFUs (total, primitive, and definite EPC-CFUs) were significantly increased. The increased UCB-CD34+ cell number enabled multiple administration. Furthermore, the enhanced vasculogenic and anti-inflammatory potential of UCB-CD34+ cells might be critical for preserving PTC integrity and reducing inflammatory cell infiltration.

There are several limitations to this study. First, the effect of tissue hypoxia was not evaluated to establish the relationship among tubular damage, interstitial fibrosis, PTC integrity, macrophage infiltration, and hypoxia in the kidney tissue of control and cell therapy-treated mice. Second, the cellular behavior of UCB-CD34+ cells (homing and staying in injured kidney) remain undetermined. Third, we could not compare the contents (mRNA, miRNA, and proteins, including growth factors and cytokines) of EVs of pre-QQc and post-QQc UCB-CD34+ cells. Therefore, the EV-mediated mechanism by which UCB-QQc-CD34+ cells showed an effective response in repairing kidney injury remains to be elucidated. We evaluated the effect of cell therapy during the progressive phase, and not the advanced ESRD stage. Therefore, it is unclear whether cell therapy might be effective at the ESRD stage. Stopping adenine diet induced recovery from dysfunction. However, it remains unknown whether stopping

adenine diet without cell therapy brings kidney function completely to normal, because we did not conduct a longer follow-up after switching adenine diet to normal chow. At the same time, we could not elucidate whether the effects of cell therapy were long-lasting since the animals were sacrificed only one week after the last dose of cells. These limitations should be addressed in future research.

CONCLUSION

In conclusion, although the more precise mechanisms should be clarified, this study provides a new insight in the field of regenerative medicine. Repetitive administration of human UCB-QQc-CD34+ cells improved kidney function and reduced histological damage in adenine-induced tubulointerstitial injury. Tubular damage and interstitial fibrosis were significantly improved by cell therapy in accordance with improved PTC integrity and reduced inflammatory cell infiltration. Hence, clinical trials using repetitive administration of regenerative CD34+ cells with enhanced proliferative and angiogenic potential might be expected for patients with progressive CKD.

ARTICLE HIGHLIGHTS

Research background

Chronic kidney Disease (CKD) constitutes a serious public health burden worldwide, and there is no established treatment to impede the progression or restore kidney function in human CKD.

Research motivation

To discover and establish a new effective treatment for CKD is urgently needed. Cell-based therapy has a potential as a new effective treatment for progressive CKD.

Research objectives

To evaluate the efficacy of cultured human cord-blood-derived CD34+ cells.

Research methods

Progressive tubulointerstitial injury with kidney dysfunction was made in mice. Human umbilical cord blood (UCB)-derived CD34+ cells were incubated in vasculogenic conditioned medium for 1 wk to increase the number of CD34+ cells and ability to form endothelial progenitor cell (EPC)-colony-forming units. These cultured human CD34+ cells were administered on days 7, 14, and 21 after the start of adenine diet.

Research results

Cell therapy significantly improved the time-course of progressive kidney dysfunction. Pathological injuries including tubular injury and interstitial fibrosis were significantly reduced in the cell therapy group compared with those in the control group. Preserved microvasculature integrity and decreased macrophage infiltration were shown in the cell therapy group.

Research conclusions

Early intervention using human cultured CD34+ cells significantly improved the progression of tubulointerstitial kidney injury.

Research perspectives

In future research, the efficacy of cultured human CD34+ cells for more advanced CKD such as end-stage renal disease (ESRD), cell behavior (homing and staying in injured kidney) after cell administration, differences in extracellular vesicles from pre- and post-incubated UCB-derived CD34+ cells, should be evaluated for further analysis of the efficacy of cell therapy. A future clinical trial of cell therapy for progressive CKD might be expected.

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FOOTNOTES

Author contributions: Ohtake T contributed to principal investigator, conception and design, provision of study material, collection and assembly of data, data analysis, data interpretation, and manuscript writing; Itaba S, Fujii S, and Kumagai H contributed to animal experiment and data analysis; Salybekov AA and Sheng Y contributed to cell culture and cell population analysis; Sato T contributed to cell preparation and cell population analysis; Yanai M and Imagawa M contributed to immunohistochemical analysis; Harata M contributed to cell preparation; Asahara T contributed to advisor of cell culture, conception and design, confirmation of analytical methods and results, data interpretation, advisor for content of manuscript, and final approval of manuscript; Kobayashi S contributed to conception and design, data interpretation, advisor for content of manuscript, and final approval of manuscript.

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